Original Article

Plasma Vitamin D Levels And Vitamin D Receptor Polymorphisms Are Associated with Survival of Non-small Cell Lung Cancer

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ABSTRACT

Objective: Vitamin D and its receptor (*VDR*) involve in multiple cellular processes and play an important role in the initiation and progression of malignancy. Thus we hypothesized that plasma vitamin D levels and single nucleotide polymorphisms (SNPs) in *VDR* may be of prognostic significance in non-small cell lung cancer (NSCLC).

Methods: We examined plasma 25-hydroxyvitamin D [25(OH)D] levels in 87 patients diagnosed with NSCLC using enzyme-linked immunosorbent assay (ELISA) and genotyped seven potentially functional SNPs in *VDR* in 568 NSCLC patients on Illumina Golden Gate platform.

Results: Patients with higher plasma 25(OH)D levels had worse survival than patients with lower ones (*P* for trend = 0.048). The SNPs of rs1544410 and rs739837 were independently associated with NSCLC survival (adjusted HR = 1.61, 95% CIs = 1.06-2.45 for rs739837 AA *vs* AC/CC and adjusted HR = 1.51, 95% CIs = 1.06-2.16 for rs1544410 AG/AA *vs* GG). A joint effect was observed between rs1544410 and rs739837 and the risk of death elevated as the number of unfavourable genotypes patients carried increased (*P* for trend = 0.003). There were no significant associations between *VDR* polymorphisms and plasma 25(OH)D levels.

Conclusion: Our findings indicate that plasma 25(OH)D levels and genetic variants of *VDR* may serve as prognostic markers for NSCLC in this Chinese population.

Key words: Vitamin D receptor polymorphisms; Non-small cell lung cancer; Chinese population; Prognosis

INTRODUCTION

Lung cancer is the leading cause of cancer death in the world[1]. Despite advances in diagnosis and treatment in the past years, the prognosis remains poor. Current clinical pathological staging system is inadequate for predicting the prognosis[2]. Several studies have explored the prognostic factors for non-small cell lung cancer (NSCLC) and have emphasized that the application of specific biomarkers, combined with traditional factors such as histological type and stage at diagnosis, could assist in predicting prognosis[3].

Dietary vitamin D mostly transforms to vitamin D_3 in the skin, which subsequently undergoes two steps of hydroxylation to yield 1, 25-dihydroxyvitamin D [1,25$(OH)_2$D][4]. Vitamin D receptor (*VDR*) is the target receptor of 1,25$(OH)_2$D[4]. Activation of 1,25$(OH)_2$D regulates more than 60 genes that exert prodifferentiating, antiproliferative and antimetastatic effects[5]. Thus both vitamin D and *VDR* participate in the process of critical cellular activity. It has been shown that vitamin D has anti-proliferative effects in a wide variety of cancers including lung cancer[6]. However, a high level of plasma vitamin D was reported to be a controversial prognostic factor for certain groups of lung cancer patients[7, 8].

Several potentially functional single nucleotide polymorphisms (SNPs) have been identified in *VDR*. For example, the C allele of rs739837 is predicted to create a binding site of miRNA-34b and may down- regulate *VDR* gene expression by post-transcriptional repression[9]. Rs1544410 is strongly linked with 3 poly(A) microstatellite repeat in the 3′UTR which may influence *VDR* mRNA stability[10]. It has been shown that immature dendritic cells

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with rs1544410-GG genotype had lower β-actin-normalized *VDR* mRNA expression compared to those with rs1544410-AA genotype[11]. The A allele of rs10735810 increases *VDR* protein by three amino acids longer, resulting in lower potency than the shorter one of G allele in transient transfection assays[12]. The A allele of rs11568820, which is located in the promoter region of the *VDR* gene, has been shown to have higher transcriptional activity than the G allele[13]. Therefore, it is biologically plausible that genetic variations in *VDR* may influence the expression as well as function of *VDR*.

