Polymorphisms of Vitamin D Receptor and Survival in Early-Stage Non–Small Cell Lung Cancer Patients

Wei Zhou,[1] Rebecca S. Heist,[1,5] Geoffrey Liu,[1,5] Donna S. Neuberg,[2,6] Kofi Asomaning,[1] Li Su,[1] John C. Wain,[7] Thomas J. Lynch,[5] Edward Giovannucci,[3,4,8] and David C. Christiani[1,4,5]

Departments of [1]Environmental Health, [2]Biostatistics, [3]Nutrition, and [4]Epidemiology, Harvard School of Public Health; [5]Department of Medicine, Massachusetts General Hospital, [6]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, [7]Thoracic Surgery Unit, Department of Surgery, Massachusetts General Hospital, and [8]Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

Abstract

Our previous analysis suggested that surgery season in the summer time and high vitamin D intake are associated with improved survival in early-stage non–small cell lung cancer (NSCLC) patients. Here, we investigated the associations of vitamin D receptor (*VDR*) polymorphisms of *Cdx-2 G>A*, *FokI C>T*, and *BsmI C>T* with overall survival (OS) and recurrence-free survival (RFS) in 373 early-stage NSCLC patients. The data were analyzed using log-rank test and Cox proportional hazards models. The median follow-up time was 71 months (range, 0.1-140 months), with 186 deaths and 127 recurrences. There was no association between *VDR* polymorphisms and survival, overall or among adenocarcinoma patients. Among squamous cell carcinoma (SCC) patients, the *G/A+A/A* genotype group of the *Cdx-2* polymorphism was associated with better OS: the 5-year OS rates were 41% [95% confidence interval (95% CI), 28-53] for the *G/G* and 55% (95% CI, 39-71) for the *G/A+A/A* genotypes, respectively (P = 0.04, log-rank test), with the adjusted hazard ratio of 0.56 (95% CI, 0.33-0.95) for *G/A+A/A* versus *G/G*. For the joint effects of the three polymorphisms, subjects with two or more ''protective'' alleles have better OS among SCC patients, with the adjusted hazard ratios of 0.20 (95% CI, 0.09-0.48), 0.40 (95% CI, 0.19-0.87), and 0.43 (95% CI, 0.19-0.97), respectively, for subjects with two, three, and four or more ''protective'' alleles when compared with subjects with zero or one ''protective'' allele (P_{trend} = 0.71). Similar associations were found in haplotype analysis and for RFS among SCC patients. In conclusion, *VDR* polymorphisms may be associated with improved survival among SCC patients of early-stage NSCLC. (Cancer Epidemiol Biomarkers Prev 2006;15(11):2239–45)

Introduction

The vitamin D endocrine system is involved in a wide variety of biological processes, including bone metabolism, modulation of the immune response, and regulation of cell proliferation and differentiation (1). Recent animal and epidemiologic studies have suggested that vitamin D may have anticancer benefits, including against progression and metastasis, in a wide spectrum of cancers, including lung cancer (2-4). The hormonal activity of vitamin D is mediated by the vitamin D receptor (VDR), a member of the steroid/retinoid receptor superfamily of nuclear receptors, which has been found in numerous tissues in different organs. The VDRs are intracellular polypeptides of 50 to 60 kDa that specifically bind $1,25(OH)_2D$ and interact with target cell nuclei to produce a variety of biological effects. Vitamin D exerts its potential tumor-suppressive functions by binding to VDR within cells. The VDR then interacts with specific regions of the DNA in cells and triggers changes in the activity of genes involved in cell division, cell adhesion, and cellular function (2).

The human *VDR* gene contains 11 exons and spans ~75 kb, and several polymorphisms are ''functional'' by *in vitro* studies. The *Cdx-2 G>A* polymorphism (rs11568820) is located within the Cdx-2-binding site in the *VDR* gene promoter, with the *A* allele being associated with significantly higher VDR transcriptional activity than the *G* allele (5, 6). The *FokI C>T* polymorphism (rs10735810) is in the translational start site of *VDR* and has distinct structural consequences for the VDR, with the *T* allele (also called ''*f*'') being less efficient in exerting $1,25(OH)_2D$ effects than the *C* allele (also called ''*F*''; ref. 7). The *BsmI C>T* polymorphism (rs1544410) is located in intron 8 at the 3′ end of *VDR* and is in high linkage disequilibrium with several other reported polymorphisms, including *ApaI*, *TaqI*, and *poly-A* (8, 9), with the *T* allele (also called ''*B*'') being associated with increased *VDR* mRNA expression and increased serum levels of $1,25(OH)_2D$ compared with the *C* allele (also called ''*b*''; refs. 10, 11). Polymorphisms of *VDR* have been associated with metastasis, recurrence, treatment response, or prognosis of breast cancer (12), malignant melanoma (13), prostate cancer (14, 15), and colorectal cancer (16); however, the results are not consistent in different studies, with little reproducibility from study to study.

