Vitamin D Receptor Gene Polymorphisms and Breast Cancer Risk

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ABSTRACT

Purpose: **The steroid hormone 1,25-dihydroxyvitamin D_3 is thought to protect against breast cancer. The actions of 1,25-dihydroxyvitamin D_3 are mediated via the vitamin D receptor (VDR), and a number of polymorphisms in the *VDR* gene have been identified. These result in distinct genotypes, some of which may alter susceptibility to breast cancer. We have investigated whether specific *VDR* gene polymorphisms are associated with breast cancer risk in a United Kingdom Caucasian population.**

Experimental Design: **In a retrospective case–control study, female breast cancer patients ($n = 398$) and control women ($n = 427$) were recruited, and three *VDR* polymorphisms were determined.**

Results: **The 3′ *VDR* polymorphisms *Bsm*I and variable-length poly(adenylate) sequence were both significantly associated with breast cancer risk; odds ratios (adjusted for age menopausal status and hormone replacement therapy usage) for *bb* genotype *versus BB* genotype = 1.92 (95% confidence interval, 1.20–3.10; $P < 0.01$) and for LL *versus* SS = 1.94 (95% confidence interval, 1.20–3.14; $P < 0.01$). A 5′ *VDR* gene variant, *Fok*I, was not associated with breast cancer risk when analyzed in isolation ($P > 0.05$). However, *Fok*I did modulate the increased risk associated with the *bb*/LL genotype such that possession of one or more *F* alleles together with the *bb*/LL genotype augmented breast cancer risk. Furthermore, the highest proportion of *bb* and *FF*LL/*Ff*LL genotypes occurred in women with metastatic breast cancer.**

Conclusions: ***VDR*** **polymorphisms are associated with breast cancer risk and may be associated with disease progression. Additional investigations into how different genotypes may affect the functional mechanisms of the VDR will provide a better strategy for identifying women at risk of breast cancer and for developing improved treatments.**

INTRODUCTION

At least 1 in 10 women in the United Kingdom will develop breast cancer at some time in their lives, and there are more than 14,000 breast cancer deaths in the United Kingdom each year (1). Five to ten percent of breast cancer cases are caused by inherited germ-line mutations of genes such as *BRCA1* and *BRCA2* (2). Although the underlying causes for the development of the remaining sporadic cases are still poorly understood, it is likely that other genes exist that increase susceptibility to breast cancer. Because breast cancer is strongly influenced by the hormonal milieu, variation in genes that are responsive to hormonal activity is a possible candidate for increased risk. Henderson and Feigelson (3) have suggested that genes involved in steroid hormone metabolism and transport could act together to provide a high-risk profile for breast cancer. Variation in these genes might modify breast cancer risk through gene–gene or gene–environment interactions.

One potential candidate is the vitamin D receptor (VDR), a member of the steroid hormone family of nuclear receptors that are responsible for the transcriptional regulation of a number of hormone-responsive genes. VDR is expressed in normal mammary tissue, and more than 80% of breast tumor specimens are VDR positive (4). The natural ligand for VDR, 1,25-dihydroxyvitamin D_3 (1,25-D_3), as well as a number of novel synthetic vitamin D analogues inhibit proliferation and induce apoptosis in breast cancer cells *in vitro* (5–8). Furthermore, in animal models of breast cancer, vitamin D analogues slow down tumor development and promote regression of established mammary tumors (reviewed in ref. 9).

Thus, the vitamin D pathway is a potential target for elucidating new breast cancer therapies. The gene encoding the VDR protein is known to display polymorphic variation. A polymorphic site in exon II at the 5′ end of the gene can be identified using the *Fok*I restriction enzyme (10). This polymorphism is a T/C transition (ATG to ACG) at the first of two possible translation initiation sites and results in VDR proteins that differ in length by three amino acids. In individuals with the ACG sequence, initiation of translation occurs at the second ATG site and the three NH_2-terminal amino acids of the full-length VDR are missing. Alleles with an absent restriction site are designated *F*. In contrast, alleles with the ATG sequence at the first initiation site synthesize the full-length (427 amino acids) VDR protein and are designated *f*. The *Fok*I *f* allele has been associated with increased breast cancer risk in African-American women (11). At the 3′ end of the *VDR* gene, three polymorphisms have been identified that do not lead to any change in either the transcribed mRNA or the translated protein.

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The first two sequences generate *Bsm*I (12) and *Apa*I (13) restriction sites and are intronic, lying between exons 8 and 9. The third polymorphism lies in exon 9, generating a *Taq*I restriction site (14), and leads to a silent codon change (from ATT to ATC); both activities result in an isoleucine at codon 352. These three polymorphisms are linked to a further gene variation, a variable-length poly(adenylate) [poly(A)] sequence within the 3′-untranslated region (3′UTR). The poly(adenylate) sequence varies in length (15) and can be segregated into two groups; "long" (L), where A = $_{18–24}$, and "short" (S), where A = $_{13–17}$. Because of their close proximity on the *VDR* gene, there is strong linkage disequilibrium between the 3′ polymorphisms, *Bsm*I, *Apa*I, and *Taq*I, and the variable-length poly(A) sequence so that, in Caucasian populations, two haplotypes are commonly observed: *baTL* (presence of *Bsm*I, *Apa*I restriction sites, absence of *Taq*I site, long poly(A) sequence), and *BAtS* (15, 16). The *baTL* haplotype has been reported to be associated with increased risk of prostatic carcinoma (17, 18) and breast cancer (19, 20), whereas the *BAtS* haplotype is associated with increased risk of osteoporosis (16, 21).