Recently, several studies reported that genetic variations of *VDR* were associated with progression and prognosis of common cancers[14-20], including lung cancer[14, 15]. Zhou et al. reported that patients with variant genotypes of rs11568820 (GA/AA) had a longer survival time than those with GG genotype in early-stage lung squamous cell carcinoma. In addition, Heist et al observed that the CC genotype of rs10735810 was significantly associated with a favorable survival in 294 advanced NSCLC patients[14, 15]. However, no studies have focused on the associations between genetic variants of *VDR* and lung cancer prognosis in Chinese population.

In this study, we investigated the associations of plasma 25-hydroxyvitamin D [25(OH)D] levels and genetic variants of *VDR* with prognosis of NSCLC in a case cohort of 568 NSCLC patients in a Chinese population.

MATERIALS AND METHODS

Study Population

The patients' enrollment was described previously[21]. Patients were recruited from Jiangsu Cancer Hospital and the First Affiliated Hospital of Nanjing Medical University, Nanjing, China, from July 2003 to April 2008. A total of 568 patients with NSCLC were included based on complete follow up data and DNA quantity and quality. Maximum follow-up time was 72 months (last follow-up date: July 2009). Our study was approved by the Institutional Review Board of Nanjing Medical University.

The demographic characteristics and clinical features of the 568 NSCLC patients have been described previously[21]. No significant associations were observed between survival and patients' characteristics (i.e., smoking status, histological type, chemotherapy or radiation treatment), except for clinical stage (Log-rank P<0.001) and surgical operation (Log-rank P<0.001). Patients with advanced stage or without surgery had significantly worse prognosis.

Plasma Vitamin D Measurements and SNPs Selection and Genotyping

Enzyme-linked immunosorbent assay (ELISA) was used to examine the plasma 25(OH)D levels in 87 randomly selected patients, which was conducted according to the manufacturer's instruction (25-Hydroxy Vitamin D EIA kit; Immunodiagnostic Systems, Limited, Bolton, Tyne and Wear, United Kingdom).

Potentially functional SNPs previously reported to be associated with gene expression and/or cancer risk/survival were included. Seven SNPs, rs739837, rs731236, rs7975232, rs757343, rs1544410, rs10735810 and rs11568820 were included and genotyped. Genotyping was performed on the Illumina Golden Gate platform at Berkeley Biotech Inc., Taizhou, China, and the information on assay conditions, primers and probes are available upon request. The quality control for the Illumina genotyping platform has been described previously[18]. All SNPs were successfully genotyped with call rates ≥98% and were followed Hardy-Weinberg equilibrium (HWE) except rs7975232 (P=1.228E-12) which was removed from further analyses (Table 1).

Statistical Analyses

Overall survival time was calculated from the date of diagnosis to the date of death or last follow-up, and median survival time (MST) was presented. Fisher's exact test was used to test HWE. The associations between survival time and demographic characteristics, clinical features, plasma 25(OH)D levels and polymorphisms in *VDR* were estimated by the Kaplan-Meier method and the log-rank test. Univariate and multivariate Cox proportional hazard models were performed to estimate the hazard ratios (HRs) and their 95% confidence intervals (95% CIs) with adjustment for age, gender, smoking status, stage, histology, surgical operation and chemotherapy or radiation treatment. Cox stepwise regression analysis was performed to evaluate the independent predictive factors for NSCLC prognosis, with a significance level of 0.05 for entering and 0.051 for removing the respective explanatory variables. The heterogeneity between stratifications was assessed with the Chi-square-based q test. T test was carried out to test the difference of plasma 25(OH)D levels among genotype groups of rs739837 or rs1544410.

