Circulating 25-Hydroxyvitamin D, VDR Polymorphisms, and Survival in Advanced Non–Small-Cell Lung Cancer

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ABSTRACT

Purpose

We showed previously that in early-stage non–small-cell lung cancer (NSCLC), serum vitamin D levels and *VDR* polymorphisms were associated with survival. We hypothesized that vitamin D levels and *VDR* polymorphisms may also affect survival among patients with advanced NSCLC.

Patients and Methods

We evaluated the relationship between circulating 25-hydroxyvitamin D levels; *VDR* polymorphisms, including *Cdx-2* *G>A* (rs11568820), *FokI* *C>T* (rs10735810), and *BsmI* *C>T* (rs144410); and overall survival among patients with advanced NSCLC. Analyses of survival outcomes were performed using the log-rank test and Cox proportional hazards models, adjusting for sex, stage, and performance status.

Results

There were 294 patients and 233 deaths, with median follow-up of 42 months. We found no difference in survival by circulating vitamin D level. The *C/C* genotype of the *FokI* polymorphism was associated with improved survival: median survival for *C/C* was 21.4 months, for *C/T* was 12.1 months, and for *T/T* was 15.6 months (log-rank *P* = .005). There were no significant effects on survival by the *Cdx-2* or *BsmI* polymorphism. However, having increasing numbers of protective alleles was associated with improved survival (adjusted hazard ratio for two or more v zero to one protective alleles, 0.57; 95% CI, 0.41 to 0.79; *P* = .0008). On haplotype analysis, the *G-T-C* (*Cdx-2-FokI-BsmI*) haplotype was associated with worse survival compared with the most common haplotype of *G-C-T* (adjusted hazard ratio, 1.61; 95% CI, 1.21 to 2.14; *P* = .001).

Conclusion

There was no main effect of vitamin D level on overall survival in the advanced NSCLC population. The *T* allele of the *VDR FokI>T* polymorphism and the *G-T-C* (*Cdx-2-FokI-BsmI*) haplotype were associated with worse survival.

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INTRODUCTION

Vitamin D is a steroid hormone that has been shown in both in vitro and in vivo experiments to block cell growth.^{1,2} In mouse models, metastatic spread of Lewis lung cancer cell lines is inhibited by high levels of vitamin D.^{3,4} A number of epidemiologic studies have suggested that vitamin D, or putative surrogates for vitamin D status, such as geographic latitude or season, are associated with incidence and mortality of a variety of cancers.⁵⁻¹⁰

We previously showed that in lung cancer, season of surgery and dietary vitamin D intake were significantly associated with survival in patients with early-stage, surgically resected non–small-cell lung cancer (NSCLC).¹¹ In addition, levels of circulating vitamin D were correlated with survival¹² in these patients.

The hormonal activity of vitamin D is mediated by vitamin D receptor (VDR), which is a steroid hormone receptor. Vitamin D binds to VDR within the nuclei of cells. This binding causes a conformational change in VDR, leading to dimerization with retinoid X receptor, interaction of the dimeric complex with vitamin D response elements in target genes, and subsequent changes in the activity of genes involved in cell division, cell adhesion, and function.¹³

Several potentially functional polymorphisms have been identified in the human *VDR* gene. The *A* allele of the *Cdx-2* *G>A* polymorphism (rs11568820), which is located in the *VDR* gene promoter region, has been shown to have higher transcriptional activity.^{14,15} The *T* allele of the *FokI* *C>T* polymorphism (rs10735810), which is located in the translational initiation site of *VDR*,

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seems to be less efficient in exerting 1,25-dihydroxyvitamin D (1,25(OH)₂D) effects as compared with the *C* allele.¹⁶ The *T* allele of the *BsmI* *C>T* polymorphism (rs1544410) has been associated with increased VDR mRNA expression and increased serum levels of 1,25(OH)₂D.^{17,18} Genotypes and haplotypes of *VDR* have been studied in relation to risk of cancer, with varying results.¹⁹⁻²⁷

