Research Article

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Vitamin D Receptor Gene Haplotypes and Polymorphisms and Risk of Breast Cancer: A Nested Case–Control Study

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Abstract

Background: Observational and experimental studies suggest that vitamin D may influence breast cancer etiology. Most known effects of vitamin D are mediated via the vitamin D receptor (VDR). Few polymorphisms in the *VDR* gene have been well studied in relation to breast cancer risk and results have been inconsistent.

Methods: We investigated *VDR* polymorphisms and haplotypes in relation to breast cancer risk by genotyping 26 single nucleotide polymorphisms (SNP) that (i) had known/suspected impact on VDR function, (ii) were tagging SNPs for the three *VDR* haplotype blocks among whites, or (iii) were previously associated with breast cancer risk. We estimated odds ratios (OR) and 95% confidence intervals (CI) in relation to breast cancer risk among 270 incident cases and 554 matched controls within the Agricultural Health Study cohort.

Results: In individual SNP analyses, homozygous carriers of the minor allele for rs2544038 had significantly increased breast cancer risk (OR = 1.5; 95% CI: 1.0–2.5) and homozygous carriers of the minor allele for rs11168287 had significantly decreased risk (OR = 0.6; 95% CI: 0.4–1.0). Carriers of the minor allele for rs2239181 exhibited marginally significant association with risk (OR = 1.4; 95% CI: 0.9–2.0). Haplotype analyses revealed three haplotype groups (blocks "A," "B," and "C"). Haplotype GTCATTTCCTA in block B was significantly associated with reduced risk (OR = 0.5; 95% CI: 0.3–0.9).

Conclusions: These results suggest that variation in *VDR* may be associated with breast cancer risk.

Impact: Our findings may help guide future research needed to define the role of vitamin D in breast cancer prevention. *Cancer Epidemiol Biomarkers Prev; 21(10); 1856–67. ©2012 AACR.*

Introduction

Evidence from observational and experimental studies suggests that vitamin D may influence breast cancer development. A growing number of epidemiologic studies have reported decreased risk of breast cancer associated with exposure to sunlight/UV radiation (1–14), which results in dermal synthesis of vitamin D and is the primary source of this vitamin for most people (15). A recent meta-analysis suggests a small inverse association between dietary vitamin D and breast cancer risk (16), although the literature on this association is inconsistent and may reflect the relatively small contribution of diet to circulating vitamin D levels in the general population (15). Serum vitamin D levels have been consistently associated with reduced risk of breast cancer in case–control studies (17, 18), but not in most nested case–control studies (17, 19). Additional evidence for the anticancer effects of vitamin D comes from *in vitro* studies showing that the biologically active form of vitamin D, 1,25-dihydroxyvitamin D, decreases proliferation and promotes differentiation and apoptosis in breast cancer cell lines (20–22).

Most of the known physiologic effects of vitamin D are mediated via binding of 1,25-dihydroxyvitamin D to the vitamin D receptor (VDR; ref. 23). The VDR, which belongs to the nuclear hormone receptor superfamily, is a transcription regulator expressed in almost all tissue, including normal breast tissue and most breast tumors (24). It regulates transcription of a range of genes, including ones involved in cellular growth, differentiation, apoptosis, angiogenesis, and metastasis (23, 25, 26). Importantly, experimental studies on mammary tumor cell lines from VDR-knockout mice show that VDR is necessary for induction of cell-cycle arrest and apoptosis in breast cancer cells by 1,25-dihydroxyvitamin D (27). Moreover, VDR-deficient mice show an enhanced susceptibility to tumorigenesis in the breast and other tissue (28). Taken together, these prior findings suggest the need

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to study the role of *VDR* polymorphisms in relation to breast cancer risk in humans.

VDR is encoded by a large gene located on chromosome 12cen-q12 (29) that contains 14 exons spanning approximately 75 kb (30, 31). Although the *VDR* gene has been well characterized and a large number of polymorphisms identified (32, 33), only a few of these have been previously examined in relation to breast cancer risk. Most have been tested with restriction enzymes and are of unknown or uncertain functional effect, although one, *FokI*, has been shown to alter the length and activity of the protein product (34). In previous linkage disequilibrium (LD) analyses of single nucleotide polymorphisms (SNP) in *VDR*, it has been shown that the SNPs constitute 3 LD blocks (32). *FokI* is not in LD with any other SNPs (32). The other commonly studied SNPs, *BsmI*, *ApaI*, and *TaqI*, are all in the same LD block and predict only 38% of the SNPs in that block with $R^2 > 0.8$, capturing no information about SNPs in the other 2 blocks in *VDR* among whites (32). Therefore, studies that genotyped only these SNPs captured no information on a large fraction of common SNPs in *VDR*. This may partially explain why epidemiologic studies to date of *VDR* polymorphisms and breast cancer risk have reported largely inconsistent results. Specifically, studies of *FokI* in relation to breast cancer risk have reported some evidence of increased risk among *ff* carriers (35–39), decreased risk among *ff* carriers (40), or no association (3, 41–45). Similarly mixed results have been observed in relation to breast cancer risk for other polymorphisms, including *BsmI*, with associations in some studies (39, 43, 45–47) but not others (35, 37–40, 44); for *ApaI*, with some findings positive (42, 48) and others null (40, 44); and for *TaqI*, with associations in some studies (36, 41, 42), but not others (3, 37, 40, 44, 49, 50). While some of these inconsistencies may result from differences in study design or study population, it is possible, given the size of the *VDR* gene, that any causal genetic variant(s) remains to be identified. The aim of the present study was to conduct an extensive analysis of haplotypes and individual polymorphisms in *VDR* in relation to risk of breast cancer in a prospective cohort.