Table 1. Genotyping results and NSCLC patient's survival

SNP	Base change	Location	No. of genotyping (%)	MAF	HWE*	Log-rank P		
						Additive model	Dominant model	Recessive model
rs739837	C>A	3' UTR	568 (100)	0.31	0.888	0.126	0.269	0.051
rs731236	A>G	Exon	560 (98.6)	0.04	0.151	0.027	0.016	0.100
rs7975232	A>C	Intron	568 (100)	0.31	1.228E-12	--	--	--
rs757343	G>A	Intron	559 (98.4)	0.26	0.825	0.872	0.731	0.773
rs1544410	G>A	Intron	562 (98.9)	0.04	0.053	0.008	0.004	0.099
rs10735810	G>A	Exon	567 (99.8)	0.40	0.607	0.526	0.326	0.810
rs11568820	A>G	5' near	564 (99.3)	0.45	0.940	0.737	0.957	0.470

*P value of Fisher's exact test of Hardy-Weinberg equilibrium

All statistical analyses were carried out on Statistical Analysis System software (version 9.1.3; SAS Institute, Cary, NC). *P* value of less than 0.05 was considered statistically significant, and all tests were two-sided.

RESULTS

Vitamin D Levels and Overall Survival

Patients with higher plasma 25(OH)D levels had worse survival than those with lower ones. The risk of death was significantly increased in patients with higher vitamin D levels (adjusted HR = 1.31, 95% CIs = 1.00-1.72, *P* for trend = 0.048) (Table 2).

Effects of Genetic Variants of *VDR* on NSCLC Survival

Variant genotypes of two SNPs, rs731236 and rs1544410, were significantly associated with survival of NSCLC in both additive model (log-rank *P* = 0.027, 0.008 for rs731236 and rs1544410, respectively) and dominant model (log-rank *P*=0.016, 0.004, respectively). In addition, the variant genotype of rs739837 was associated with prognosis with borderline significance in recessive genetic model (log-rank *P*=0.051) (Table 1). Cox regression analyses revealed that the survival was significantly associated with rs739837 in recessive model (HR=1.82, 95% CIs=1.21-2.72), with rs731236 in dominant model (HR = 1.49, 95% CIs = 1.07-2.08) and with rs1544410 in dominant model (HR = 1.64, 95% CIs = 1.16-2.31) after adjustment for age, gender, smoking status, stage, histology, surgical operation and chemotherapy or radiation treatment (Table 3).

Stepwise Cox Regression Analysis for NSCLC Survival

We further conducted stepwise Cox proportional hazard analysis to evaluate the effects of demographic characteristics, clinical features and the three SNPs in *VDR* on NSCLC survival. Four variables (stage, surgical operation, SNPs rs739837 and rs1544410) were included in the final regression model (*P*< 0.001 for stage and surgical operation; *P*=0.023, 0.002 for rs739837 and rs1544410, respectively) (Table 4).

Combined Effects of rs739837 and rs1544410

We further assessed combined effects of the two SNPs of rs739837 and rs1544410 on NSCLC survival. The results indicated that the more unfavourable genotypes the patients carried, the shorter MST they had, suggesting a locus-

Table 2. Plasma 25-hydroxyvitamin D levels and NSCLC patients survival

Group	Vitamin D Level (nnmol/L)	N	Ajusted HR (95% CIs)*	*P*
1	< 25.36	22	1.00	
2	25.36 - 37.72	22	1.47 (0.58-3.73)	0.415
3	37.72 - 56.54	22	1.59 (0.75-3.39)	0.225
4	≥ 56.54	21	2.54 (1.01-6.41)	0.048
Trend			1.31 (1.00-1.72)	0.048

*Adjusted for age, gender, smoking status, stage, histology, surgical operation and chemotherapy or radiation treatment.

Table 3. Genotypes of *VDR* polymorphisms and NSCLC patients survival

Genotype	Cases	Deaths	MST (Months)	Crude HR (95% CIs)	Adjusted HR (95% CIs)*
rs739837	n=568	n=311			
CC	309	164	26.2	1.00	1.00
AC	219	120	23.5	1.07 (0.85-1.36)	1.26 (0.99-1.61)
AA	40	27	21.4	1.52 (1.01-2.29)	2.00 (1.32-3.04)
AA *vs* AC/CC	40/528	27/284	21.4/25.1	1.48 (1.00-2.20)	1.82 (1.21-2.72)
rs731236	n = 560	n =307			
AA	496	265	26.2	1.00	1.00
AG	60	39	19.1	1.44 (1.03-2.02)	1.41 (1.00-1.99)
GG	4	3	11.4	2.63 (0.84-8.28)	4.26 (1.32-13.8)
AG/GG *vs* AA	64/496	42/265	18.4/26.2	1.49 (1.07-2.07)	1.49 (1.07-2.08)
rs1544410	n = 562	n = 307			
GG	505	268	26.4	1.00	1.00
AG	53	36	18.2	1.59 (1.12-2.26)	1.55(1.09-2.21)
AA	4	3	11.4	2.66 (0.85-8.25)	4.33(1.34-14.0)
AG/AA *vs* GG	57/505	39/268	18.2/26.4	1.64 (1.17-2.31)	1.64(1.16-2.31)