We showed previously that polymorphisms in the *VDR* gene may be associated with survival in patients with early-stage NSCLC. Specifically, among patients with squamous cell carcinoma, carrying the *A* allele of the *Cdx-2* *G>A* polymorphism, as well as increasing numbers of protective alleles in the *Cdx-2*, *FokI*,

and *BsmI* polymorphisms, were associated with better survival. The *G-T-C* (*Cdx-2-FokI-BsmI*) haplotype, hypothesized to be associated with the lowest VDR expression or function, was also associated with worse survival in the squamous cell group.²⁸

We investigated whether vitamin D levels or *VDR* gene polymorphisms were associated with survival outcomes in advanced-stage NSCLC. On the basis of functional data cited above, we hypothesized that the variant *A* allele of the *Cdx-2* polymorphism, wild-type *C* allele of the *FokI* polymorphism, and the variant *T* allele of the *BsmI* polymorphism, either individually or combined in joint effects or haplotypes, would be associated with better survival. We also hypothesized that higher circulating vitamin D levels would be associated with improved survival. Because of our earlier findings of differences by stage or histology, we investigated these subgroups as well.

Table 1. Patient Characteristics

Characteristic	No.	%
Age, years		
Median	62	
Range	33-85	
Sex		
Male	141	48
Female	153	52
Stage		
IIIA	66	22
IIIB	62	21
IV	166	57
Histologic subtype		
Adenocarcinoma	156	53
Squamous cell	51	17
Large cell	44	15
NSCLC NOS	43	15
Smoking status		
Never	25	8
Former	131	45
Current	138	47
ECOG performance status		
0 to 1	276	94
≥ 2	18	6
Pack-years		
Median	45	
Range	0-183	
Platinum chemotherapy		
Cisplatin	78	27
Carboplatin	216	73
<i>BsmI</i>		
<i>C/C</i>	95	32
<i>C/T</i>	142	48
<i>T/T</i>	57	20
<i>Cdx-2</i>		
<i>G/G</i>	176	60
<i>G/A</i>	93	32
<i>A/A</i>	25	8
<i>FokI</i>		
<i>C/C</i>	125	42
<i>C/T</i>	128	44
<i>T/T</i>	41	14
Circulating 25(OH)D levels, ng/mL		
Mean	20.6	
SD	10.2	

Abbreviations: NSCLC, non-small-cell lung cancer not otherwise specified; ECOG, Eastern Cooperative Oncology Group; 25(OH)D, 25-hydroxyvitamin D; SD, standard deviation.

Table 2. Univariate Analysis

Variable	Overall Survival		
	Crude HR	95% CI	P
Age	1.005	0.99 to 1.02	.47
Male sex	1.36	1.05 to 1.76	.02
ECOG PS			
0 to 1	Reference		
≥ 2	2.49	1.49 to 4.18	.0005
Stage			
IIIA	Reference		
IIIB	1.43	0.97 to 2.12	.07
IV	2.10	1.49 to 2.95	< .0001
Histologic subtype			
Adenocarcinoma	Reference		
Squamous	1.15	0.79 to 1.66	.47
Large cell	0.96	0.43 to 2.15	.92
NSCLC NOS	1.18	0.73 to 1.89	.51
Season of diagnosis			
Winter	Reference		
Spring/fall	0.91	0.66 to 1.24	.54
Summer	1.03	0.74 to 1.42	.88
Smoking status			
Never	Reference		
Former	1.28	0.78 to 2.08	.33
Current	1.21	0.74 to 1.97	.45
Pack-years	1.002	0.99 to 1.01	.24
<i>BsmI</i>			
<i>C/C</i>	Reference		
<i>C/T</i>	0.89	0.66 to 1.19	.43
<i>T/T</i>	0.99	0.69 to 1.44	.99
<i>Cdx-2</i>			
<i>G/G</i>	Reference		
<i>G/A</i>	0.97	0.73 to 1.29	.81
<i>A/A</i>	1.13	0.71 to 1.81	.61
<i>FokI</i>			
<i>C/C</i>	Reference		
<i>C/T</i>	1.58	1.19 to 2.09	.002
<i>T/T</i>	1.47	1.0 to 2.15	.05

Abbreviations: HR, hazard ratio; ECOG PS, Eastern Cooperative Oncology Group performance status; NSCLC NOS, non-small-cell lung cancer not otherwise specified.