Materials and Methods

Study population

The Agricultural Health Study cohort was established between 1993 and 1997 and includes private pesticide applicators (primarily farmers) and their wives from Iowa and North Carolina (51). All 43,475 male private pesticide applicators who indicated at enrollment that they were married were requested to ask their wives to complete 2 take-home questionnaires. One questionnaire elicited information on the wives' farm exposures and general health ("spouse enrollment questionnaire"), whereas the other focused on their reproductive health history ("female and family health questionnaire"). A total of 32,127 wives (74% of eligible wives) enrolled in the cohort. Of these, 19,578 (61% of those enrolled) completed both questionnaires, whereas 12,549 (39% of those enrolled) completed only the spouse enrollment questionnaire. In addition, 23,676 wives (74% of those enrolled) completed a detailed follow-up telephone interview approximately 5 years after enrollment, at which time they were asked to provide a mouthwash rinse sample for extraction of DNA from buccal cells; approximately 60% of these participants returned a sample. Over 98% of the wives in this cohort are white.

Participant follow-up and case ascertainment

Breast cancer cases were identified through population-based cancer registries in Iowa and North Carolina. Estrogen receptor and progesterone receptor status of the tumor was available from the registries for 74.4% to 75.4% of cases. Cases had no cancer diagnoses before enrollment and were diagnosed with malignant breast cancer (International Classification of Diseases for Oncology second edition, C50.0–C50.9) between enrollment and December 31, 2004 (i.e., incident cases, $n = 578$). Only 263 cohort members (0.8%) moved out of state and were consequently lost to follow-up during the study period.

All eligible breast cancer cases and potential controls who had previously provided a mouthwash rinse sample for extraction of DNA as part of the parent study were eligible for the present study. Of the 578 incident cases in the cohort, 293 (50.7%) had provided mouthwash samples and were, therefore, selected as cases for this study. Importantly, previous analyses found little difference in demographic, lifestyle, occupational, or medical factors between members of the cohort who returned a mouthwash sample and those who did not, suggesting that selection bias related to provision of this sample is unlikely (52). Two controls were randomly matched with replacement to each case by race (white, other), state (Iowa, North Carolina), age at enrollment (5 year age groups), and enrollment period (1993–1995, 1996–1997); controls had to be alive, have no cancer diagnoses, and be living in state at the date of diagnosis of their corresponding case. A total of 879 cases and controls were selected. Because of controls being selected with replacement, which provides an unbiased sample from the cohort (53), 19 subjects were each selected as controls for 2 cases and 4 subjects were each selected as both a control and, at a later time point, a case. Informed consent was obtained and the study protocol was reviewed by all relevant Institutional Review Boards.

DNA extraction and genotyping

DNA was extracted from buccal cells using the QIAamp 96 DNA Blood Kit (QIAGEN Inc). DNA concentration was measured by spectrophotometry at 260 nm and DNA quality was determined by the A260/A280 ratio. Sufficient DNA for genotyping, without consuming all available DNA, was obtained from 802 (93.7%) samples. These represented 270 cases and 554 controls, including 18 subjects selected as controls for 2 cases and 4 subjects

who were each selected as both a control and, at a later date, a case.

We selected 27 SNPs, based partially on the results of Nejentsev and colleagues (32), who conducted sequencing and extensive genotyping of the *VDR* gene among multiple populations of Europeans and Africans and identified the minimal set of haplotype tag SNPs (htSNP). These SNPs were selected as htSNPs to capture the genetic variation within the 3 haplotype blocks among whites (32), and also included SNPs with known or suspected impact on VDR function or that had been associated with breast cancer risk in previous studies. They included rs2544038, rs739837, rs731236 (TaqI), rs2239182, rs2107301, rs2239181, rs2238139, rs2189480, rs3782905, rs7974708, rs11168275, rs2408876, rs1989969, rs2238135, rs10875694, rs3922882, rs11168287, rs7299460, rs11168314, rs4303288, rs4073729, rs3923693, rs4760674, rs6823, rs2071358, rs7975232 (ApaI), and rs2228570 (FokI, formerly reported as rs10735810). One SNP, rs4303288, could not be successfully genotyped because of poor clustering and nonspecific signals and, therefore, is not included in the following analyses. One of the SNPs (rs2228570), at a FokI restriction site, was included because it is known to alter the VDR protein (34) but has no detectable linkage with any other SNP and is, therefore, not in a haplotype block. The SNPs rs731236 (TaqI) and rs7975232 (ApaI) had previously been studied in relation to breast cancer and other diseases (3, 36, 37, 40–42, 44, 48–50). All selected SNPs have a minor allele frequency of at least 10% in whites. To facilitate comparison with previous literature, the following provides the correspondence between nucleotides and restriction fragment length polymorphism nomenclature for major and minor alleles genotyped in this study: *FokI* (rs2228570): $C = F$, $T = f$; *ApaI* (rs7975232): $A = A$, $C = a$; and *TaqI* (rs731236): $T = T$, $C = t$.