*Adjusted for age, gender, smoking status, stage, histology, surgical operation and chemotherapy or radiation treatment.

Table 4. Stepwise Cox regression model on NSCLC survival

Variables	B	SE	HR	95% CIs	*P*
Stage	0.50	0.09	1.65	1.40-1.95	<0.001
Surgical Operation (Yes *vs* No)	-0.63	0.14	0.53	0.40-0.71	<0.001
rs739837 in recessive model	0.48	0.21	1.61	1.06-2.45	0.023
rs1544410 in dominant model	0.41	0.18	1.51	1.06-2.16	0.002

Table 5. Combined effects of polymorphisms of *VDR* on NSCLC survival

Combined Genotypes (Unfavorable Genotypes)	Patients	Deaths	MST (Months)	Crude HR (95% CIs)	Adjusted HR (95% CIs)*
0	485	257	26.4	1.00	1.00
1	69	42	20.0	1.39 (1.01-1.93)	1.54(1.10-2.14)
2	14	12	17.4	2.16 (1.21-3.86)	2.27(1.26-4.08)
Locus trend	P for trend = 0.003			1.44 (1.14-1.80)	1.52(1.21-1.91)

*Adjusted for age, gender, smoking status, stage, histology, surgical operation and chemotherapy or radiation treatment.

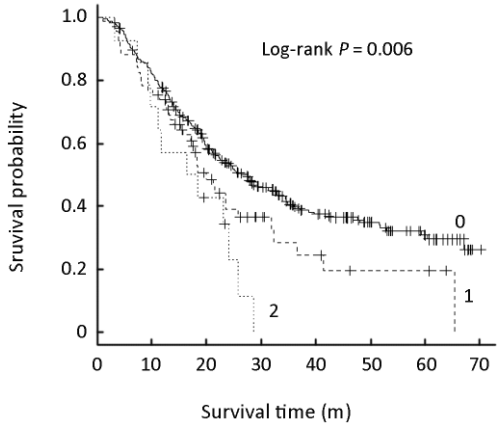

Figure 1. Kaplain-Meier plot of survival by combination of rs739837 and rs1544410 in NSCLC-specific survival (log-rank *P*=0.006). 0: patients carrying no unfavourable genotypes; 1: patients carrying one unfavourable genotype; 2: patients carrying two unfavourable genotypes.

dosage effect between combined genotypes and NSCLC survival (*P* for trend=0.003). Patients carrying two unfavourable genotypes (AG/AA of rs1544410 and AA of rs739837, MST: 17.4 months) and one unfavourable genotype (AG/AA of rs1544410 or AA of rs739837, MST: 20.0 months) had worse survival than patients without unfavourable genotypes (MST: 26.4 months) (Figure1). The risk of death was significantly increased in patients carrying one unfavourable genotype (adjusted HR = 1.54, 95% CIs = 1.10-2.14), and it was more evident in those carrying two unfavourable genotypes (adjusted HR = 2.27, 95% CIs = 1.26-4.08) (Table 5).

Stratified Analysis

The associations between rs1544410 and rs739837 and NSCLC survival were also evaluated by stratifying smoking status, histology, stage, surgical operation and chemotherapy or radiation treatment. The increased risk of rs1544410 variant genotypes were more prominent among patients received surgical operations (HR = 2.25, 95% CIs = 1.43-3.55, *P* for heterogeneity test = 0.043) than those without surgical operations. There were no significant differences of the associations between rs739837 and rs1544410 and NSCLC survival among different subgroups of smoking status, histology, stage, and chemotherapy or radiation treatment.