Our previous data suggested that high vitamin D levels (the joint effects of summer surgery season and high recent vitamin D intake) at the time of treatment initiation may be associated with improved survival of early-stage non–small cell lung cancer (NSCLC) patients (17). Based on the ''functional'' data of *VDR* polymorphisms, we hypothesized that the variant *A* allele of the *Cdx-2* polymorphism, the wild-type *C* allele of the *FokI* polymorphism, and the variant *T* allele of the *BsmI* polymorphism, which are thought to be associated with higher *VDR* transcriptional activities or higher vitamin D levels, are associated with improved survival of NSCLC and have joint effects with vitamin D status at the time of treatment initiation. Furthermore, we hypothesized that the joint effects of the three polymorphisms are stronger than the individual effect. Because histologic difference has been reported for the effects

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Requests for reprints: Wei Zhou, Occupational Health Program, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115. Phone: 617-432-1641. Fax: 617-432-6981. E-mail: wzhou@hsph.harvard.edu

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of $1,25(OH)_2D$ and *VDR* on lung cancer (18), we investigated the above a priori hypotheses in our cohort of early-stage NSCLC patients, overall and in different histologic cell types of NSCLC.

Materials and Methods

Study Population. The study population is based on an ongoing hospital-based case follow-up study initiated in 1992, with details described previously (17). In brief, all participants were histologically confirmed and consecutively recruited incident early-stage (stages IA to IIB) NSCLC patients from Massachusetts General Hospital (MGH; Boston, MA). Histologic review of all samples was done by pathologists at the MGH Pathology Department at the time of surgical resection. Fresh tumor tissue was obtained at the time of surgery and cut into slides, and representative H&E-stained sections were examined to evaluate the pathologic type of the tumor. We limited the analysis to those patients recruited before year 2000, ensuring a follow-up time of at least 4 years (outcome data were collected in year 2004). All of the patients had their surgical resection at MGH, with outpatient records available and detailed demographic information collected by interviewer-administered questionnaires. More than 85% of eligible patients participated in this study, and 96% were Caucasians.

Among the 456 eligible subjects, 83 patients were excluded because they did not have adequate blood or DNA samples for genotyping, leaving a total of 373 subjects. There were no statistically significant differences in patient characteristics between those with and those without genotype data. Characteristics examined include age, gender, histologic cell type, stage, radiotherapy, and chemotherapy after surgery. There were also no differences in overall survival (OS) between the groups, neither. All patients had surgical resection as the initial treatment, including wedge (24%), lobectomy (62%), bilobectomy (2%), pneumonectomy (6%), sleeve lobectomy (3%), and lobectomy plus wedge (1%). Additionally, 30 (8%) patients received postoperative radiation and/or adjuvant chemotherapy. The study was approved by the Human Subjects Committee of MGH and Harvard School of Public Health, and informed consent was collected from each participant.

***VDR* Genotyping.** DNA was extracted from peripheral blood samples using the Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN). The three *VDR* polymorphisms were genotyped by the 5′ nuclease assay (Taqman) using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primers, probes, and reaction conditions were available upon request. Genotyping was done by laboratory personnel blinded to case-control status, and a random 5% of the samples were repeated to validate genotyping procedures. Two authors reviewed independently all genotyping results.

Outcome Data Collection. OS and recurrence-free survival (RFS) were the end points in this study. OS was calculated from the date of surgery to the date of last follow-up or death from any cause. Dates of death were obtained and cross-checked using at least one of the following four methods: (*a*) inpatient and outpatient medical records, (*b*) MGH tumor registry, (*c*) Social Security Death Index, and (*d*) confirmation with the patient's primary care physician and/or family. Patients who were not deceased were censored at the last date they were known to be alive based on date of last contact. This date was verified by methods (*b*) and/or (*d*) as described above. Median follow-up time for this cohort was computed among alive subjects. RFS was defined as the time from the date of surgery to the first date of recurrence of cancer or death from any cause or the date of last follow-up. Date of recurrence was obtained by reviewing the hospital and outpatient records of all patients. For those 14% of patients who had their primary follow-up outside of the MGH system, we contacted the primary physician to obtain follow-up information.

Statistical Analysis. Demographic, clinical, and histologic information was compared across different genotype groups using Pearson χ^2 tests (for categorical variables) and Kruskal-Wallis tests (for continuous variables), where appropriate. Hardy-Weinberg equilibrium of each polymorphism was tested using the χ^2 test, and detection of linkage disequilibrium between the three polymorphisms was based on Lewontin's D′. The associations between individual *VDR* polymorphisms (or the total number of ''protective'' alleles from the three polymorphisms) and OS and RFS were estimated using the method of Kaplan and Meier and assessed using the log-rank test. Cox proportional hazards models were used as our primary analyses, controlling for multiple possible covariates simultaneously, including age, gender, stage, smoking status, and surgery season, where appropriate, with each covariate, including genotype groups or number of ''protective'' alleles represented by indicator variables. In addition to the overall analysis, we also investigated the effects of *VDR* polymorphisms on NSCLC in different histologic cell types [i.e., adenocarcinoma and squamous cell carcinoma (SCC), respectively].