In 2001, we carried out a pilot study in 422 Caucasian women to assess whether *VDR* polymorphisms are associated with breast cancer risk in a United Kingdom population (22). The pilot study was limited because there were very few volunteers with *VDR* genotype *BB*/SS, and so it was difficult to perform statistical analyses. In this report, we have extended the number of women in the study to 825, and determined the *Bsm*I, *Fok*I, and poly(A) genotypes of each individual. The aim is to assess more accurately whether specific genotypes are related to breast cancer risk in this Caucasian population, and to determine whether certain genotypes are associated with disease progression, or with pathological factors such as estrogen receptor status and tumor grade. We have also calculated association with breast cancer risk using a "cross-genotyping'" method similar to that described by Whitfield *et al.* (23), to investigate whether combining the 5′ *Fok*I genotype with the 3′ *Bsm*I/poly(A) genotype would reveal a more positive association with breast cancer risk than with each individually.

MATERIALS AND METHODS

A retrospective case-control study was undertaken to investigate whether specific *VDR* gene polymorphisms are associated with breast cancer risk. The study was carried out exclusively on Caucasian subjects, because the *VDR* polymorphism distribution varies between different ethnic populations. St. George's Hospital Medical School Ethics Committee approved the study, and written informed consent was obtained from participants in both control and cancer groups before interview and sampling.

Control Volunteers. Control women (n = 427) were recruited from the United Kingdom National Breast Screening Programme for South-West London. Women ages 50–65 years are invited for routine mammography screening every 3 years, and women under 50 or over 65 may attend by self-referral. Women recruited from the screening program all had a recent mammogram, confirming that there was no detectable breast cancer at the time of sampling, and all had no personal history of breast cancer. Mammography revealed that 147 women in the study had benign breast disease, the majority of these were benign calcifications, fibrocystic disease, fibroadenoma or other benign lump. These benign conditions are not conventionally associated with increased breast cancer risk. The age range of the control women was 36–80 years, with a median age of 54 years, at the time of sampling (Table 1). There were no age-related differences in VDR gene frequency between the oldest and youngest women. The majority of the control subjects (325 women, 76.1%) were postmenopausal and 229 women (53.6%) were current or past users of hormone replacement therapy (HRT; Table 1).

Breast Cancer Volunteers. Of a possible 821 female breast cancer patients, 398 (48.5%) were recruited between the dates July 1, 1998, and October 31, 2002, through the Combined Breast Clinic at St. George's Hospital. Not all patients were recruited because researchers attended the clinic only on an intermittent basis. Ninety-nine percent of the women who were asked to take part in the study consented. Cases were recruited

Table 1 Difference in characteristics of controls and cases

	Controls	Cases	OR (CI)	P value of difference
Age (yrs), median (range)*	54 (36–80)	60.5 (27–90)		<0.001†
Age (yrs), median (range)‡	54 (36–80)	56 (26–89)		0.72
Family history, n (%)*				<0.001†
None	346 (81.0)	285 (71.6)	1.00	
Weak	41 (9.6)	34 (8.5)	1.01 (0.62–1.63)	
Strong	40 (9.4)	79 (19.9)	2.40 (1.59–3.62)	
Menopausal status, n (%)‡				<0.001†
Pre	49 (11.5)	123 (30.9)	3.14 (2.17–4.54)	
Peri	53 (12.4)	15 (3.8)	0.35 (0.20–0.64)	
Post	325 (76.1)	260 (65.3)	1.00	
HRT use, n (%)*				
Never	198 (46.4)	285 (71.6)		<0.001†
Current or past	229 (53.6)	113 (28.4)	0.34 (0.26–0.46)	

* At sampling for control and cases.
† Statistically significant.
‡ At sampling for controls and at diagnosis for cases.

without prior knowledge of diagnosis or tumor stage, so that as a result, some patients on the study had been diagnosed with their primary breast cancer before 1998 (prevalent cases), whereas some patients were newly diagnosed (incident cases). Details of primary tumor diagnosis, recurrences, and metastases were subsequently obtained from histopathology reports and medical records. Personal information such as number of children, age at menarche, HRT use, alcohol intake, and smoking status was obtained by asking the patient, but, when possible, this was also confirmed by medical records. The median age of the breast cancer volunteers was 56.0 (range, 26–89) years at diagnosis, and 60.5 (range, 27–90) years at the time of sampling (Table 1). Median time from diagnosis to sampling was 3.0 years (range, 0–26 years). A total of 260 women (65.3%) in this group were postmenopausal, and 113 (28.4%) were current or past users of HRT (Table 1). The majority of these women (99.1%) underwent surgery (wide local excision or mastectomy) with or without postoperative radiotherapy. In addition, 295 women were treated with hormonal therapy, 39 with chemotherapy, and 131 with a combination of both. The four women who did not undergo surgery presented with metastases. The characteristics of the tumors were validated from histopathological reports of biopsy and/or resection specimens (shown in Table 2). Tumor grade, estrogen receptor status, and lymph node involvement are presented for patients with invasive breast cancer. Thirty-four women reported a weak family history of breast cancer (defined as possessing one second-degree relative with breast cancer), and 79 women reported a strong family history (defined as having at least one first-degree relative, or two or more second-degree relatives with breast cancer; Table 1).