PATIENTS AND METHODS

Study Population

Since 1992, patients with histologically confirmed NSCLC have been recruited prospectively into a molecular epidemiology study at Massachusetts General Hospital (MGH; Boston, MA). Blood samples for genotyping and patient demographic information (including age, sex, and smoking status) were collected at the time of recruitment. Informed consent was obtained to collect follow-up data. More than 85% of eligible patients were recruited in this cohort.

We limited our analysis to all patients with incident cases of stage III or IV NSCLC, enrolled between December 1992 and July 2004, who received first-line platinum-based treatment at MGH and had follow-up data, and who had adequate DNA and serum/plasma samples for genotyping and circulating 25-hydroxyvitamin D [25(OH)D] measurement. This allowed us to study a relatively homogeneously treated group of patients where differences in treatment style or practice would not be significant confounding factors. We identified 294 patients who met these criteria. There were no differences in age, sex, stage, histology, smoking status, or pack-years of smoking among those patients with or without adequate DNA and serum/plasma samples for inclusion in the study (χ^2 test and Kruskal-Wallis test). The study was approved by the institutional review boards of MGH and Harvard School of Public Health (Boston, MA).

Genotyping and Vitamin D Measurements

Blood samples were collected from all study participants at the time of recruitment. The majority of patients had blood drawn within 2 months of initial diagnosis of lung cancer. Serum/plasma samples were separated and stored at -80°C . Circulating 25(OH)D levels were measured using a published radioimmunoassay.²⁹

DNA was extracted from peripheral-blood samples using the PureGene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). The *VDR* polymorphisms *Cdx-2* G>A (rs11568820), *FokI* C>T (rs10735810), and *BsmI* C>T (rs144410) were genotyped by the 5'-nuclease assay (TaqMan) using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Genotyping was performed by laboratory personnel blinded to case status, and 5% of the total samples were randomly selected as replicates for genotyping quality check. Two authors independently reviewed the genotyping results, data entry, and statistical analyses.

Outcomes Collection

Overall survival (OS) was the end point in this analysis. OS was calculated from date of diagnosis to date of death, or last known date alive. Data were collected from at least one of the following sources: (1) MGH inpatient and outpatient records, (2) MGH tumor registry, (3) Social Security Death Index, (4) primary physician's office, and (5) patient or family contact. Permission to contact patients and their families to obtain follow-up information was included in our original consent form; in the vast majority of cases, we were able to obtain the information through the other four sources.

Statistical Analysis

Demographic and clinical information was compared across genotype and variables such as age, stage, histology, and smoking, using Pearson χ^2 tests (for categoric variables) and Kruskal-Wallis tests (for continuous variables), where appropriate. To investigate the effect of circulating 25(OH)D levels on OS, we separated the population into four groups by quartiles of 25(OH)D levels. The associations between *VDR* polymorphism status or 25(OH)D levels and survival were estimated using the method of Kaplan and Meier and assessed using the log-rank test. Cox proportional hazards models were used to adjust for potential confounders, including sex, stage, and performance status,