Haplotype frequencies and individual haplotypes were generated using the expectation-maximization algorithm, which reconstruct individual probabilities for individual phasing accuracy based on unphased genotype data, as well as estimates on the overall haplotype frequencies and their SEs (19-22). The associations between *VDR* haplotypes and survival were estimated using the ''expectation substitution'' approach (19, 20, 22), which treats expected haplotype scores (calculated under additive model) as observed covariates in a standard Cox proportional hazards model, instead of assigning each subject with the most likely haplotype pair. All reported *P*s are from two-sided tests. *P*s < 0.05 were considered statistically significant. All analyses were done using Statistical Analysis System software version 9 (SAS Institute, Cary, NC).

Results

Demographic, Stage, and Treatment Characteristics. Among the 373 NSCLC patients, median age was 69 years (range, 31-89), 49% were females and 39% were current smokers. Adenocarcinoma, SCC, large cell, and bronchioloalveolar carcinoma represented 48%, 29%, 5%, and 12% of the tumor histologies, respectively. Fifty-two percent were stage IA, 29% were stage IB, 4% were stage IIA, and 15% were stage IIB. There were 186 deaths and 127 recurrences, including 98 deaths that occurred in the absence of reported recurrence and 39 recurrences without death. The median follow-up time for the 225 patients still alive was 71 months (range, 0.1-140 months).

Detailed demographic, clinical, and treatment information by different histologic cell types is presented in Table 1. Compared with adenocarcinoma, SCC patients had lower frequencies of females (37% versus 54%), stage IA (37% versus 58%), and never smokers (1% versus 9%). Surgery seasons were similar between different histologic cell types. Because of the small sample sizes of bronchioloalveolar carcinoma and large cell carcinoma, we limited the genotype survival analysis to adenocarcinoma and SCC in the subgroup analysis.

Genotype Information. The three *VDR* polymorphisms were all in Hardy-Weinberg equilibrium. The variant allele frequency was 0.27 for the *Cdx-2* polymorphism (the *A* allele), 0.40 for the *FokI* polymorphism (the *T* allele), and 0.44 for the

Table 1. Demographic, treatment, and genotype characteristics in different histologic cell types of lung cancer patients

Characteristic	Histologic cell types				
	All patients (N = 373), n (%)	Adenocarcinoma (N = 180), n (%)	Squamous (N = 108), n (%)	Others (N = 85), n (%)	P
Age (y)*	69 (31-89)	69 (35-86)	70 (47-89)	69 (31-88)	0.32
Gender, female	182 (49)	97 (54)	40 (37)	45 (53)	0.01
Smoking status					
Never	26 (7)	17 (9)	1 (1)	8 (9)	<0.001
Ex-smokers	202 (54)	89 (49)	54 (50)	59 (69)	
Current smokers	145 (39)	74 (41)	53 (49)	18 (21)	
Clinical stage					
IA	194 (52)	104 (58)	40 (37)	50 (59)	<0.01
IB	109 (29)	43 (24)	41 (38)	25 (29)	
IIA	14 (4)	10 (5)	3	1 (1)	
IIB	56 (15)	23 (13)	24 (22)	9 (11)	
Surgery type					
Wedge	90 (24)	50 (28)	23 (21)	17 (20)	<0.001
Lobectomy	232 (62)	117 (65)	57 (53)	58 (68)	
Others	51 (14)	13 (7)	28 (26)	10 (12)	
Radiation/chemotherapy	30 (8)	12 (7)	11 (10)	7 (8)	0.57
Surgery season†					
Winter	107 (29)	53 (29)	32 (30)	22 (26)	0.98
Spring/fall	143 (38)	68 (38)	41 (38)	34 (40)	
Summer	123 (33)	59 (33)	35 (32)	29 (34)	
VDR Cdx-2 G>A polymorphism					
G/G	201 (54)	87 (48)	66 (61)	48 (57)	0.20
G/A	144 (39)	78 (44)	37 (34)	29 (34)	
A/A	28 (7)	15 (8)	5 (5)	8 (9)	
VDR FokI C>T polymorphism					
C/C	141 (38)	68 (38)	44 (41)	29 (34)	0.63
C/T	170 (45)	78 (43)	48 (44)	44 (52)	
T/T	62 (17)	34 (19)	16 (15)	12 (14)	
VDR BsmI C>T polymorphism					
C/C	119 (32)	54 (30)	33 (31)	32 (38)	0.54
C/T	182 (49)	92 (51)	50 (46)	40 (47)	
T/T	72 (19)	34 (19)	25 (23)	13 (15)	

NOTE: Among the population, 340 patients were Caucasians, 12 patients were non-Caucasians, and 21 patients were missing the race information. Except for age, all of the other values are tested by χ^2 test or Fisher's exact test.
*Median (range), tested by Kruskal-Wallis test.
† Seasons include winter (the low sun exposure months of November-February), spring/fall (the intermediate sun exposure months of March, April, September, and October), and summer (the high sun exposure months of May-August).

BsmI polymorphism (the *T* allele). The three *VDR* polymorphisms were not in linkage disequilibrium, with the D′ of *Cdx-2_A-FokI_T* of 0.10, *Cdx-2_A-BsmI_C* of 0.09, and *FokI_T-BsmI_C* of 0.03. None of the evaluated genotypes was associated with histologic cell types (Table 1), age, gender, stage, smoking, treatment, or surgery season (data not shown).