Table 2 Characteristics of 398 cancer patients

	n (%)	Time, mo (range)
Histology		
DCIS only	31 (7.8)	
Invasive ductal carcinoma	334 (83.9)	
Invasive lobular carcinoma	33 (8.3)	
Tumor grade (invasive types only)		
I	87 (23.7)	
II	143 (39.0)	
III	118 (32.1)	
Unknown/cannot be assessed	19 (5.2)	
Lymph node status (invasive types only)		
Lymph node involvement	118 (32.2)	
No lymph node involvement	249 (67.8)	
Estrogen receptor status (invasive types only)		
Positive	263 (71.7)	
Negative	97 (26.4)	
Unavailable	7 (1.9)	
Disease progression at end of recruitment		
Local recurrence only	29	
Metastatic disease	58	
New primary breast tumors	12	
New primary tumors (not breast)	2	
Deaths	43	
Median time		
From diagnosis to local recurrence		43 (2–205)
From diagnosis to metastases		35 (0–205)
From diagnosis to death		65 (1–221)
Disease-free interval		60 (0–346)

Polymorphism analysis for breast cancer risk was carried out using status at sampling, and all preceding data were collected at sampling. In addition, to carry out a preliminary investigation into the association of *VDR* polymorphisms with disease progression, the status of cancer patients was also reexamined at the end of the recruitment period (October 31, 2002), and these results are shown in Table 2. At this point, 40 women had local recurrence, (this number includes 11 women who also had metastases), 12 women had new primary breast tumors, and 58 had metastatic disease. Forty-three patients in the study were deceased at the end of recruitment, 38 from breast cancer. All of the women in the control group were still breast cancer free on October 31, 2002.

Genotyping. For all of the control women and for the 373 women in the cancer group, a 10-ml blood sample was collected into a lithium heparin tube, and whole blood was used for the extraction of DNA. In the remaining 26 cancer patients, DNA was extracted from a sample of their tumor tissue stored in liquid nitrogen. DNA extractions from blood and tissue were carried out using the GenElute mammalian genomic DNA kit (Sigma, Poole, United Kingdom). Genomic DNA was amplified by PCR using primers as described previously: *Bsm*I (16), *Fok*I (10), poly(A) (18). For *Bsm*I and *Fok*I genotyping, PCR product was digested with the appropriate restriction endonuclease (New England Biolabs Ltd, Hitching, United Kingdom), was separated on 1× 5 agarose gels, and was visualized by ethidium bromide staining. *Bsm*I and *Fok*I genotypes were defined by capital letters (*B* and *F*, respectively) in the absence of the restriction site and by small letters (*b* and *f*) where the restriction site was present. For the poly(A) analysis, a 425-bp PCR product was separated on a 6% PAGE-urea gel and was visualized by staining with a 0.1% solution of silver nitrate (Sigma). Under these conditions, the poly(A) region resolved into two distinct bands, long (L, A_{18}–A_{24}) and short (S, A_{13}–A_{17}).

Statistical Analysis. The χ^2 test was used to assess any association between *VDR* polymorphisms and breast cancer risk, and to calculate whether allele frequencies deviated from expected Hardy-Weinberg equilibrium. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to determine the risk of breast cancer associated with a given *VDR* genotype. Logistic regression was used to adjust the risk of breast cancer for age, menopausal status and HRT use. Odds ratios were adjusted primarily by using age of controls at sampling, and age of cases at diagnosis because age at diagnosis is related to breast cancer risk. To investigate whether results were biased by recall or time period effects, analysis was repeated and odds ratios were adjusted using age of controls and cases at sampling, because subjects were recruited on the basis of their status at time of sampling. A P value of ≤ 0.05 was considered as significant. All analysis was undertaken in Stata 7.0 (24).

RESULTS

Characteristics. There is a significant difference in the median age of the cases and controls at sampling (60.5 *versus* 54 years; $P < 0.001$; Table 1). However there is no difference in age of controls at sampling and age of cases at diagnosis, (54 *versus* 56 years; $P = 0.72$; Table 1). Significant differences were seen in menopausal status (premenopausal, 11.5% *versus*

30.9%) and HRT use (past or present use, 53.6% *versus* 28.4%) between the controls and cases ($P < 0.001$; Table 1); however, the reason that a higher proportion of cases are premenopausal is due to the broader age range of the cancer population. Nevertheless, among only 50–65 year olds, an unbiased sample excluding self-referrals, the distribution is very similar with 55% and 38% of controls and cases, respectively, having currently or previously used HRT. HRT use is lower in the cancer group because HRT use is usually ceased at diagnosis, and a smaller percentage of women will be prescribed HRT after having breast cancer than in the control population.

***Bsm*I and Poly(A) Polymorphisms Are Associated with Breast Cancer Risk.** The frequencies of *VDR* polymorphisms found in our sample populations are shown in Table 3. There was no deviation from the expected Hardy-Weinberg frequency ($P > 0.05$) in either the control or cancer population. The 3′ *VDR* polymorphism *Bsm*I and the variable-length poly(A) sequence are in linkage disequilibrium, such that, in this Caucasian population, *bb* genotype cosegregated with long poly(A) genotype, LL, in 97.7% of cases and controls (806 of 825). We found that both *Bsm*I and poly(A) genotypes were significantly associated with breast cancer risk; the odds of developing breast cancer for a woman of genotype *bb* or LL were nearly twice that for a woman of genotype *BB* or SS, (*bb versus BB* genotype, OR = 1.75, 95% CI, 1.15–2.66, $P < 0.01$; LL *versus* SS, OR = 1.70, 95% CI, 1.11–2.60, $P < 0.05$; Table 3). Results were adjusted for age, HRT use, and menopausal status, which did not affect statistical significance of the *Bsm*I OR, but slightly increased the significance of the poly(A) OR (Table 3). In addition, no significant difference was observed between genotype frequencies in control subjects with or without benign disease.