Twenty-four SNPs were genotyped by mass spectrometry and 2 SNPs were genotyped with pyrosequencing. Primer sequences are described in detail in the Supplementary Material (Tables S1 and S2). The genotyping by mass spectrometry was done on the Sequenom MassARRAY iPLEX genotyping Platform (Sequenom Inc) in 7 multiplex assays. Experiments were designed with the RealSNP Assay Database and the MassArray software v.3.1. Twenty nanograms of genomic DNA were amplified using specific primers, reagents, and cycling conditions detailed in the Supplementary Material (Table S1). The products were desalted and then spotted onto a 384 SpectroCHIP bioarray (Sequenom). Cluster plots were evaluated with the TyperAnalyzer application (MassARRAY v.3.4). Assays were considered optimal according to degree of clustering, absence of signal in the blanks, and when sequencing of representative samples within the clusters confirmed the genotypes. Two SNPs that failed to cluster or showed suboptimal performance on mass array (rs3923693 and rs7975232) were tested with pyrosequencing (54) using the AB PSQ™ MA instrument (Qiagen Inc). Specific primers, reaction, and cycling conditions are available in Table S2. All reactions contained known internal controls, and standard precautions to avoid contamination were followed. Quality control included review of clusters and specificity, 5% randomly selected repeats, as well as interpretation of results and verification of data and data entry by an independent laboratory member. The genotype frequencies among controls did not differ from the expected Hardy–Weinberg equilibrium proportions.

Data analysis

We used conditional logistic regression to estimate odds ratios (OR) and 95% confidence intervals (CI) to account for the matched design of the study. All analyses were adjusted for known breast cancer risk factors, including age at menopause (premenopausal and postmenopausal in the following age groups: <45, 45–49, 50–54, and ≥55 years), first degree family history of breast cancer (yes and no), parity, and age at first birth. Parity and age at first birth were combined (1 birth by age 30 years; ≥2 births, first of which was by age 30 years; nulliparous or all births after age 30 years), with nulliparous women and those with first births after age of 30 years combined because of the small number of nulliparous women in this study (4 cases and 7 controls). Body mass index, age at menarche, smoking status, and education were examined as potential confounders but were not included in the final models because they did not materially change the risk estimates. We used last-reported values before diagnosis for cases or reference date for controls for all time-varying covariates.

Because some members of matched case–control sets (1 case and 2 controls per set) had missing information on genotype or haplotype, we used the missing-indicator method (55) to retain all subjects and maintain case–control matching. This method produces an OR estimate that is a compromise between the estimate by a matched analysis of the complete sets and by an unmatched analysis of the incomplete sets. Missing covariate data were imputed using the IVEware program (56). This program simultaneously imputes values for specified variables by fitting a sequence of regression models and drawing values from the corresponding predictive distributions. Missing values were imputed for race (3.2%), family history of breast cancer (5.0%), parity (17.6%), age at menopause (21.8%), and use of hormone replacement therapy (9.8%). Risk estimates that included imputed data were not materially different from those that included only observed data, so we present risk estimates adjusted using these imputed and observed data.

Our primary interest in this article is in the evaluation of haplotypes. We first provide results for the individual SNP analyses, followed by the haplotype analyses. For each SNP, conditional logistic regression analysis was used to estimate the OR and 95% CI of the heterozygous and homozygous (for the minor allele) genotype groups. For the haplotype analyses, we first determined the haplotype structure, particularly the haplotype blocks, of our study population using haploview v4.1 (57) among control subjects. Because the genotype data were unphased,

we estimated the haplotype frequencies and the expected haplotypes using the haplo.stats software package (58) in R v2.3 (59) and used these as independent variables in regression models, as described by Kraft and colleagues (60). Only the most common 50% of haplotypes for each haplotype block, ranging in prevalence from 4.7% to 20.1%, are shown in the tables, although all haplotypes were included in the analyses.

We also conducted analyses stratified by menopausal status at diagnosis, family history of breast cancer, state of residence, and estrogen receptor (ER; $N_{ER+} = 170$, $N_{ER-} = 51$) and progesterone receptor (PR; $N_{PR+} = 150$, $N_{PR-} = 68$) status of the tumor. These stratified case–control analyses were conducted using unconditional logistic regression.

All statistical analyses were conducted using SAS v9.1 (61), except where otherwise noted. All statistical significance was assessed at the 5% level. We did not adjust the *P*-values for multiple comparisons because of the exploratory nature of our extensive evaluations of the individual SNPs and haplotypes. All data used in these analyses were based on Agricultural Health Study data releases P1REL0506.01 and P2REL0506.04.

Results

Selected characteristics of the subjects in this case–control study are provided in Table 1. The mean age was 58.3 ± 10.0 years for cases at diagnosis and 62.8 ± 8.9 years for their matched controls. The large majority of cases and controls were white (~98% of each). Only 1.4% of cases and 1.2% of controls were nulliparous. About 67.8% of cases at diagnosis and 68.1% of controls were postmenopausal. Risk of breast cancer was significantly increased among women reporting a family history of breast cancer (OR = 1.8; 95% CI: 1.2–2.6). Risk was also increased among women who either were nulliparous or had a first birth after age 30 years compared with women with 2 or more births, the first of which was by age 30 years (OR = 2.1; 95% CI: 1.2–3.8). Among premenopausal women, breast cancer risk was significantly reduced among those with BMI of 30 kg/m^2 or above (OR = 0.3; 95% CI: 0.1–0.8) compared with those with BMI less than 25 kg/m^2; however, this was based on only 9 cases in the high BMI category. Breast cancer risk was elevated among former and current smokers relative to never smokers, although the increases were not statistically significant [OR = 1.8 (95% CI: 0.9–3.6) among former smokers and OR = 2.0 (95% CI: 0.9–4.2) among current smokers].