Individual *VDR* Polymorphisms and OS and RFS. The results of log-rank test and Cox proportional hazards models for *VDR* polymorphisms are presented in Table 2 (for OS). No association was found between the *VDR Cdx-2* polymorphism and OS in the whole population or in adenocarcinoma patients. For the *Cdx-2* polymorphism in SCC, due to the small number of patients with *A/A* genotype (n = 5) and similar survival effects with the *G/A* genotype (Table 2), we combined the *G/A* and *A/A* genotype groups in the analysis. The *VDR Cdx-2* polymorphism was associated with statistically significant better survival among SCC patients. The 5-year OS survival rates [95% confidence interval (95% CI)] of the *Cdx-2* polymorphisms were 41% (28-53) for the *G/G* genotype and 55% (39-71) for the *G/A+A/A* genotypes, respectively (P = 0.04, log-rank test; Fig. 1A). In the Cox proportional hazards model, the adjusted hazard ratio (AHR) was 0.56 (95% CI, 0.33-0.95) for the *G/A+A/A* versus *G/G*. Similar improved survival effects of the *Cdx-2* polymorphism were found for RFS in SCC: the 5-year RFS survival rates were 34% (95% CI, 22-46) for the *G/G* genotype and 50% (95% CI, 34-66) for the *G/A+A/A* genotypes, respectively (P = 0.03, log-rank test; Fig. 1B), with the AHR of 0.57 (95% CI, 0.34-0.94) for *G/A+A/A* versus *G/G*. Further analysis showed that the interaction between *Cdx-2* polymorphism (*G/A+A/A* versus *G/G*) and histologic cell types (SCC versus adenocarcinoma) was statistically significant (P = 0.02 for both OS and RFS) in the Cox proportional hazards model. Similar results were observed in the Caucasian-only analysis.

In the secondary analysis, we investigated the joint effects between surgery season or dietary vitamin D intake and *Cdx-2* polymorphism. Similar to the results of *Cdx-2* polymorphism alone, the joint effects of summer season and *G/A+A/A* genotype and of higher vitamin D intake and *G/A+A/A* genotype were associated with better OS or RFS among SCC patients, respectively, although the trend test was not always statistically significant (Table 3).

There was no statistically significant association between *VDR FokI* or *BsmI* polymorphism and OS (Table 2) or RFS, overall or by histologic cell types. Similar results were found when the three polymorphisms were included in the same model in the Cox proportional hazards models.

Combined *VDR* Polymorphisms and OS and RFS. We also analyzed the joint effects of the three *VDR* polymorphisms based on the ''function'' of each polymorphism and total number of ''protective'' alleles. For the purposes of this analysis, the *Cdx-2 G/G*, *FokI T/T*, and *BsmI C/C* genotypes, which are associated with lower VDR transcriptional activity, lower VDR mRNA levels, or lower circulating vitamin D levels, were assigned as having zero ''protective'' allele; conversely, the *Cdx-2 A/A*, *FokI C/C*, and *BsmI T/T* genotypes were assigned as having two ''protective'' alleles. A total of four groups was generated: group 1, the reference group, with

Table 2. Five-year OS rates (95% CI) and AHRs (95% CI) for individual *VDR* polymorphism

Genotypes	All patients				Adenocarcinoma				SCC			
	n	Death	5-year survival	AHR	*n*	Death	5-year survival	AHR	*n*	Death	5-year survival	AHR
VDR Cdx-2 G>A polymorphism												
G/G	201	105	54% (47-61%)	1.00	87	39	64% (53-74%)	1.00	66	44	41% (28-53%)	1.00
G/A	144	69	58% (50-67%)	0.84 (0.62-1.14)	78	39	62% (51-74%)	1.02 (0.64-1.62)	37	19	55% (38-72%)	0.55 (0.32-0.95)
A/A	28	12	53% (33-73%)	0.92 (0.50-1.68)	15	9	47% (21-72%)	1.71 (0.81-3.60)	5	2	60% (17-100%)	0.69 (0.16-2.96)
*P**			0.40	0.37			0.57	0.33			0.12	0.05
G/A+A/A	172	81	59% (51-67%)	0.85 (0.63-1.14)	93	48	60% (50-70%)	1.11 (0.72-1.73)	42	21	55% (39-71%)	0.56 (0.33-0.95)
VDR FokI C>T polymorphism												
C/C	141	76	54% (45-63%)	1.00	68	30	62% (50-75%)	1.00	44	30	40% (24-56%)	1.00
C/T	170	79	59% (52-67%)	0.84 (0.61-1.16)	78	41	59% (47-70%)	1.13 (0.67-1.88)	48	25	52% (37-66%)	0.75 (0.44-1.28)
T/T	62	31	55% (42-68%)	1.13 (0.74-1.74)	34	16	62% (45-79%)	1.31 (0.70-2.46)	16	10	45% (19-71%)	0.98 (0.47-2.03)
*P**			0.47	0.93			0.75	0.40			0.49	0.64
VDR BsmI C>T polymorphism												
C/C	119	61	52% (42-62%)	1.00	54	25	59% (45-73%)	1.00	33	22	38% (21-55%)	1.00
C/T	182	89	64% (57-72%)	0.83 (0.59-1.16)	92	44	67% (57-77%)	0.88 (0.52-1.48)	50	26	59% (44-74%)	0.59 (0.33-1.05)
T/T	72	40	44% (32-56%)	1.35 (0.90-2.03)	34	18	47% (29-66%)	1.52 (0.81-2.83)	25	17	31% (11-51%)	1.18 (0.62-2.23)
*P**			0.20	0.31			0.63	0.30			0.08	0.83