The *Fok*I Polymorphism Is Associated with Breast Cancer Risk When Analyzed in Conjunction with the Poly(A) Variant. The 5′ *VDR* polymorphism, *Fok*I, was not in linkage disequilibrium with the *Bsm*I or poly(A) polymorphisms, as shown in other studies (15, 25), suggesting that they segregate independently. *Fok*I genotype was not associated with breast cancer risk when analyzed in isolation (Table 3). However, some studies have shown that analysis of two *VDR* genotypes simultaneously can increase the statistical significance of any correlations found (reviewed in ref. 23). One example of this is in the study by Ferrari *et al.* (26), who used combined *VDR* genotype analysis in relation to bone mineral density and found stronger correlations in their study population. Therefore, we have investigated whether a combination of the *Fok*I and poly(A) genotypes is associated with breast cancer risk. Fig. 1 shows the distribution of the combined genotypes, and illustrates that *ff*SS is the least common genotype in both cancer and control subjects, whereas the heterozygous *Ff*LS is the most common in the control population, and *Ff*LL is the most common in the cancer population. A χ^2 test including all genotypes showed that the distribution of *Fok*I/poly(A) genotypes is significantly different in women with cancer compared with control women ($P = 0.01$; Fig. 1). Representing the genotype distributions graphically in this way suggested that the genotypes *FF*LL and *Ff*LL are overrepresented in the cancer group (Fig. 1). Combining results from *Fok*I and poly(A) analysis, 26% of controls and 38% of cases have genotypes *FF*LL or *Ff*LL (Fig. 2). There was a highly significant association between *FF*LL/*Ff*LL genotypes and breast cancer risk compared with all other genotypes ($P < 0.001$; Table 4). The odds of developing breast cancer for a woman of genotype *FF*/LL or *Ff*/LL were almost twice (OR = 1.82; CI, 1.35–2.45; Table 4) those of a woman of any other *Fok*I/poly(A) combination, and this was slightly strengthened once the data were adjusted for age, HRT, and menopausal status (Table 4).

The *Fok*I Polymorphism May Be Associated with Breast Cancer Progression When Analyzed in Conjunction with the Poly(A) Variant. We divided the total cancer group ($n = 398$) into women with metastatic disease ($n = 58$) and women without metastatic disease ($n = 340$) to determine whether there was any correlation between combined VDR genotypes and cancer progression. Statistical analysis for disease progression was carried out using patient status at the end of the recruitment period (Table 2). This revealed that compared with controls, the adjusted OR for women with cancer but no metastases was 2.01 (95% CI, 1.43–2.84; $P < 0.001$) and this was increased to 3.35 (95% CI, 1.82–6.16; $P < 0.001$) in cancer

Table 3 VDR polymorphism frequencies

	Controls *n* (%)	Allele frequency	Cases *n* (%)	Allele frequency	Odds ratio (95% CI)	Odds ratio* (95% CI)	Odds ratio† (95% CI)
*Bsm*I							
bb	139 (32.8)	*b* 0.58	173 (42.7)	*b* 0.65	1.75 (1.15–2.66)‡	1.88 (1.19–2.95)‡	1.92 (1.20–3.10)‡
Bb	215 (50.1)	*B* 0.42	173 (44.5)	*B* 0.35	1.13 (0.75–1.70)	1.07 (0.69–1.65)	1.00 (0.63–1.59)
BB	73 (17.1)		52 (12.8)		1.00	1.00	1.00
Poly(A)							
LL	141 (33.0)	*L* 0.58	169 (42.4)	*L* 0.65	1.70 (1.11–2.60)§	1.90 (1.20–3.01)‡	1.94 (1.20–3.14)‡
LS	215 (50.4)	*S* 0.42	179 (45.0)	*S* 0.35	1.18 (0.78–1.79)	1.17 (0.75–1.82)	1.10 (0.70–1.77)
SS	71 (16.6)		50 (12.6)		1.00	1.00	1.00
*Fok*I							
FF	159 (37.2)	*F* 0.60	163 (40.7)	*F* 0.64	1.42 (0.93–2.16)	1.31 (0.84–2.06)	1.33 (0.83–2.14)
Ff	196 (45.9)	*f* 0.40	183 (46.0)	*f* 0.36	1.29 (0.86–1.95)	1.26 (0.81–1.95)	1.28 (0.81–2.04)
ff	72 (16.9)		52 (13.3)		1.00	1.00	1.00

* Adjusted odds ratio for age (of controls at sampling and cases at diagnosis), HRT usage, and menopausal status.
† Adjusted odds ratio for age (of controls at sampling and cases at sampling), HRT usage, and menopausal status.
‡ $P < 0.01$, statistically significant.
§ $P < 0.05$, statistically significant.