Table 3. Vitamin D Levels and Overall Survival

Vitamin D Quartiles (ng/mL)	Patients		Median OS	95% CI	<i>P</i>	AHR	95% CI	<i>P</i> _{trend} *
	Deaths	Total						
All NSCLC								
Quartile 1, < 12.6	57	73	19.8	16.4 to 27.3		Reference		
Quartile 2, 12.6 to 20.2	59	74	13.8	10.5 to 16.7		1.09	0.75 to 1.57	
Quartile 3, 20.3 to 27.6	57	73	16.4	11.1 to 24.5		1.03	0.71 to 1.50	
Quartile 4, $\geq$ 27.7	60	74	13.3	11.5 to 19.9	.76	1.08	0.75 to 1.57	.76
Adenocarcinoma								
Quartile 1, < 12.6	31	39	20.8	18.1 to 32.9		Reference		
Quartile 2, 12.6 to 20.2	28	34	13.8	10.4 to 22.6		1.13	0.67 to 1.91	
Quartile 3, 20.3 to 27.6	31	42	24.3	16.1 to 30.2		0.82	0.49 to 1.37	
Quartile 4, $\geq$ 27.7	35	41	13.3	11.5 to 19.9	.20	1.34	0.81 to 2.19	.51
Squamous								
Quartile 1, < 12.6	10	15	28.5	17.1 to 46.3		Reference		
Quartile 2, 12.6 to 20.2	10	15	14.9	9.4 to . . .		1.67	0.65 to 4.28	
Quartile 3, 20.3 to 27.6	10	10	8.1	6.4 to 15.2		3.04	1.05 to 8.84	
Quartile 4, $\geq$ 27.7	7	11	21.4	6.3 to . . .	.09	1.60	0.55 to 4.66	.14
Stage III disease								
Quartile 1, < 12.6	33	39	26.1	18.3 to 41.7		Reference		
Quartile 2, 12.6 to 20.2	21	30	20.3	13.7 to 55.9		0.87	0.50 to 1.52	
Quartile 3, 20.3 to 27.6	28	35	21.1	9.4 to 29.7		1.17	0.70 to 1.96	
Quartile 4, $\geq$ 27.7	19	24	21.4	12.4 to 69.8	.78	0.88	0.49 to 1.57	.98
Stage IV disease								
Quartile 1, < 12.6	24	34	17.1	9.0 to 20.3		Reference		
Quartile 2, 12.6 to 20.2	38	44	10.4	9.5 to 14.9		1.32	0.79 to 2.21	
Quartile 3, 20.3 to 27.6	29	39	16.4	8.0 to 24.3		0.99	0.58 to 1.72	
Quartile 4, $\geq$ 27.7	41	49	12.5	10.3 to 17.2	.47	1.29	0.78 to 2.15	.56

Abbreviations: OS, overall survival; AHR, adjusted hazard ratio; NSCLC, non-small-cell lung cancer.

*Adjusted for sex, stage, and performance status.

with VDR genotypes or quartiles of vitamin D levels fitted as indicator variables. Joint effects of polymorphisms were analyzed based on the number of protective alleles from the three polymorphisms, where the A allele of the *Cdx-2* G>A polymorphism, the C allele of the *FokI* C>T polymorphism, and the T allele of the *BsmI* C>T polymorphism were considered protective. Haplotype frequencies and individual haplotypes were generated using the macro happpy. All statistical testing was done at the two-sided .05 level, and SAS software version 9.1 (SAS Institute, Cary, NC) was used.

RESULTS

Patient Characteristics

There were 294 patients and 233 deaths. Median follow-up time among censored observations was 42 months (range, 2 to 146 months). Follow-up data were very complete among the censored group, with only 14 patients who had 2 years or fewer of follow-up, and 23 patients who had 3 years or fewer of follow-up. Demographic, tumor, and treatment characteristics are listed in Table 1. Genotype frequencies of the VDR polymorphisms are as follows: for *Cdx-2*: G/G, 176 (60%); G/A, 93 (32%); and A/A, 25 (8%); for *FokI*: C/C, 125 (42%); C/T, 128 (44%); and T/T, 41 (14%); for *BsmI*: C/C, 95 (32%); C/T, 142 (48%); and T/T, 57 (20%). The three VDR polymorphisms

were not in high linkage disequilibrium, with the D' of *Cdx-2_A-FokI_T* of 0.09, *Cdx-2_A-BsmI_C* of 0.25, and *FokI_T-BsmI_C* of 0.14. Genotype was not associated with patient- or tumor-related factors such as sex, smoking status, stage, or histologic subtype for any of the polymorphisms. On univariate analysis, sex, stage, performance status, and the *FokI* polymorphism were significantly associated with survival (Table 2).

Vitamin D and Overall Survival

Median circulating vitamin D level was 20.25 ng/mL, with a range from 1.0 to 55.5 ng/mL. For the analysis, we divided the population into quartiles of vitamin D levels: less than 12.6 ng/mL, 12.6 to 20.2 ng/mL, 20.3 to 27.6 ng/mL, and ≥ 27.7 ng/mL. Vitamin D levels were not significantly associated with survival. Table 3 shows median OS and adjusted hazard ratios for death by quartile of vitamin D level; there was no difference in the overall population or in subgroups of histology or stage. In the population as a whole, the adjusted hazard ratio (AHR) for the highest quartile of vitamin D was 1.09 (95% CI, 0.75 to 1.58), as compared with the lowest quartile of vitamin D ($P_{\text{trend}} = .74$). There were no differences by stage or histology.

