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Plasma 25-hydroxy vitamin D concentrations, vitamin D receptor genotype and breast cancer risk in a UK Caucasian population

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Abstract

Low levels of 25-hydroxy vitamin D (25(OH)D) and polymorphisms in the vitamin D receptor gene (*VDR*) have been found separately to increase risk of breast cancer. The aim of this study was to determine whether low 25(OH)D levels, alone and in combination with *Bsm*I *VDR* genotype, increased breast cancer risk in a United Kingdom (UK) Caucasian population. Breast cancer patients ($n = 179$) and control women ($n = 179$) were recruited and 25(OH)D levels measured by enzyme-linked immunosorbent assay (ELISA). *VDR* genotype was determined by polymerase chain reaction (PCR) and restriction enzyme digest. Analysis showed that subjects with 25(OH)D levels <50 nM and the *bb Bsm*I *VDR* genotype are 6.82 times more likely to have breast cancer than subjects with levels of 25(OH)D > 50 nM and either the *BB* or *Bb* genotype (95% confidence interval (CI) 2.31–14.7, $P < 0.001$). This study indicates that low levels of circulating 25(OH)D, both alone and in combination with *Bsm*I *VDR* genotype, may increase risk of breast cancer in a UK Caucasian population.

Keywords: Vitamin D; Breast cancer; Risk; Vitamin D receptor; Polymorphism

1. Introduction

In addition to its well-established role in maintenance of calcium homeostasis, the active form of the hormone vitamin D, 1α, 25-dihydroxy vitamin D ($1,25(OH)_2D$) is known to have potent anti-proliferative effects in many cancer cell types, including breast and prostate cancers [1]. The anti-cancer properties of $1,25(OH)_2D$ include induction of differentiation and apoptosis in addition to inhibition of cancer cell growth. $1,25(OH)_2D$ is produced by hydroxylation of the major circulating form of vitamin D, 25-hydroxy vitamin D (25(OH)D), a reaction catalysed by the renal enzyme 25-hydroxy vitamin D-1α-hydroxylase, which has recently been found to be expressed in a variety of tissues such as colon [2], breast, cervix and ovary [3]. This presents the possibility that the non-classical effects of vitamin D may be linked to extra-renal expression of this enzyme and paracrine/autocrine production of $1,25(OH)_2D$.

Laboratory studies have demonstrated that $1,25(OH)_2D$ and its synthetic analogues inhibit growth and induce apoptosis in cultured breast cancer cells and in animal models of breast cancer [4]. Epidemiological

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and laboratory studies have suggested that adequate vitamin D status may play a role in reducing breast cancer risk. Vitamin D status is dependent upon cutaneous synthesis initiated by solar radiation and also on dietary intake; a reduction in one or both sources leads to vitamin D insufficiency. Population studies have suggested that risk of fatal breast cancer in the major urban areas is inversely related to the intensity of local sunlight [5] and reduced risk of breast cancer has been observed in women with high dietary vitamin D intake [6]. Circulating levels of 25(OH)D are directly related to dietary vitamin D intake and cutaneous synthesis. The capacity to generate $1,25(OH)_2D$ locally in the breast will be dependent on the availability of 25(OH)D as substrate for 1α-hydroxylase. Thus it could be postulated that low circulating concentrations of 25(OH)D impair generation of $1,25(OH)_2D$ within breast tissue, increasing risk of tumour development.

$1,25(OH)_2D$ exerts both its anti-cancer effects and its effects on calcium homeostasis by binding to its intracellular receptor, the vitamin D receptor (VDR). Upon ligand binding VDR undergoes a conformational change that causes its dissociation from transcriptional repressors and binding to transcriptional activators. VDR then acts as a transcription factor that binds to specific elements within target genes, which ultimately leads to changes in gene transcription [7]. There are a number of polymorphisms in the gene encoding the VDR (*VDR*), some of which have been linked to risk of certain types of cancer, including prostate [8,9], colorectal [10] and renal carcinoma [11]. A single nucleotide polymorphism (SNP) in the 3′ end of the gene, *Bsm*I, present in two forms denoted *B* and *b*, has previously been linked to breast cancer risk; in a study of Caucasian women, we have found that women with the *bb* genotype had almost twice the risk of breast cancer compared with those with the *BB* genotype [12]. This study group has been substantially enlarged, and a significant relationship between the *VDR* polymorphism *Bsm*I and breast cancer risk confirmed [13]. In the present study, we have quantitated circulating 25(OH)D concentrations in matched cases and controls in order to assess the combined effect of the *VDR* polymorphism and circulating concentrations of 25(OH)D on risk of breast cancer in our population.

2. Materials and methods

2.1. Volunteers

Ethical approval for this study was received from the St. George's Hospital Ethics Committee. Breast cancer patients and control volunteers were recruited as part of a study investigating *VDR* genotype and risk of breast cancer. Only Caucasian women were recruited. All gave written informed consent before providing a 10-ml blood sample and answering a brief questionnaire. The questionnaire was used to determine personal information, such as use of hormone replacement therapy (HRT) and menopausal status. In addition to this, volunteers were asked whether they were vegetarians or vegans. No detailed dietary information was requested.