The haplotype structure of our study population (Fig. 1) was comparable to that observed among whites by Nejentsev and colleagues (32). Therefore, blocks were defined using the naming convention of Nejentsev and colleagues (32), with htSNPs in the following positional order—Block A: rs2544038; Block B: rs739837, rs731236, rs7975232, rs2239182, rs2107301, rs2239181, rs2238139, rs2189480, rs3782905, rs7974708, and rs11168275; Block C: rs2408876, rs1989969, rs2238135, rs10875694, rs3922882, rs11168287, rs7299460, rs11168314, rs4073729, rs3923693, rs4760674, rs6823, and rs2071358.

The locus rs2228570 (FokI), which was not in LD with any other SNP, was not significantly associated with altered risk of breast cancer [OR = 1.3 (0.9, 1.7) for C/T and 1.2 (0.7, 1.9) for T/T, relative to C/C; *P* for trend = 0.31; Table 2]. The SNP rs2544038 (in haplotype block A) was associated with increased risk of breast cancer [OR = 1.3 (0.9, 1.8) for C/T and 1.5 (1.0, 2.5) for C/C, relative to T/T; *P* for trend = 0.05], although the risk for heterozygous carriers was only marginally significant. In addition, rs11168287 (in haplotype block C) was associated with decreased risk [OR = 0.7 (0.5, 1.1) for A/G and 0.6 (0.4, 1.0) for G/G, relative to A/A; *P* for trend = 0.05], but the risk among heterozygous carriers was only marginally significant. Finally, rs2239181 (in haplotype block B) exhibited a marginally significant association with breast cancer risk [OR = 1.4 (0.9, 2.0) for G/T or G/G relative to T/T; *P* = 0.09].

The haplotype GTCATTTCCTA in LD block B (denoted haplotype "B4") was significantly associated with decreased risk of breast cancer, with OR = 0.5 (0.3, 0.9; Table 3). In statistical models that included all of the htSNPs in block B simultaneously, rs2239181 had a significantly increased risk [1.6 (1.0, 2.7) for G/G or G/T vs. T/T]; no other htSNP in block B approached statistical significance.

Results did not differ substantively between subgroups defined by family history of breast cancer or in subanalyses restricted to whites (data not shown). Results were similar between Iowa and North Carolina (data not shown). In analyses stratified by menopausal status, the risk associated with rs2408876 appeared to differ for premenopausal versus postmenopausal women [premenopausal: OR = 0.5 (0.3, 0.9) for T/C and OR = 0.7 (0.3, 1.6) for C/C, relative to T/T; postmenopausal: OR = 1.6 (1.1, 2.4) for T/C and OR = 1.8 (1.0, 3.2) for C/C, relative to T/T]. When analyses were stratified by ER status and PR status, the risk associated with rs2544038 was apparently stronger in ER− and PR− cases, although CIs were wide and overlapping [ER−: OR = 2.3 (1.0, 4.9) for C/T and OR = 2.9 (1.2, 7.2) for C/C, relative to T/T; ER+: OR = 1.2 (0.8, 1.8) for C/T and OR = 1.4 (0.8, 2.3) for C/C, relative to T/T; PR−: OR = 2.3 (1.2, 4.5) for C/T and OR = 2.7 (1.2, 6.1) for C/C, relative to T/T; PR+: OR = 1.1 (0.7, 1.7) for C/T and OR = 1.3 (0.8, 2.3) for C/C, relative to T/T].

Discussion

Results from this case–control study nested within a large, prospective cohort provide limited evidence that variants of the *VDR* gene may be related to risk of breast cancer. Haplotype GTCATTTCCTA in LD block B was observed to be associated with a reduced risk. Among individual SNPs, breast cancer risk was observed to be significantly associated with rs2544038 (block A) and rs11168287 (block C), and to have a marginally significant association with rs2239181 (block B).

Table 1. Selected characteristics of breast cancer cases and controls among wives in the Agricultural Health Study

	Cases (n = 293)[a]		Controls (*n* = 586)[a]			
Characteristic	**No[c]**	**%**	**No[c]**	**%**	**Adjusted OR[b]**	**95% CI**
Age (years)						
18–39	30	10.2	69	11.8	NA	
40–49	67	22.9	142	24.2	NA	
50–59	122	41.6	234	39.9	NA	
60–69	60	20.5	119	20.3	NA	
70–86	14	4.8	22	3.8	NA	
Race						
White	281	97.9	574	98.0	NA	
Other	6	2.1	12	2.0	NA	
State of residence						
Iowa	196	66.9	392	66.9	NA	
North Carolina	97	33.1	194	33.1	NA	
Highest educational level						
<high school	10	3.9	33	6.3	0.5	0.2, 1.1
High school	107	41.3	229	43.4	0.9	0.6, 1.2
>high school	142	54.8	266	50.4	1	ref
Smoking						
Never	207	74.5	417	73.7	1	ref
Former	59	21.2	110	19.4	1.8	0.9, 3.6
Current	12	4.3	39	6.9	2.0	0.9, 4.2
First degree family history of breast cancer	62	22.1	81	14.2	**1.8**	**1.2, 2.6**
BMI (kg/m^2), premenopausal						
<25.0	43	48.3	52	29.1	1	ref
25.0–29.9	20	22.5	43	24.0	0.6	0.3, 1.5
≥30.0	9	10.1	34	19.0	**0.3**	**0.1, 0.8**
BMI (kg/m^2), postmenopausal						
<25.0	61	32.6	135	35.3	1	ref
25.0–29.9	53	28.3	123	32.1	0.9	0.5, 1.4
≥30.0	35	18.7	62	16.2	1.2	0.7, 2.1
Age at menarche (years)						
<12	27	12.2	59	14.6	1	ref
12–14	178	80.2	307	75.8	1.2	0.7, 2.0
≥15	17	7.7	39	9.6	0.9	0.4, 1.8
Parity and age at first birth, combined						
Nulliparous, or first birth after age 30 years	27	12.8	27	7.1	**2.1**	**1.2, 3.8**
1 birth by age 30 years	15	7.1	25	6.6	1.1	0.5, 2.2
≥2 births, first of which was by age 30 years	169	80.1	326	86.2	1	ref
Age at menopause (years)						
Premenopausal	89	30.4	179	30.6	1.0	0.6, 1.7
<45	65	22.2	138	23.6	1	ref
45–49	42	14.3	92	15.7	0.8	0.5, 1.4
50–54	64	21.8	110	18.8	1.1	0.7, 1.8
≥55	15	5.1	38	6.5	0.9	0.5, 1.8
Tumor estrogen/progesterone receptor status						
ER+	170	76.9	N/A			
ER−	51	23.1	N/A			
PR+	150	68.8	N/A			
PR−	68	31.2	N/A			