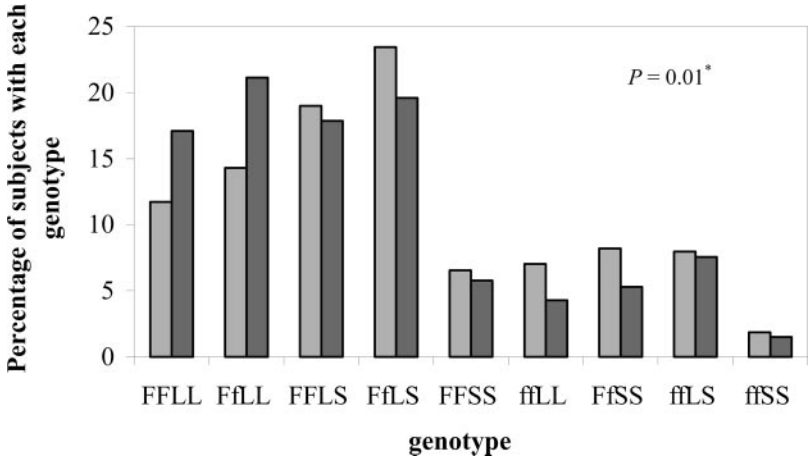

Fig. 1 Analysis of combined *Fok*I and poly-(adenylate) genotypes in control women compared with cancer patients. Percentages of control women ($n = 427$) and women with cancer ($n = 398$) with each of the combined *Fok*I and poly-(adenylate) genotypes. The *FF*LL and *Ff*LL genotypes are overrepresented in the cancer group. The distribution of all genotypes is significantly different in women with cancer (*dark gray bars*) compared with control women (*light gray bars*; $P = 0.01$), as assessed by the χ^2 test, (degrees of freedom = 8). *FF*LL and *Ff*LL genotypes have a more significant association with breast cancer than the other genotype combinations ($P < 0.001$; degrees of freedom = 1; see Table 4). □, Control; ■, Cancer.

patients with metastatic disease (Table 5). The difference in *FF*LL/*Ff*LL genotypes between the cancer patients who have metastatic disease and those who do not is not statistically significant ($P = 0.09$). However, this metastatic group is relatively small and results suggest that the *FF*LL/*Ff*LL genotypes are associated not only with the risk of breast cancer, but might also be associated with disease progression. It must also be remembered that patients in the cancer-without-metastases subgroup may well develop metastases subsequently.

No Association Was Seen between VDR Polymorphisms and Clinical Parameters. *Bsm*I, *Fok*I and poly(A) genotypes were not associated with tumor grade, lymph node involvement, or estrogen receptor status in patients with invasive forms of breast cancer, either when assessed in isolation (data not shown) or when *Fok*I and poly(A) were combined, (Table 6). Adjusting for age, HRT use, and menopausal status does not make any difference to these results (Table 6).

The *bb* and *FF*LL/*Ff*LL Genotypes Are Overrepresented in Breast Cancer Cell Lines. Twenty-five commonly used breast cancer cell lines were genotyped for *VDR* polymorphisms (Table 7); 56% were found to have the *bb* genotype (Fig. 2). This is a high proportion when compared with our volunteer control group (33% *bb*) and the total cancer patient group (43% *bb*; Fig. 2). In addition, analysis using the *Fok*I/poly(A) cross-genotype method showed that 40% of the cell lines express the *FF*LL/*Ff*LL combined genotype compared with 26% in the control volunteers, and 38% in the total cancer patient group, (Fig. 2). We have also observed that the highest proportion of *bb* and *FF*LL/*Ff*LL genotypes in our case–control study (47% and 46.5%, respectively) occur in women with metastatic breast cancer (Fig. 2), although this is not statistically significant, possibly because of the relatively low number of patients with metastatic disease. It is notable then that most of the cell lines are derived from metastatic breast cancers (see Table 7).

DISCUSSION

We have investigated the association between VDR genotype and breast cancer risk in a United Kingdom Caucasian

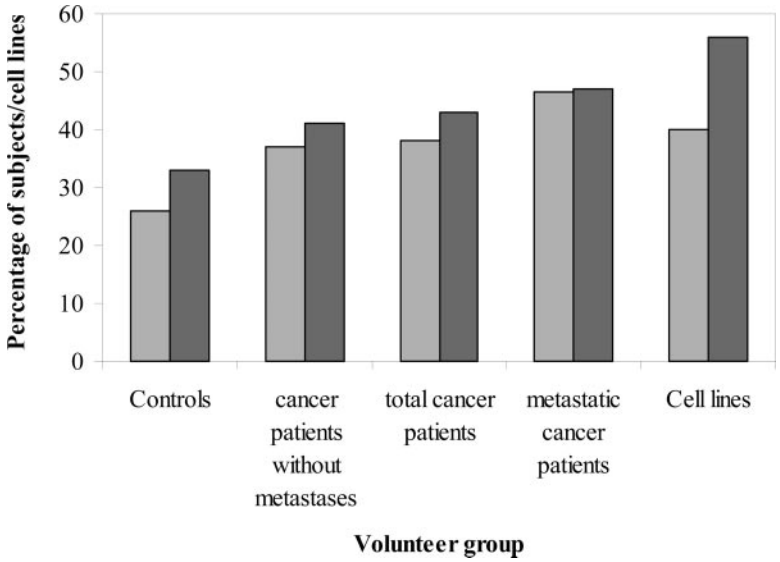

Fig. 2 Percentages of subjects with *bb* or *FF*LL/*Ff*LL genotypes in different volunteer groups, and in common breast cancer cell lines. The control group has the lowest proportion of subjects with genotype *bb* (*dark gray bars*) or genotype *FF*LL/*Ff*LL (*light gray bars*). This proportion increases in cancer patients without metastases and in the total cancer patient group. When patients with metastases were separated from the total cancer cases, the percentage of subjects with genotypes *bb* or *FF*LL/*Ff*LL was higher. The breast cancer cell lines, many of which are derived from patients with breast cancer metastases, displayed the highest percentage of *bb* genotypes and also a high percentage of *FF*LL/*Ff*LL genotypes. □, *FF*LL/*Ff*LL; ■, *bb*.