NOTE: AHRs, adjusting for age, gender, smoking status, stage, and surgery season, where appropriate.
*The *P*s were for log-rank test or trend test in Cox proportional hazards models, respectively.

zero or one ''protective'' allele; group 2, with two ''protective'' alleles; group 3, with three ''protective'' alleles; and group 4, with four or more ''protective'' alleles. Results of log-rank test and Cox proportional hazards models are shown in Table 4. There was no statistically significant association between the joint polymorphisms and OS or RFS, overall or in adenocarcinoma patients. Among SCC patients, those with two or more ''protective'' alleles have statistically better survival when compared with the reference group in Cox proportional hazards models; however, no clear ''dose-response'' effect was observed. For OS, the AHRs were 0.20 (95% CI, 0.09-0.48), 0.40 (95% CI, 0.19-0.87), and 0.43 (95% CI, 0.19-0.97) for subjects with two, three, and four or more ''protective'' alleles (P_{trend} = 0.71); for RFS, the corresponding AHRs were 0.23 (95% CI, 0.10-0.51), 0.43 (95% CI, 0.20-0.89), and 0.44 (95% CI, 0.20-0.97), respectively (P_{trend} = 0.70). Similar results were observed in the Caucasian-only analysis.

***VDR* Haplotypes and OS and RFS.** Finally, we investigated the associations between *VDR* haplotypes and OS and RFS in Cox proportional hazards models. A total of eight haplotypes was generated (Table 5). Among these, the *G-T-C* haplotype (15%, *Cdx-2-FokI-BsmI*) was hypothesized to be associated with the lowest *VDR* expression or VDR function levels based on the ''functional'' data and was therefore used as reference group in the analysis. Similar to the results of genotype analysis, there was no statistically significant association between *VDR* haplotypes and OS or RFS, overall or among adenocarcinoma patients. Among SCC patients, the majority of the haplotypes were associated with lower AHRs when compared with the *G-T-C* haplotype, and the *A-C-T* haplotype (6% of frequency) that was hypothesized to be associated with the best function was associated with better OS (statistically significant) and RFS (borderline significant): the AHR of *A-C-T* versus *G-T-C* was 0.22 (95% CI, 0.05-0.96; *P* = 0.04) for OS and 0.27 (95% CI, 0.07-1.12; *P* = 0.07) for RFS, respectively (Table 5). Similar results were also observed in the Caucasian-only analysis.

Discussion

Vitamin D has potential antiproliferative, anti-invasive, and proapoptotic properties against a wide spectrum of cancers and may be beneficial for the survival of cancers, including lung cancer (2, 4, 17). In this study, we investigated the associations between three ''functional'' *VDR* polymorphisms of *Cdx-2 G>A*, *FokI C>T*, and *BsmI C>T* and the survival of early-stage NSCLC patients and found that the *G/A+A/A* genotypes of the *Cdx-2* polymorphism, the combined ''protective'' genotypes, and the *A-C-T* haplotype (*Cdx-2-FokI-BsmI*), which are associated with higher VDR expressions, transcription, or higher vitamin D levels, were all associated with better OS and RFS among SCC lung cancer patients. The results were also confirmed by the joint effects of surgery season, dietary

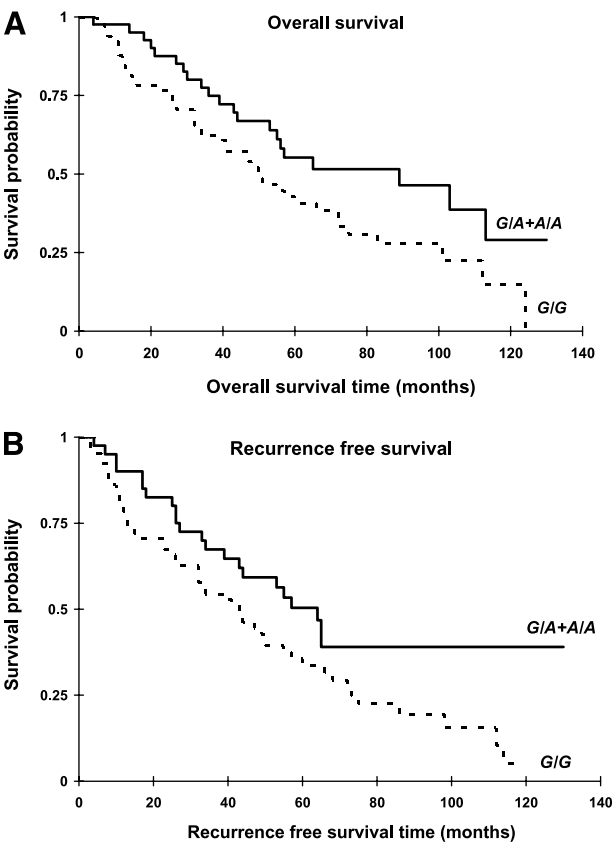

Figure 1. Kaplan-Meier curves of *VDR Cdx-2 G>A* polymorphism in OS (*P* = 0.04, log-rank test; **A**) and RFS (*P* = 0.03, log-rank test; **B**) among SCC patients, where the *G/A* and *A/A* genotypes are combined. Log-rank test was based on the full data of SCC patients.