[a]Controls were randomly selected with replacement and include 19 subjects who were each selected as controls for 2 cases and 4 subjects who were each selected as both a control and, at a later time, a case.

[b]All factors are adjusted for the other factors in the table, except where indicated and except for matching factors—age at enrollment (5-year age groups), enrollment period (1993–1995, 1996–1997), race (white, other), and state of residence (Iowa, North Carolina).

[c]Number of cases or controls indicated for some factors may be less than the total number of cases or controls because of missing data.

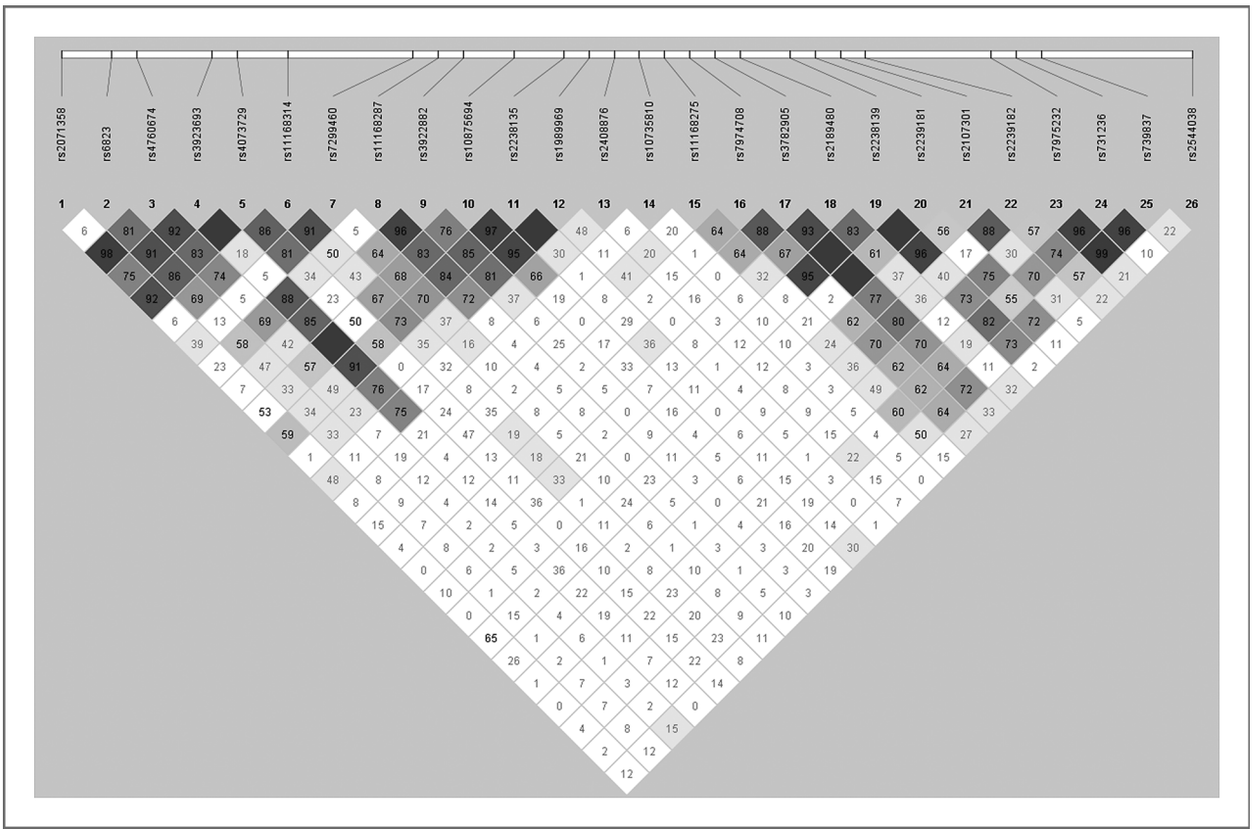

Figure 1. Linkage disequilibrium plot of genotyped SNPs in *VDR* gene, among whites, Agricultural Health Study.