Table 4 Frequency of *FF*LL and *Ff*LL genotypes *versus* all other *Fok*I and poly(adenylate) combinations

	Control *n*	Total cancer patients, n	Odds ratio (95% CI)	Odds ratio* (95% CI)	Odds ratio† (95% CI)
FFLL or FfLL	109	153	1.82 (1.35–2.45)‡	1.99 (1.44–2.75)‡	2.13 (1.52–2.99)‡
All other *Fok*I/poly(adenylate) combinations	318	245	1.00	1.00	1.00
Total	427	398			

* Adjusted odds ratio for age (of controls at sampling and cases at diagnosis), HRT usage, and menopausal status.
† Adjusted odds ratio for age (of controls at sampling and cases at sampling), HRT usage, and menopausal status.
‡ $P < 0.001$; statistically significant.

Table 5 Frequency of *FF*LL and *Ff*LL genotypes *versus* all other *Fok*I and poly(A) combinations in controls and cancer patients with and without metastases

	*FF*LL or *Ff*LL	All other *Fok*I/poly(A) combinations	Total	Odds ratio (95% CI)	Odds ratio* (95% CI)	Odds ratio† (95% CI)
Control	109	318	427	1.00	1.00	1.00
Cancer patients (no metastases)	126	214	340	1.72 (1.26–2.34)‡	1.87 (1.34–2.61)‡	2.01 (1.43–2.84)‡
Cancer patients (with metastases)	27	31	58	2.54 (1.45–4.45)‡	3.07 (1.70–5.54)‡	3.35 (1.82–6.16)‡

* Adjusted odds ratio for age (of controls at sampling and cases at diagnosis), HRT usage, and menopausal status.
† Adjusted odds ratio for age (of controls at sampling and cases at sampling), HRT usage, and menopausal status.
‡ $P < 0.001$.

population. Results of this study show that, in accordance with the original pilot study (22), the 3′ polymorphisms *Bsm*I and variable-length poly(A) are significantly associated with breast cancer risk, because the *bb* and LL variants are overrepresented in the cancer group.

Previous investigations of *VDR* polymorphisms and breast cancer risk by other groups have produced inconsistent results. Curran *et al.,* (19) showed that the *VDR* polymorphisms *Apa*I and *Taq*I were significantly associated with breast cancer risk in a Caucasian population, such that haplotype *aT* increased risk. This corresponds with our findings because *baTL* alleles are in strong linkage disequilibrium in Caucasians (15). A similar association was reported in a study on a Japanese population, in which the *bb* genotype conferred an almost 4-fold increase in the risk of breast cancer (27). However, in contrast to our findings, four studies have reported no link between *VDR* polymorphisms and breast cancer risk in Caucasian subjects (20, 28–30). Three of these studies determined only the *Taq*I genotype, and it is not apparent whether the control women were assessed for the absence of breast cancer by mammography (20, 29, 30). Furthermore, the study by Lundin *et al.* (20) was carried out exclusively in premenopausal women (age range, 24–36 years), making it more likely that these cases were familial, and, therefore, cannot be directly compared with our results. Ruggiero *et al.,* (28) found no difference between *bb* and *BB* frequencies in patients with primary breast cancer, although the number of subjects in each group was relatively small ($n = 50$ cases, 167 controls). Our study has looked at both prevalent and incident cases, and such a study design may lead to some survival bias in the prevalent cases; unfortunately, however, the number of incident cases ($n = 184$) would not be enough to analyze on its own.

Table 6 Frequency of *FF*LL and *Ff*LL genotypes *versus* all other *Fok*I and poly(adenylate) combinations; comparison of odds ratios in breast cancer patients by tumor grade, lymph node involvement, and estrogen receptor (ER) status

	*FF*LL or *Ff*LL	All other *Fok*I/poly(adenylate) combinations	Odds ratio (95% CI)	Odds ratio* (95% CI)	Odds ratio† (95% CI)
Tumor grade (invasive types only)					
I	58	29	1.00	1.00	1.00
II	80	63	1.58 (0.90–2.74)	1.57 (0.90–2.75)	1.55 (0.88–2.71)
III	74	44	1.19 (0.66–2.13)	1.17 (0.65–2.11)	1.12 (0.62–2.04)
Total	212	136			
Lymph node (invasive types only)					
Involvement	155	94	1.00	1.00	1.00
No involvement	68	50	1.21 (0.78–1.89)	1.21 (0.76–1.91)	1.18 (0.75–1.87)
Total	223	144			
ER status (invasive types only)					
Negative	58	39	1.00	1.00	1.00
Positive	161	102	0.94 (0.59–1.52)	0.95 (0.58–1.53)	0.95 (0.59–1.54)
Total	219	141			

* Adjusted odds ratio for age (of controls at sampling and cases at diagnosis), HRT usage, and menopausal status.
† Adjusted odds ratio for age (of controls at sampling and cases at sampling), HRT usage, and menopausal status.