Table 3. AHRs (95% CI) for the joint effects between surgery season or dietary vitamin D intake and *VDR Cdx-2* polymorphism

VDR groups	All patients			Adenocarcinoma			SCC		
	n	Events	AHR	*n*	Events	AHR	*n*	Events	AHR
Joint effects of surgery season and *VDR Cdx-2 G>A* polymorphism									
OS									
Winter + *G/G*	60	33	1.00	30	15	1.00	20	13	1.00
Others	257	125	0.99 (0.67-1.46)	121	54	1.01 (0.56-1.81)	73	44	0.83 (0.43-1.60)
Summer + *A−**	56	28	0.90 (0.54-1.50)	29	18	1.43 (0.70-2.92)	15	8	0.52 (0.21-1.27)
RFS									
Winter + *G/G*	60	40	1.00	30	19	1.00	20	16	1.00
Others	257	152	1.00 (0.70-1.42)	121	70	1.10 (0.65-1.86)	73	48	0.59 (0.32-1.10)
Summer + *A−**	56	33	0.90 (0.57-1.44)	29	21	1.38 (0.72-2.66)	15	8	0.35 (0.15-0.85)
Joint effects of dietary vitamin D intake and *VDR Cdx-2 G>A* polymorphism†									
OS									
Low intake + *G/G*	73	33	1.00	33	12	1.00	23	14	1.00
Others	148	82	1.06 (0.70-1.61)	82	43	1.23 (0.63-2.41)	39	25	0.82 (0.41-1.66)
High intake + *A−**	63	27	0.78 (0.47-1.32)	28	12	1.04 (0.46-2.35)	20	10	0.44 (0.19-1.04)
RFS									
Low intake + *G/G*	73	42	1.00	33	19	1.00	23	15	1.00
Others	148	90	0.95 (0.65-1.39)	82	48	0.92 (0.52-1.61)	39	26	1.01 (0.51-2.02)
High intake + *A−**	63	37	1.02 (0.65-1.61)	28	16	0.99 (0.50-1.96)	20	11	0.57 (0.25-1.28)

NOTE: AHRs, adjusting for age, gender, smoking status, and stage, where appropriate, with winter season + *G/G* genotype or low vitamin D intake + *G/G* genotype as the reference group, respectively.
**G/A+A/A* genotype.
† Dietary data were available for 284 patients in this study, with low and high dietary vitamin D intake stratified by mean vitamin D intake (273 IU/d) in the whole population.

vitamin D intake, and *Cdx-2* polymorphism. The findings were observed only for SCC and thus could be due to chance. However, the consistency of the findings for SCC observed for the individual genotypes, combined analysis, haplotype analysis, and joint analysis with surgery season and vitamin D intake argues against a chance finding.

The associations between VDR and cancer metastases and survival are supported by animal models. Using cells isolated from VDR knockout and wild-type mice, both normal and transformed mammary cells derived from wild-type mice are growth inhibited by $1,25(OH)_2D$. However, cells derived from VDR knockout mice are completely unresponsive to $1,25(OH)_2D$ (23). An inverse relationship between VDR levels and both colonic hyperproliferation and oxidative stress has been found in a mouse model (24). In humans, significant higher expression levels of VDR have been found in lung cancer tissues than in normal tissues (25, 26), and VDR activity may be enhanced by β-catenin (27), which makes cells in the tumor mass more adherent to each other and reduces the likelihood of mobilization of large numbers of malignant cells into the lymphatic or blood circulation.

VDR is a highly polymorphic gene. Although functional significances between different genotypes of *VDR* polymorphisms have been reported based on *in vitro* studies, the associations between *VDR* polymorphisms and cancer survival are not consistent across studies, and there is no report on the association between *VDR* genotypes or haplotypes and lung cancer survival (28). The *BsmI C>T* polymorphism is the most widely studied *VDR* polymorphism in cancer prognosis. The *T/T* genotype may protect the development of metastases among breast cancer patient (29, 30) and may affect the prognosis of rectal cancer by influencing erbB-2 oncogene expression, although it alone did not influence survival (16). The *BsmI T* allele has also been associated with reduced acute

Table 4. Five-year survival rates (95% CI) and AHRs (95% CI) for combined *VDR* polymorphisms