To our knowledge, this study is the first to examine comprehensive haplotypes in the *VDR* gene in relation to breast cancer risk. Most previous studies have examined individual polymorphisms, focusing primarily on the restriction fragment length polymorphisms *BsmI* (rs1544410), *FokI* (rs2228570), *ApaI* (rs7975232), and *TaqI* (rs731236). Results of these studies have been inconsistent. The weight of evidence tends to be strongest for an association with *FokI*. Among the studies that examined *FokI* in relation to breast cancer risk (3, 35–45), several provided some evidence of increased risk among carriers of the *ff* genotype (35–39), with ORs ranging between 1.16 and 2.34. Apart from 1 case–control study that reported a significantly decreased relative risk of 0.71 among *ff* carriers (40), the remaining studies found no association. The reasons for this discrepancy remain unclear, although it may be due in part to differences across studies in sample size, race/ethnicity of study populations, or control selection. *FokI* is not in LD with any other SNPs (32). A meta-analysis that included most of the above studies (i.e., all studies published through October 2008; ref. 62) suggested a small, but significantly, increased risk of breast cancer among *Fok1 ff* carriers compared with *FF* carriers (summary OR = 1.15; 95% CI = 1.03, 1.28). Our estimated OR of 1.2 was consistent in direction and magnitude with this summary OR, but was not statistically significant. This SNP did not depart from Hardy–Weinberg equilibrium among controls in our study and the minor allele frequency was similar to that of other white populations. An effect of this SNP is plausible, given that the *f* allele results in production of a VDR protein that is less effective as a transcriptional activator (34), the consequences of which would be expected to mimic that of lower vitamin D status.

Two studies that examined *VDR* haplotypes (41, 44) used only a small number of polymorphisms that likely did not capture a large fraction of the variation in this gene (32). One of these studies inferred haplotypes from only *BsmI, ApaI, TaqI*, and a poly(A) repeat and found no association between any haplotype and breast cancer risk (44). The other study inferred haplotypes from the *FokI*, *TaqI*, *VDR*-5132, and *Cdx2* polymorphisms, and found increased risk of breast cancer associated with the haplotype containing *FokI F*, *TaqI t*, *VDR-5132* C, and *Cdx2* A (41). These results and those of the present study cannot be directly compared because of differences in haplotype definitions (32). Moreover, while some *in vitro* studies of VDR haplotypes have reported higher mRNA expression for the *BsmI–ApaI–TaqI* haplotype BAt (rs1544410-A/rs7975232-A/rs731236-C) than for the haplotype baT (rs1544410-G/rs7975232-C/rs731236-T), other studies have observed the opposite pattern not only for mRNA expression, but also for mRNA stability and

Table 2. Genetic polymorphisms and risk of breast cancer among wives in the Agricultural Health Study

Haplotype block[b]	SNP	Genotype	Cases ($n = 270$)[a] No.[c]	Cases ($n = 270$)[a] %	Controls ($n = 554$)[a] No.[c]	Controls ($n = 554$)[a] %	Adjusted OR[d]	95% CI	*P* for trend
A	rs2544038	T/T	75	28.4	182	33.9	1	ref	
		C/T	136	51.5	266	49.5	1.3	0.9, 1.8	
		C/C	53	20.1	89	16.6	1.5	1.0, 2.5	0.05
B	rs739837	T/T	87	32.3	159	29.0	1	ref	
		G/T	120	44.6	246	44.9	0.9	0.6, 1.2	
		G/G	62	23.0	143	26.1	0.8	0.5, 1.1	0.19
B	rs731236 (TaqI)	T/T	98	36.4	211	38.6	1	ref	
		T/C	117	43.5	226	41.4	1.1	0.8, 1.6	
		C/C	54	20.1	109	20.0	1.1	0.7, 1.6	0.65
B	rs7975232 (ApaI)	A/A	88	32.8	160	29.0	1	ref	
		C/A	120	44.8	251	45.5	0.9	0.6, 1.2	
		C/C	60	22.4	141	25.5	0.7	0.5, 1.1	0.16
B	rs2239182	G/G	77	28.7	163	29.7	1	ref	
		G/A	124	46.3	244	44.5	1.1	0.8, 1.5	
		A/A	67	25.0	141	25.7	1.0	0.7, 1.5	0.99
B	rs2107301	C/C	153	57.1	290	52.8	1	ref	
		T/C	93	34.7	219	39.9	0.8	0.6, 1.1	
		T/T	22	8.2	40	7.3	1.1	0.6, 2.0	0.57
B	rs2239181	T/T	212	79.4	460	83.9	1	ref	
		G/T or GG	55	20.6	88	16.1	1.4	0.9, 2.0	0.09
B	rs2238139	T/T	167	61.9	361	65.6	1	ref	
		C/T	89	33.0	161	29.3	1.1	0.8, 1.6	
		C/C	14	5.2	28	5.1	1.0	0.5, 2.1	0.55
B	rs2189480	C/C	111	41.4	218	39.6	1	ref	
		C/A	119	44.4	243	44.1	1.0	0.7, 1.3	
		A/A	38	14.2	90	16.3	0.8	0.5, 1.3	0.47
B	rs3782905	C/C	132	49.3	259	47.5	1	ref	
		G/C	103	38.4	228	41.8	0.9	0.7, 1.3	
		G/G	33	12.3	58	10.6	1.1	0.7, 1.8	0.84
B	rs7974708	T/T	131	48.7	259	47.3	1	ref	
		C/T	101	37.5	211	38.6	1.0	0.7, 1.3	
		C/C	37	13.8	77	14.1	0.9	0.6, 1.5	0.76
B	rs11168275	A/A	155	57.4	316	57.4	1	ref	
		G/A	98	36.3	191	34.7	1.0	0.8, 1.4	
		G/G	17	6.3	44	8.0	0.8	0.4, 1.4	0.61
-	rs2228570 (FokI)	C/C	93	34.6	218	39.5	1	ref	
		C/T	136	50.6	257	46.6	1.3	0.9, 1.7	
		T/T	40	14.9	77	13.9	1.2	0.7, 1.9	0.31
C	rs2408876	T/T	97	36.1	212	38.8	1	ref	
		T/C	128	47.6	261	47.8	1.1	0.8, 1.5	
		C/C	44	16.4	73	13.4	1.4	0.8, 2.2	0.23
C	rs1989969	C/C	94	35.2	175	31.9	1	ref	
		C/T	123	46.1	274	49.9	0.8	0.6, 1.2	
		T/T	50	18.7	100	18.2	0.9	0.6, 1.4	0.59
C	rs2238135	G/G	160	59.5	329	60.0	1	ref	
		G/C	93	34.6	180	32.8	1.0	0.8, 1.4	
		C/C	16	5.9	39	7.1	0.9	0.5, 1.6	0.90
C	rs10875694	T/T	196	72.6	402	73.1	1	ref	
		T/A or A/A	74	27.4	148	26.9	1.0	0.8, 1.4	0.83