Table 7 VDR genotypes of commonly used breast cancer cell lines

Breast cancer cell line	Origin	Ethnic origin of patient	Genotype
MCF7	Metastatic pleural effusion	Caucasian	*bb FF* LL
MDA 361	Brain metastases	Caucasian	*bb FF* LL
ZR75–30	Malignant ascitic effusion	Negroid	*bb FF* LL
MDA 231	Pleural effusion	Caucasian	*bb FF* LL
HBL100	From milk of a nursing mother, tumorigenic	Caucasian	*bb FF* LL
MDA 134	Metastatic pleural effusion	Caucasian	*bb Ff* LL
MDA 175	Metastatic pleural effusion	Negroid	*bb Ff* LL
PMC42	Unknown	Unknown	*bb Ff* LL
MDA 330	Metastatic pleural effusion	Caucasian	*bb Ff* LL
SKBR3	Metastatic pleural effusion	Caucasian	*bb Ff* LL
MDA 157	Metastatic pleural effusion	Negroid	*bb ff* LL
MDA 468	Metastatic pleural effusion	Negroid	*bb ff* LL
ZR75–1	Malignant ascitic effusion	Caucasian	*bb ff* LL
BT20	Breast carcinoma	Caucasian	*bb ff* LL
BT474	Invasive ductal breast carcinoma	Caucasian	*Bb FF* LS
HMT 3552	Benign breast tumor	Unknown	*Bb Ff* LS
T47D	Metastatic pleural effusion	Unknown	*Bb Ff* LS
MDA 453	Metastatic pleural effusion	Caucasian	*Bb Ff* LS
SKBR5	Unknown	Unknown	*Bb Ff* LS
CAMA1	Metastatic pleural effusion	Caucasian	*Bb Ff* SS
GI 101	Invasive ductal carcinoma	Unknown	*Bb Ff* SS
SKBR7	Unknown	Unknown	*BB Ff* SS
CAZ51	Unknown	Unknown	*BB FF* SS
MDA 435	Invasive ductal carcinoma	Caucasian	*BB FF* SS
Hs578T	Invasive ductal breast carcinoma	Caucasian	*BB FF* SS

The studies by Ruggiero *et al.* (28) and Lundin *et al.* (20) did however report that progression of breast cancer was associated with *VDR* polymorphisms; the *bb* genotype gave a four times higher risk of developing metastases compared with *BB* (28), whereas the *TT* genotype was associated with an increased risk of 1.8 for lymph node metastases (20). These results substantiate our preliminary finding that the "at risk" haplotype *baTL* is more prevalent in women who have developed metastatic disease, suggesting that, in Caucasian women, *VDR* polymorphisms are associated with progression of breast cancer. Routine *VDR* genotyping of women with breast cancer may, therefore, be a useful prognostic tool. Interestingly, although we suggest that the *b* allele is associated with increased breast cancer risk, other studies have reported a positive association with bone mineral density (16, 31), and this is concordant with an observed increased rate of breast cancer among women with high bone mass (32).

It has also been reported that low levels of circulating 1,25-D_3 are related to breast cancer risk (33) and development of bone metastases (34), which corresponds with the putative antitumorigenic role of 1,25-D_3. It is plausible that polymorphisms in the *VDR* gene alter the ability of 1,25-D_3 to interact with the *VDR* gene product so that, even if a woman is not deficient in 1,25-D_3, the ligand is not able to effectively induce transcription of genes via the VDR. The interaction of 1,25-D_3 with the VDR produces a conformational change in the VDR, permitting heterodimerization with the retinoid X receptor and leading to interaction with vitamin D response elements, and transcriptional regulation of vitamin D target genes. At least 26 genes are thought to be regulated in this way by 1,25-D_3 (35), and some of these are involved in cell cycle regulation, differentiation, and apoptosis, such as *WAF1/CIP1* and *c-myc*. We hypothesize that polymorphisms in the *VDR* gene might lead to differential responsiveness of target cells to the action of 1,25-D_3. Specifically the *baTL* haplotype might modify the ability of 1,25-D_3 to regulate its target genes in a normal manner, by altering the expression or stability of the VDR, or by the ability of the VDR to interact with 1,25-D_3, retinoid X receptor, or vitamin D response elements.

The *Fok*I polymorphism results in two differently sized proteins ($F = 424$ and $f = 427$ amino acids in length), and this may have an effect on VDR function. Although the *bbFFLL* genotype is associated with greater breast cancer risk and faster progression of breast cancer in this study, suggesting that *F* is less active than *f*, this is not reflected in other studies that have used different cell systems. For instance, functional studies have suggested that the *F* allele is the more active *VDR* haplotype, being more efficient in bringing about the effects of 1,25-D_3 than is *f*, in transfected HeLa cells (36) and peripheral blood mononuclear cells (37). In addition, by transfecting *F*-VDR and *f*-VDR into VDR-null monkey fibroblasts (COS-7) it has been shown that the *F* variant binds more efficiently with the transcription cofactor TFIIB, which elevates its transcriptional activity compared with *f* (38). By contrast, using transfected COS-7 cells, Gross *et al.*, (39) found no difference in the ability of *f* or *F* to bind either 1,25-D_3, or DNA, or to transactivate the expression of vitamin D responsive genes. It should be noted that all of these studies (36–39) investigated the *FokI* polymorphism in isolation. Whitfield *et al.*, (23) also found that there was no difference in transcriptional activity when assessing VDR encoded by *Fok*I variants in human fibroblast cell lines. However, differences were seen when *Fok*I and poly(A) variants were assessed in combination; these workers designated an "allele score" (where *FF*LL = 4 and *ff*SS = 0) and found a positive association with transcriptional activity induced by 1,25-D_3 in which *F*/L was more active than *f*/S. Although we

have found a significant association with breast cancer risk using cross-genotyping analysis of *Fok*I and poly(A) variants, our results oppose those of Whitfield *et al.,* (23), because we find that the *FF*LL genotype is more common in women with breast cancer and advanced disease. This would suggest that in breast cancer cells the *F* variant, when in combination with the long form L, of poly(A), is less active and, therefore, less able to exert the antitumorigenic effects of 1,25-D_3. To support this theory, one recent study has shown that the *FF* genotype is also more prevalent in aggressive prostate cancers than *ff* (40). It is, therefore, conceivable that *F* and L might be less active in breast (and prostate) cells if the functional effects of *VDR* polymorphisms were cell-type specific, although to our knowledge no functional experiments have been carried out on breast or breast cancer cells. However, because much of the literature does not support our hypothesis that *F*/L are less active *VDR* alleles, it is more likely that *F* and L may increase cancer risk in breast and prostate tissues via an unknown mechanism.