Table 4. Individual and Combined VDR Polymorphisms and Survival

Variable	Patients		Median OS	95% CI	<i>P</i>	AHR	95% CI	<i>P</i> _{trend} [*]
	Deaths	Total						
Individual polymorphisms								
All NSCLC								
<i>BsmI</i>								
<i>C/C</i>	77	95	14.9	11.1 to 22.6		Reference		
<i>C/T</i>	110	142	17.1	13.7 to 19.9		0.89	0.66 to 1.19	
<i>T/T</i>	46	57	13.3	10.1 to 21.1	.67	0.93	0.64 to 1.35	.61
<i>Cdx-2</i>								
<i>G/G</i>	142	176	16.4	12.8 to 20.4		Reference		
<i>G/A</i>	71	93	15.8	11.4 to 21.4		0.85	0.63 to 1.15	
<i>A/A</i>	20	25	14.2	10.5 to 19.3	.82	1.02	0.64 to 1.63	.59
<i>FokI</i>								
<i>C/C</i>	89	125	21.4	16.1 to 25.7		Reference		
<i>C/T</i>	107	128	12.1	10.3 to 16.6		1.32	0.98 to 1.77	
<i>T/T</i>	37	41	15.6	9.7 to 22.2	.005	1.41	0.96 to 2.07	.04
No. of protective alleles across all three polymorphisms								
All NSCLC								
0 to 1	44	46	10.8	6.3 to 18.1		Reference		
≥ 2	189	248	17.0	13.5 to 20.3	.002	0.57	0.41 to 0.79	.0008
Adenocarcinoma								
0 to 1	23/	24	14.4	5.7 to 27.2		Reference		
≥ 2	102/	132	19.8	14.6 to 22.2	.08	0.63	0.40 to 0.99	.05
Squamous								
0 to 1	6	6	8.4	6.3 to 16.4		Reference		
≥ 2	31	45	19.4	11.5 to 29.6	.07	0.40	0.16 to 1.03	.06
Stage III								
0 to 1	19	20	17.3	6.4 to 27.5		Reference		
≥ 2	82	108	23.6	19.4 to 29.6	.03	0.57	0.34 to 0.95	.03
Stage IV								
0 to 1	25	26	9.0	5.0 to 12.8		Reference		
≥ 2	107	140	13.2	11.2 to 16.7	.02	0.56	0.36 to 0.87	.01

Abbreviations: OS, overall survival; AHR, adjusted hazard ratio; NSCLC, non-small-cell lung cancer.

*Adjusted for sex, stage, and performance status.

Individual and Combined VDR Polymorphisms and OS

Individually, neither the *Cdx-2* nor *BsmI* polymorphism was significantly associated with survival (Table 4). However, carrying the *T* allele of the *FokI* polymorphism was associated with worse survival: median survival was 21.4 months for patients who were *C/C*, compared with 12.1 and 15.6 months for patients who were *C/T* and *T/T*, respectively (log-rank $P = .005$); AHR for death compared with the reference group of *C/C* was 1.32 (95% CI, 0.98 to 1.77) for *C/T* and 1.41 (95% CI, 0.96 to 2.07) for *T/T* ($P_{\text{trend}} = .04$; Fig 1A and Table 4). Similar findings were noted when we analyzed by histology (adenocarcinoma and squamous subgroups) or stage (III or IV). Vitamin D levels by better prognosis (*FokI C/C*) and worse prognosis (*FokI C/T + T/T*) genotypes did not affect survival.

Fig 1. (A) *VDR FokI* polymorphism and overall survival. (B) Number of protective *VDR* alleles and overall survival.