(Continued on the following page)

Table 2. Genetic polymorphisms and risk of breast cancer among wives in the Agricultural Health Study (Cont'd)

Haplotype block[b]	SNP	Genotype	Cases ($n = 270$)[a] No.[c]	Cases ($n = 270$)[a] %	Controls ($n = 554$)[a] No.[c]	Controls ($n = 554$)[a] %	Adjusted OR[d]	95% CI	*P* for trend
C	rs3922882	C/C	98	36.4	197	35.8	1	ref	
		C/G	123	45.7	276	50.1	0.9	0.7, 1.3	
		G/G	48	17.8	78	14.2	1.3	0.8, 2.0	0.51
C	rs11168287	A/A	85	32.2	134	25.2	1	ref	
		A/G	134	50.8	285	53.7	0.7	0.5, 1.1	
		G/G	45	17.0	112	21.1	0.6	0.4, 1.0	0.05
C	rs7299460	C/C	132	49.1	279	51.1	1	ref	
		C/T	108	40.1	212	38.8	1.1	0.8, 1.5	
		T/T	29	10.8	55	10.1	1.1	0.7, 1.9	0.53
C	rs11168314	G/G	172	63.9	373	67.7	1	ref	
		G/A or A/A	97	36.1	178	32.3	1.2	0.9, 1.7	0.21
C	rs4073729	C/C	200	74.1	407	74.1	1	ref	
		T/C or T/T	70	25.9	142	25.9	1.0	0.7, 1.4	0.99
C	rs3923693	C/C	215	81.4	434	79.1	1	ref	
		C/T or T/T	49	18.6	115	21.0	0.9	0.6, 1.3	0.47
C	rs4760674	C/C	92	34.3	189	34.7	1	ref	
		C/A	121	45.1	270	49.5	0.9	0.7, 1.3	
		A/A	55	20.5	86	15.8	1.3	0.8, 2.0	0.37
C	rs6823	C/C	74	27.6	140	25.6	1	ref	
		C/G	133	49.6	280	51.3	0.8	0.6, 1.2	
		G/G	61	22.8	126	23.1	0.9	0.6, 1.4	0.59
C	rs2071358	C/C	181	68.3	362	65.9	1	ref	
		C/A	71	26.8	164	29.9	0.9	0.6, 1.2	
		A/A	13	4.9	23	4.2	1.1	0.6, 2.3	0.71

[a]Controls were randomly selected with replacement and include 18 subjects who were each selected as controls for 2 cases and 4 subjects who were each selected as both a control and, at a later time, a case. Because some members of matched case–control sets had missing information on genotype or haplotype, the missing-indicator method was used to retain all subjects ($N_{cases} = 293$, $N_{controls} = 586$) and maintain case–control matching (see text).

[b]Nejentsev S and colleagues (32).

[c]Number of cases or controls indicated for some SNPs may be less than the total number of cases or controls because of missing genotype data.

[d]Cases and controls were matched on age at enrollment, race, and state; analyses were adjusted for age at menopause (premenopausal, <45, 45–49, 50–54, and ≥55 years), combined parity and age at first birth (1 birth by age 30 years; ≥2 births, first of which was by age 30 years; nulliparous or all births after age 30 years), and first degree family history of breast cancer (yes and no).

transactivation, which could be due in part to differences in the cell lines used (63).

In the present study, we identified 1 haplotype, GTCATTTCCTA in LD block B, that was significantly associated with a 50% reduced risk of breast cancer. While we are unaware of comparable haplotype data from other studies, a number of studies have investigated several individual SNPs within this block. Our results for SNPs within block B are consistent with a meta-analysis of commonly examined *VDR* SNPs (62), which found no significant associations between *Bsm1, Apa1,* or *Taq1,* which are in this block, and risk of breast cancer. Reports published since this meta-analysis are also largely consistent with these findings (36, 37, 39, 40, 45). Although we did not test *BsmI*, we did test rs731236, which is in strong LD ($r^2 = 0.97$) with *BsmI* among whites (64) and which showed no association with breast cancer risk. In addition, a recent case–control study (40) that included several of the less commonly studied SNPs assessed in the present study also supports our findings of no effect associated with rs739837, rs1989969, rs2107301, and rs2238135, which are found in blocks B and C. Our haplotype results, if confirmed, would suggest that another polymorphism or polymorphisms in strong LD with rs2239181 may be responsible for the reduced risk associated with this haplotype.

We found only limited evidence of differences in risk by menopausal status or by ER/PR status of the tumor.