Vitamin D Levels and Genotypes of rs739837 and rs1544410

We tested the differences of plasma 25(OH)D levels between favourable and unfavourable genotype groups of rs739837 and rs1544410, but no significant differences were detected.

DISCUSSION

In the current study, we evaluated the associations between plasma 25(OH)D levels and potentially functional SNPs in *VDR* and NSCLC survival in Chinese population and found that patients with higher plasma 25(OH)D levels had worse survival than those with lower levels. Besides, rs739837 and rs1544410 were identified as independent prognostic factors for NSCLC survival along with stage and surgical operation. A significant locus-dosage effect was present between combined genotypes of rs739837 and rs1544410 and NSCLC survival. No differences of plasma 25(OH)D levels were observed between favorable and unfavorable genotypes of rs739837 or rs1544410.

The prognostic impact of vitamin D levels on cancer has been extensively investigated, but the results are controversial. Freedman et al.[8] examined relationship of baseline plasma 25(OH)D with total cancer mortality and found that risks were increased at higher 25(OH)D levels in men but decreased in women. Though we didn't perform the stratified analysis due to small sample size, 78.2% of participants (68/87) were men in our study, indicating that our observations in NSCLC were consistent with that of Freedman. However, Ng et al.[19] found that higher prediagnosis plasma 25(OH)D levels were associated with significant improvement in overall survival among patients with colorectal cancer. Goodwin et al.[22] suggested that vitamin D deficiency was associated with poor outcomes in breast cancer. Further analyses with large sample size are required to clarify the significance of vitamin D levels on NSCLC survival.

Rs1544410 is strongly linked with 3 poly(A) microstatellite repeat in the 3'UTR, which may influence *VDR* mRNA stability[10]. Besides, it has been shown that immature dendritic cells with rs1544410-GG genotype exhibited lower *VDR* mRNA expression compared to those with rs1544410-AA genotype[11]. Thus it is biologically plausible that rs1544410 serves as a prognostic biomarker for NSCLC. Given that rs739837 is located in the 3'UTR of *VDR*, it is possible that such a variation may lead to altered binding affinity to microRNAs and down-regulate *VDR* gene expression by post- transcriptional repression[9]. We predicted that miRNA-34b binds to the wild C allele of rs739837 and rs739837-CC genotype may be linked with lower *VDR* expression. However, further functional assays were warranted to unravel the biological significance of the

variant. In addition, rs731236, a missense variant with an amino acid change of isoleucine to methionine, was significantly associated with NSCLC survival but was removed from the multivariate Cox model in our population. This variant was in high linkage disequilibrium (LD) with rs1544410 (r^2: 0.75) and may be functional since that the amino acid substitution may result in *VDR* function change and therefore may modify the development and prognosis of cancer.

Christiani and his colleagues[14, 15] investigated the role of genetic variations of *VDR* on NSCLC and found that AG/AA genotype of rs11568820 was associated with better overall survival among early stage lung squamous cell carcinoma patients[14] while CC genotype of rs10735810 was associated with improved survival in advanced stage NSCLC patients[15]. In our study, neither rs11568820 nor rs10735810 variant was significantly related to survival of NSCLC patients in this Chinese population. Data from the public HapMap SNPs database showed that the variant allele frequencies of rs731236 and rs1544410 were both less than 5% in Chinese population, but common in Caucasian population. The C allele of rs739837 is more common (C allele frequence = 0.69) in Chinese population than that in Caucasian population (C allele frequence = 0.43). Therefore, ethnic differences may contribute to part of the inconsistent findings, especially if these markers were not causal ones by themselves, which warrants additional studies in diverse ethnic populations.

In conclusion, our results indicate that both plasma vitamin D level and genetic variants of *VDR* may serve as prognostic biomarkers for NSCLC in Chinese population. These findings require additional validations and the role of these variants needs to be clarified by further functional assays.

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