When we combined the number of protective alleles in the *Cdx-2*, *FokI*, and *BsmI* polymorphisms, we found that compared with a reference group of patients with only zero to one protective alleles, all other groups had improved survival (AHR for two protective alleles, 0.53 [95% CI, 0.39 to 0.78]; for three protective alleles, 0.60 [95% CI, 0.41 to 0.88]; and for $\geq$ four protective alleles, 0.58 [95% CI, 0.39 to 0.87]; $P = .08$). Because the hazards ratios were similar for the groups with two or more protective alleles, we combined these groups to compare with the reference group of only zero to one protective alleles. We found a significantly improved survival among those carrying two or more protective alleles as compared with those carrying zero to one protective alleles, with a median OS of 17.0 months (95% CI, 13.5 to 20.3) versus 10.8 months (95% CI, 6.3 to 18.1; $P = .002$); AHR, 0.57 (95% CI, 0.41 to 0.79; $P = .0008$). These findings were observed when analyzed by histology and stage as well (Fig 1B and Table 4).

VDR Haplotypes and OS

We investigated the associations between *VDR* haplotypes and OS in Cox proportional hazards models. A total of six common haplotypes were identified in the population (Table 5). Among these, the *G-T-C* haplotype (16%, *Cdx-2-FokI-BsmI*) was hypothesized to be associated with the lowest *VDR* expression or function based on the functional data. Compared with the reference *G-C-T* haplotype, which was the most common haplotype, the *G-T-C* haplotype had significantly worse survival on both crude and adjusted analysis (Table 5). This finding was consistent across all subgroups examined—adenocarcinoma histology, squamous histology, stage III disease, and stage IV disease. Vitamin D levels by worse or better prognosis haplotypes were not associated with survival.

DISCUSSION

We previously reported in our patient population with early-stage surgically resected NSCLC that increasing levels of circulating vitamin D and certain *VDR* gene polymorphisms and haplotypes were associated with better survival outcome. We investigated whether the same held true in our patients with advanced-stage NSCLC. We found no difference in survival by vitamin D level. However, we found that patients carrying the *C/C* genotype of the *FokI* polymorphism seemed to have improved survival as compared with patients who were either *C/T* or *T/T*. This would be keeping with the hypothesized function of the polymorphism, as the *T* allele is thought to be less efficient in exerting $1,25(\text{OH})_2\text{D}$ effects as compared with the *C* allele.¹⁶

In addition, we found an effect of *VDR* haplotypes on survival, with the *G-T-C* (*Cdx-2-FokI-BsmI*) haplotype being associated with worse survival among patients with advanced-stage NSCLC. This finding is consistent with the functional data of the individual polymorphisms, where the *Cdx-2 G* allele, *FokI T* allele, and *BsmI C* allele are all thought to be associated with decreased *VDR* functional activity.

Although there are multiple studies investigating the role of *VDR* polymorphisms and risk of developing cancer, little is known about the potential prognostic impact of these polymorphisms. We showed previously that in patients with early-stage resected NSCLC, *VDR* polymorphisms may be associated with survival.²⁸

Table 5. VDR Haplotype Frequencies and Hazard Ratios in Cox Proportional Hazards Model

Haplotype	%	SE	Crude		Adjusted*	
			HR	95% CI	HR	95% CI
G-C-T	26	0.02	Reference		Reference	
G-C-C	24	0.02	0.82	0.63 to 1.07	0.86	0.66 to 1.12
G-T-C	16	0.02	1.52	1.15 to 2.00	1.61	1.21 to 2.14
G-T-T	10	0.01	0.82	0.54 to 1.2	0.80	0.52 to 1.23
A-C-C	10	0.01	0.94	0.63 to 1.39	0.94	0.63 to 1.41
A-T-C	7	0.01	0.94	0.62 to 1.41	0.85	0.55 to 1.31
P				.02		< .0001

Abbreviation: HR, hazard ratio.

*Adjusted for sex, stage, and performance status.

Among patients with squamous cell carcinoma, carrying the A allele of the *Cdx-2* G>A polymorphism, as well as increasing numbers of protective alleles in the *Cdx-2*, *FokI*, and *BsmI* polymorphisms, was associated with better survival. The G-T-C (*Cdx-2-FokI-BsmI*) haplotype, hypothesized to be associated with the lowest VDR expression or function, was also associated with worse survival in early-stage squamous carcinoma.¹⁶

In our analysis of patients with advanced NSCLC, we found associations between specific polymorphisms and haplotypes in the population as a whole. Although trends were similar in the various histology and subgroups studied, these subgroup analyses should be viewed with caution, because the numbers are quite small. Therefore, analysis by subgroups, particularly in subgroups such as squamous histology where there were only 51 cases, is not conclusive.