Table 3. Selected haplotypes and risk of breast cancer among wives in the Agricultural Health Study

Common haplotypes[c]	Cases ($n = 270$)[a] Mean probability	SD	Controls ($n = 554$)[a] Mean probability	SD	Adjusted OR[b]	95% CI
Block B[d]						
B1: G T C A C T C C C T A	0.19	0.39	0.17	0.37	1.2	0.8, 1.9
B2: G T C A C T T A C T A	0.09	0.29	0.11	0.29	0.8	0.5, 1.4
B3: G T C A T T T A C T G	0.10	0.28	0.12	0.30	0.8	0.5, 1.3
B4: G T C A T T T C C T A	0.06	0.22	0.11	0.31	0.5	0.3, 0.9
B5: T C A G C T T A C T A	0.14	0.36	0.16	0.40	0.9	0.6, 1.3
B6: T C A G C T T C G C A	0.39	0.57	0.41	0.58	0.9	0.7, 1.2
Block C[d]						
C1: C C C A C G C G C C C C C	0.18	0.39	0.17	0.38	1.1	0.8, 1.6
C2: C C G T C A T G C C A G C	0.16	0.40	0.13	0.35	1.1	0.8, 1.7
C3: C T G T G A C G C C A G C	0.10	0.28	0.09	0.27	1.1	0.6, 1.9
C4: T C C T C G C G C C C C C	0.10	0.30	0.12	0.33	0.8	0.5, 1.3
C5: T C G T C G C G C T C C A	0.09	0.27	0.09	0.29	0.9	0.5, 1.6
C6: T T G T G A C G C C A G C	0.27	0.46	0.29	0.46	0.9	0.6, 1.3
C7: T T G T G A C G C C C G A	0.09	0.29	0.10	0.30	0.8	0.5, 1.3

[a]See corresponding footnote in Table 2.

[b]Cases and controls were matched on age at enrollment, race, and state; analyses were adjusted for age at menopause (premenopausal, <45, 45–49, 50–54,and ≥55 years), combined parity and age at first birth (1 birth by age 30 years; ≥2 births, first of which was by age 30 years; nulliparous or all births after age 30 years), and first degree family history of breast cancer (yes and no).

[c]Results are presented for only the most common 50% of haplotypes in each block (from among 123 in Block B and 178 in Block C), although all haplotypes were included in analyses.

[d]Blocks based on Nejentsev and colleagues (32) as follows, with the order of SNPs as listed—Block B: rs739837, rs731236, rs7975232, rs2239182, rs2107301, rs2239181, rs2238139, rs2189480, rs3782905, rs7974708, rs11168275; Block C: rs2408876, rs1989969, rs2238135, rs10875694, rs3922882, rs11168287, rs7299460, rs11168314, rs4073729, rs3923693, rs4760674, rs6823, rs2071358.

Previous studies that investigated interactions between menopausal status and *VDR* variants on breast cancer risk have found little evidence of effect modification (35, 39, 40, 45, 46). At the same time, evidence is limited and inconsistent for a modification of effect of *VDR* variants by ER or PR status. *FokI ff* was found to have a marginally and nonsignificantly stronger association with ER−/PR− tumors than ER+/PR+ tumors in one study (39) and the *TaqI t* allele was associated with a significantly increased breast cancer risk only in ER+ tumors in another study (41); however, no modification of effect by ER or PR status was found in other studies in which it was investigated (35, 36, 46). We observed no differences in risk for *FokI* or *TaqI* by ER or PR status and we are unaware of other studies that examined modification of the association of rs2544038 and breast cancer risk by ER or PR status.

The heterogeneity of observed associations across studies may be due in part to differences in vitamin D status of study populations. Persons living on farms may receive more UV exposure and have different diets than the general population, which could influence circulating vitamin D levels during certain periods. In contrast to the static genetic variation investigated herein, these exposures are complex and time-varying. The interaction between markers of vitamin D status and *VDR* variants on breast cancer risk in this cohort will be investigated in detail in the future.

The observed association between smoking and breast cancer risk is consistent with the conclusions of a recent expert panel on tobacco smoke and breast cancer risk, which concluded that the relationship between active smoking and breast cancer is consistent with causality (65). Results from 2 large prospective cohort studies published subsequent to this report (66, 67) support this conclusion. Although this association remains controversial, further discussion is beyond the scope of this paper.

Limitations of this study include the availability of DNA for only about 51% of the cases in the cohort. However, previous analyses suggest that this was unlikely to introduce selection bias (52). The study sample size was relatively small, especially for stratified analyses, though we had sufficient power to detect modest associations in the full sample. Also, there were too few nonwhites in this cohort to separately examine associations among these subjects. Exclusion of nonwhites from analyses had minimal impact on risk estimates. Finally, given the number of comparisons that were conducted, we cannot exclude the possibility that the observed associations occurred by chance.

This study had a number of strengths. These include detailed data at baseline and at 5-year follow-up on potential confounding factors. The availability of data collected before disease diagnosis mitigated concerns about survival or reporting bias. Moreover, reliability of these data was shown to be good to excellent for a range of factors among cohort members who completed the same questionnaire at least 1 year apart (68). We were able to capture and incorporate into our analyses much of the variation in the *VDR* gene by using an extensive set of htSNPs. Finally, we were able to compare results between both states in this cohort, which provided some evidence of internal consistency.

These results provide limited evidence that variation in the *VDR* gene may be associated with risk of breast cancer. The modest magnitude and borderline significance of most of these associations necessitate caution in their interpretation. Further years of follow-up of this cohort, with inclusion of additional incident cases and follow-up data in future analyses, should help to clarify the relationship between *VDR* variants and the risk of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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