VDR groups	All patients				Adenocarcinoma				SCC			
	n	Events	5-year survival	AHR	*n*	Events	5-year survival	AHR	*n*	Events	5-year survival	AHR
OS												
Group 1	55	27	52% (38-66%)	1.00	24	9	64% (44-84%)	1.00	15	12	25% (2-48%)	1.00
Group 2	123	60	61% (52-71%)	0.71 (0.45-1.13)	61	30	64% (51-76%)	0.90 (0.41-1.97)	35	18	59% (42-75%)	0.20 (0.09-0.48)
Group 3	109	55	53% (43-63%)	0.86 (0.53-1.38)	50	26	58% (43-72%)	0.98 (0.43-2.22)	35	19	40% (22-59%)	0.40 (0.19-0.87)
Group 4	86	44	55% (44-66%)	0.90 (0.55-1.46)	45	22	59% (44-75%)	1.11 (0.50-2.47)	23	16	44% (22-65%)	0.43 (0.19-0.97)
*P**			0.63	0.76			0.63	0.51			0.18	0.71
RFS												
Group 1	55	35	40% (26-54%)	1.00	24	14	45% (24-66%)	1.00	15	13	20% (0-40%)	1.00
Group 2	123	71	54% (44-63%)	0.69 (0.46-1.04)	61	36	53% (40-66%)	0.76 (0.40-1.46)	35	21	51% (34-67%)	0.23 (0.10-0.51)
Group 3	109	66	42% (32-52%)	0.88 (0.58-1.33)	50	32	43% (28-57%)	0.87 (0.44-1.71)	35	22	34% (16-53%)	0.43 (0.20-0.89)
Group 4	86	53	42% (31-53%)	0.92 (0.60-1.41)	45	28	41% (26-57%)	1.01 (0.52-1.97)	23	16	39% (18-60%)	0.44 (0.20-0.97)
*P**			0.46	0.62			0.78	0.58			0.26	0.70

NOTE: The joint effects of the three *VDR* polymorphisms were based on the total number of ''protective'' alleles, where the *Cdx-2 G/G* genotype, the *FokI T/T* genotype, and *BsmI C/C* genotype have zero ''protective'' allele. For the joint groups, group 1, the reference group, with zero or one ''protective'' allele; group 2, with two ''protective'' alleles; group 3, with three ''protective'' alleles; and group 4, with four or more ''protective'' alleles. Covariates included in the Cox models are age, gender, smoking status, stage, and surgery season, where appropriate.
*The *P*s were for log-rank test or trend test in Cox proportional hazards models, respectively.

Table 5. *VDR* haplotype frequencies and AHRs (95% CI) in Cox proportional hazards model

Haplotype	All patients				Adenocarcinoma		SCC	
	n	SE	AHR for OS	AHR for RFS	AHR for OS	AHR for RFS	AHR for OS	AHR for RFS
G-T-C	15%	1%	1.00	1.00	1.00	1.00	1.00	1.00
G-C-C	25%	2%	0.96 (0.61-1.50)	0.90 (0.60-1.35)	0.85 (0.39-1.89)	0.71 (0.37-1.35)	0.66 (0.29-1.51)	0.72 (0.33-1.57)
G-C-T	21%	1%	1.16 (0.78-1.73)	1.04 (0.72-1.49)	1.01 (0.50-2.07)	0.83 (0.47-1.48)	0.97 (0.47-1.98)	0.91 (0.46-1.78)
G-T-T	12%	1%	0.91 (0.49-1.70)	0.79 (0.45-1.38)	1.04 (0.39-2.82)	0.70 (0.30-1.65)	0.58 (0.20-1.69)	0.55 (0.20-1.51)
A-C-C	9%	1%	0.82 (0.45-1.50)	0.97 (0.58-1.61)	0.94 (0.37-2.38)	0.89 (0.41-1.93)	0.62 (0.22-1.73)	0.63 (0.25-1.61)
A-T-C	7%	1%	0.93 (0.46-1.89)	0.80 (0.42-1.54)	1.09 (0.36-3.32)	0.71 (0.28-1.84)	0.29 (0.05-1.81)	0.24 (0.04-1.41)
A-C-T	6%	1%	0.77 (0.38-1.55)	0.79 (0.44-1.44)	1.17 (0.44-3.11)	0.92 (0.42-2.01)	0.22 (0.05-0.96)	0.27 (0.07-1.12)
A-T-T	5%	1%	1.45 (0.61-3.42)	1.12 (0.51-2.48)	1.89 (0.60-5.96)	1.37 (0.48-3.92)	1.20 (0.19-7.77)	1.32 (0.22-7.86)

NOTE: Haplotype frequencies were presented by the sequences of *Cdx-2 G>A*, *FokI C>T*, and *BsmI C>T*. The AHRs were collected from Cox proportional hazards model, where the expected haplotype scores (calculated under additive model) were treated as a continuous variable (range, 0-2), with the *G-T-C* haplotype as the reference group. Covariates included age, gender, smoking status, stage, and surgery season, where appropriate.

graft-versus-host disease when present in the patient's genotype (31) and protected against recurrence of locally advanced prostate cancer (15). In a small study with 191 mostly Caucasian prostate cancer patients, the *C* allele of the *FokI C>T* polymorphism was associated with an increased risk of aggressive prostate cancer and could therefore be associated with worse prognosis (14). We did not observe the association between the individual *BsmI* or *FokI* polymorphism and NSCLC survival, whereas the joint effects of the three polymorphisms and haplotype analysis suggested better survival is associated with the ''protective'' alleles of the three polymorphisms, although no ''dose-response'' relationship was observed for the number of ''protective'' alleles (Table 4). Therefore, all of the three investigated *VDR* polymorphisms may contribute to better survival of NSCLC patients, with the *Cdx-2* polymorphism having the strongest effect.