Because *Bsm*I, *Taq*I, and *Apa*I polymorphisms do not lead to changes in the VDR protein amino acid sequence, it remains difficult to explain how these variations might influence VDR function. To date, most studies have shown no correlation between these 3′ *VDR* polymorphisms individually and the abundance of *VDR* message (41, 42), VDR protein expression and function (43), or mRNA stability (41, 44). However, Carling *et al.,* (45) reported that Caucasian patients with parathyroid tumors are more often homozygous for the *baT* genotype than in controls and that *VDR* mRNA levels were lower in these patients compared with patients with a *BBAAtt* genotype.

It might be the case that in Caucasian subjects the *Bsm*I/*Apa*I/*Taq*I cluster does not influence VDR function, but because of genetic linkage, it merely acts as a marker for the poly(A) sequence within the 3′ UTR. 3′ UTR sequences are often critical for determining transcript stability (reviewed in ref. 46), and the 3′ UTR regions of the steroid hormone receptor family are unusually long and highly conserved (47), suggesting that they play an important functional role. It is, therefore, possible that the area making up the internal poly(A) polymorphism in the VDR might be important for the regulation of mRNA stability. One possible mechanism could involve adenylate/uridylate-rich elements found to be present in the 3′ UTR. It is known that the 3′UTR of several genes (cited in ref. 47), including members of the steroid hormone receptor family, possess adenylate/uridylate-rich elements. Adenylate/uridylate-rich elements contain multiple copies of the motif AUUUA, which act as destabilizing elements by recruiting a set of adenylate/uridylate-rich element-binding proteins that facilitate mRNA degradation (reviewed in ref. 48). In a published sequence of the *VDR* gene (GenBank accession no. 004466) we have found three AUUUA motifs present in the 3′ UTR, near to the poly(A) sequence, leading us to speculate that the differences in the length of the poly(A) sequence might affect the binding of adenylate/uridylate-rich element-binding proteins. However, it has not yet been shown that adenylate/uridylate-rich elements are implicated in regulation of *VDR* mRNA stability, and there does not appear to be any literature regarding the potential importance of internal poly(A) sequences. Furthermore, Whitfield *et al.,* (23) surmise that poly(A) binding proteins might bind differentially to the L and S forms of the poly(A) sequence within the 3′UTR, producing different functional consequences. Poly(A) binding proteins are commonly known to bind to the poly(A)+ tails of RNA and may be implicated in positively regulating translatability of mRNAs via a possible interaction with the 5′ end of genes (49). Interestingly, it has been shown that poly(A) binding proteins can bind to a region of adenylate residues 11–25 in length (50) and may, therefore, have the ability to bind to internal poly(A) tracts, such as the one in the *VDR* 3′ UTR.

In the future, we wish to establish *in vitro* models to further assess the functional significance of the polymorphisms; therefore, we assessed the *VDR* genotype of 25 commonly used breast cancer cells lines. Similar to the results from the patient study, the genotypes *bb* and *FF*LL/*Ff*LL were overrepresented in the breast cancer cell lines providing support for the hypothesis that these genotypes increase the risk of breast cancer. We have found a range of *VDR* haplotypes in the breast cancer cell lines, which in future will enable us to investigate how VDR polymorphisms may influence the function of the *VDR* in breast cancer cells.

When analyzed in isolation, the 5′ *Fok*I polymorphism (variants *FF*, *Ff*, *ff*) was not associated with breast cancer risk. However, we have found that by examining the 5′ and 3′ polymorphisms simultaneously, a statistically significant increase in risk of breast cancer occurs in which a subject possesses one or more *F* alleles together with the LL genotype. Many studies have investigated individual *VDR* polymorphisms in isolation when assessing the association with different disease states, but these results are often nonsignificant or conflicting (reviewed in ref. 51). However, a small number of studies have carried out cross-genotyping analysis on the *VDR* polymorphisms and have found that this can reveal a positive association with disease status. For example, Ingles *et al.,* (11) observed that in African-American women, the LS and LL poly(A) variants had a 50% decreased risk of breast cancer compared with women of genotype SS, but that this protective effect was limited to women who have the *FF Fok*I variant. Whitfield *et al.,* (23) transfected 20 human fibroblast cell lines of varying *VDR* genotypes with a 1,25-D_3-responsive reporter-plasmid, and assessed their endogenous VDR activity. They found that results became statistically significant only when the *Fok*I and poly(A) genotypes were examined simultaneously. It appears, from these studies and the present study, that it is not sufficient to analyze only one *VDR* polymorphism in relation to disease, because the 5′ and 3′ polymorphisms may be functionally linked either through their independent influences on VDR activity, or possibly by physical interaction.

In summary, this study has provided further support for a significant association between specific *VDR* gene polymorphisms and breast cancer risk. This finding may be useful in predicting the likelihood of women developing breast cancer, or whether a woman with breast cancer is likely to develop metastases. At diagnosis, patients at higher risk might then be targeted for more aggressive treatments. To this end, we will continue to monitor the 825 women in the study for any new breast tumor development, recurrence or metastatic spread, so that we can examine further whether the “at risk” genotypes are linked to risk and progression.

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