Of note, we found a significant association with the *FokI* polymorphism, but not the *Cdx-2* polymorphism, as reported for early-stage NSCLC, in our current study. Why there is a discrepancy in these single-nucleotide polymorphisms (SNPs) is unclear. However, there was concordance between the prior study in early-stage NSCLC and our current study in advanced NSCLC on the effects of combined polymorphisms and haplotype analysis. This suggests that there is an effect of VDR genetic variation on survival in lung cancer, in both early and advanced stages. It is possible that haplotypes or combined effects of multiple polymorphisms will be more informative than single SNPs alone, as these can capture more of the genetic variation of a gene than one single SNP.

Interestingly, we did not find an association of circulating vitamin D with survival in this population. In contrast, in the early-stage population, we had seen a significant effect of vitamin D on survival, with an AHR of 0.74 (95% CI, 0.50 to 1.10) for the highest versus lowest quartile of vitamin D levels. There are several potential reasons for why this survival benefit was seen in the early-stage but not advanced-stage populations. First, survival in advanced-stage NSCLC is quite poor, and any differences by vitamin D level may be too small to affect the overall course of disease. Second, the vitamin D levels present in this population are low, and it is possible that at these levels, there is no meaningful impact on prognosis—it may be that significantly higher levels of vitamin D are needed to have any impact in this poor prognosis population. Indeed, researchers have proposed a level of 20 ng/mL to define vitamin D deficiency and 32 ng/mL to define insufficiency;²⁵ our median vitamin D level was 20.25 ng/mL, and the highest quartile was more than 27.75 ng/mL. Only 39 patients (13%) of our population had vitamin D levels that were ≥ 32 ng/mL. Hence our patient population was vitamin D deficient, as might be expected

from an ill, northeastern United States population of patients with advanced lung cancer. In addition, malignant cells may develop mechanisms to escape the antiproliferative actions of vitamin D, including decreased VDR expression or decreased conversion of 25(OH)D to 1,25(OH)₂D, among others.¹ One could hypothesize that this capacity may be more prevalent among advanced-stage cancers than earlier stages, thereby making vitamin D a more potent modifier of survival in the early-stage NSCLC population rather than the late-stage population. And finally, it is possible that vitamin D has no true impact on survival in this advanced NSCLC population.

There are several limitations to our study. Overall the sample size is large for an outcomes study of this kind, but in the various subgroups, numbers become smaller and therefore are more difficult to interpret. In addition, adjustment for smoking status was based on information provided by patients at the time of enrollment; we do not have complete information on postdiagnosis smoking, which may also be a factor to consider in future studies designed to collect such information. There are also limitations with choice of candidate polymorphisms—we chose to investigate three VDR polymorphisms that are thought to be functional based on in vitro data and that have clinical outcomes data from our prior studies. There are multiple other reported VDR polymorphisms, however, and a more comprehensive analysis of the VDR gene would be a future goal. More genotype-phenotype correlations would be useful as well, to further elucidate the functional significance of these polymorphisms. We did not find an association between genotype and vitamin D level in this study, which is not entirely unexpected. We are planning in future studies to assess VDR expression status in tumor tissue, investigating how genotype affects VDR expression. These studies are difficult to perform in patients with advanced NSCLC, as there is often limited tissue available from needle biopsies, which are often used to diagnosis NSCLC in the late stages. We therefore plan to do this in early-stage patients, where tumor tissue is more readily available. Finally, there are multiple other genes involved in the pathway of vitamin D metabolism, and future studies should incorporate these as well.

In conclusion, we found that in patients with advanced-stage NSCLC, the C/C genotype of the *FokI* polymorphism, which functionally is hypothesized to have higher VDR activity, was associated with improved survival. In addition, the G-T-C (*Cdx-2-FokI-BsmI*) haplotype in VDR, which functionally is hypothesized to have lower VDR activity, is associated with worse survival. Further studies are warranted to confirm these findings. Clinical trials are ongoing that are investigating the addition of vitamin D or vitamin D analogs to the

treatment of patients with various cancers, including lung cancer. Investigating these polymorphisms in these clinical trials will be important for associations with treatment outcomes.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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