In this study, the association with *VDR* polymorphisms was observed among SCC patients whereas not among adenocarcinoma patients. In our previous analysis, we also observed a slightly stronger effect of surgery season and/or vitamin D intake on NSCLC survival among SCC patients than adenocarcinoma patients, although the difference was not statistically significant (data not shown). *In vitro* studies have suggested that vitamin D inhibits growth of a lung SCC cell line whereas not adenocarcinoma cell line, and the mRNA levels of *VDR* were much higher in the SCC cell line than the adenocarcinoma cell line (18). The mechanism for this histologic difference of *VDR* polymorphisms may be associated with the effect of retinoic acid, retinoic acid receptors (RAR), or retinoid X receptors (RXR). VDR is a member of the steroid/retinoid receptor superfamily of nuclear receptors. In the cell, $1,25(OH)_2D$ binds to the VDR, and the VDR*$1,25(OH)_2D$ complex then interacts with the RXR to form a $1,25(OH)_2D$*VDR*RXR heterodimer complex, which then interacts with vitamin D–responsive elements. RARs also function as heterodimers with RXR proteins (2). Because both retinol and vitamin D require RXR proteins for their actions, high doses of retinol may antagonize vitamin D actions (32, 33). In lung cancer, the expression levels of *RARβ* gene have been found to be highly expressed in adenocarcinoma cell lines whereas not detectable in SCC cell lines (34). Retinoic acid has also shown its growth-inhibitory activity when applied to the adenocarcinoma cell line of human lung and stomach whereas is inactive on the esophageal SCC cell lines (35). Therefore, in adenocarcinoma patients, the effect of *VDR* may be overwhelmed by the highly expressed RAR levels, which may be one reason that we did not observe a significant effect of *VDR* polymorphism on survival among adenocarcinoma patients.

There are several limitations for this study. (*a*) Sample size. Although this is a case follow-up study with moderate sample size, the numbers are small in the SCC subgroup and for the gene-environment and gene-gene joint analyses. (*b*) Selection of candidate polymorphisms. We investigated three ''functional'' *VDR* polymorphisms based on *in vitro* data, and it is possible that these polymorphisms may not be truly functional. We did not investigate the other reported *VDR* polymorphisms, including *ApaI, TaqI*, and *poly-A*, which may introduce bias in the results, especially in the haplotype analysis. However, adding more polymorphisms in the results may also introduce multiple comparisons in the genotype analysis. Given the high linkage disequilibrium of these polymorphisms with the *BsmI* polymorphism, their association with NSCLC survival will be similar to the results of the *BsmI* polymorphism. (*c*) Missing data. In our population, 83 patients were excluded because they did not have adequate blood or DNA samples for genotyping. However, the distributions of demographic, histologic, treatment, and survival characteristics were very similar between patients with and without genotype information. (*d*) Survival data collection. In this population, recurrence data were collected retrospectively and patients were not on a prescribed surveillance schedule. We attempted to contact local physicians whenever patients were followed outside of the MGH system (14%). We collected the vital status data for each patient. However, we could not distinguish between the death from lung cancer and the death from other causes. Because the 5-year OS rates in this population were 65%, 53%, 34%, and 38%, respectively, for stages IA to IIB, the vast majority of these patients likely died from lung cancer. (*e*) We did not have complete information on either performance status or weight loss in our early-stage patients. However, in a random sample of 100 patients who had performance status data available, surgical resectability is a reasonable surrogate measure for good performance status. We also had very incomplete weight loss information, with most of the available information dichotomized into weight loss present or absent. Our preliminary data in a random sample of 40 stage IA patients and 10 stage II patients suggested that disease stage (in early-stage patients) may be crudely correlated with weight loss. In our models, we do adjust for disease stage. These factors are unlikely to be associated with *VDR* polymorphisms and will not introduce systematic bias in the results. (*f*) We lack information on the serum levels of vitamin D or other genes that are involved in vitamin D metabolism, which may limit the generalizability of our results.

In conclusion, this is the first study suggesting that *VDR* polymorphisms may be associated with improved survival in early-stage NSCLC patients. Our results suggest that the *A* allele (*G/A+A/A* genotypes) of the *Cdx-2* polymorphism, the combined ''protective'' genotypes of the *Cdx-2*, *FokI*, and *BsmI* polymorphisms, and the *A-C-T* haplotype (*Cdx-2-FokI-BsmI*) are associated with better OS and RFS among SCC patients whereas not among adenocarcinoma patients. The results are consistent with our previous findings on surgery season and

vitamin D intake, further suggesting that vitamin D may be associated with improved survival in early-stage NSCLC patients. These results need to be confirmed by independent prospective studies or clinical trials.

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