2.1.1. Breast cancer volunteers

Breast cancer patients ($n = 179$) were recruited from the Combined Breast Clinic in St. George's Hospital between 13th August 1998 and 15 October 2003. A wide variety of patients participated in the study, such that some patients were newly diagnosed ($n = 47$), others had been diagnosed many years before and were attending 6 month or yearly follow-up clinics ($n = 89$) while others were patients with recurrent ($n = 14$) or metastatic disease ($n = 29$). In each case information on tumour grade, receptor status and lymph node involvement was taken from histopathology reports and/or patient notes. Family history of breast cancer was determined using the questionnaire and defined as: none (no family history), weak (one second-degree relative, defined as aunt, cousin or grandmother with breast cancer) and strong (one or more first-degree relative, defined as mother or sister, or more than one second-degree relative with breast cancer).

2.1.2. Control volunteers

A total of 131 control volunteers were recruited from the Duchess of Kent breast-screening clinic on hospital grounds after having a negative mammogram. A total of 48 control volunteers were recruited from the same clinic where they were being investigated for benign disease, the majority of which were benign calcifications, fibrocystic disease and fibroadenoma, none of which increase risk of breast cancer.

2.1.3. Matching volunteers

Breast cancer patients were each matched to one control volunteer. The main criteria for matching was time of year the blood sample was taken, since vitamin D is derived from cutaneous synthesis in response to sunlight exposure. The year was divided into summer (April–September) and winter months (October–March). Each case was matched to a control subject where the sample was obtained in the same half of the year and also within 2 months of each other. The majority of patients was matched to a control that had the same menopausal status, although 14 were not. The majority was also matched closely for age at sampling. The median difference in age at sampling was 2 years (range 0–12 years). No patient or control volunteers were vegetarians or vegans.

2.2. Quantitation of plasma 25-hydroxy vitamin D concentration

Aliquots of whole blood were taken for *VDR* genotyping and the remaining blood was separated into plasma and buffy coat by centrifugation. The plasma was then used to measure circulating 25(OH)D concentration by enzyme immunoassay (ImmunoDiagnostics Services, UK). This is a competitive assay in which biotin-labelled 25(OH)D is added to standards and samples which are then incubated in microtitre wells coated with a highly specific sheep 25(OH)D antibody. This antibody is 100% specific for 25-hydroxy vitamin D_3 and 75% specific for 25-hydroxy vitamin D_2. Horseradish peroxidase-labelled avidin is added, which binds to complexed biotin and colour is developed using a chromogenic substrate. The absorbance of each well was determined in a microtitre plate reader at 450 nm (reference 650 nm). Each sample was measured in duplicate and 25(OH)D levels were determined with a standard curve using standards supplied with the kit. Patient and control samples were run on the same plates.

2.3. Genotyping

Genomic DNA was extracted from whole blood samples using a GenElute blood genomic DNA kit (Sigma). *Bsm*I genotype was determined by performing a PCR reaction followed by a restriction digest. PCR was performed using a forward primer (5′ CAACCAAGACTACAAGTACCGCGTCAGTGA 3′) and reverse primer (5′ AACCAGCGGGAAGAGGTCAAGGG 3′), both specific for the *Bsm*I region of *VDR*. The PCR product was digested using the *Bsm*I enzyme (BioLabs). Restriction digest products were separated on a 1.5% agarose gel and visualised using ethidium bromide.

2.4. Statistical analysis

The χ^2 test was used to calculate whether allele frequencies deviated from expected Hardy–Weinberg equilibrium. Conditional logistic regression was used to determine the risk of breast cancer associated with a given level of 25(OH)D in this matched case control study. Odds ratios and 95% confidence intervals (CI) were adjusted for matching variables; time of year, age at sampling and menopausal status. A P value $\leqslant 0.05$ was considered significant. All analysis was undertaken in Stata 8.2.

3. Results

3.1. Characteristics of cases

The median age of the cases was 58 (range 34–84) years. A total of 160 (89.4%) women were postmenopausal, 117 (65.4%) had never used HRT and 126 (70.4%) had no family history of breast cancer. A total of 167 women (93%) had invasive ductal carcinoma and 140 (78%) were oestrogen receptor (ER) positive. The remaining 12 patients had ductal carcinoma *in situ* (DCIS). A total of 124 (69%) patients received postoperative radiotherapy. Fourteen patients had a local recurrence only and 29 had metastatic disease, of which 18 had metastases at the time of sampling and 11 developed metastases after sampling.

3.2. Characteristics of controls

The median age of the control group was also 58 (range 36–80) years, 164 women (91.6%) were postmenopausal, 90 (50.3%) had never used HRT and 150 (83.8%) had no family history of breast cancer.

3.3. Circulating 25-hydroxy vitamin D concentrations in cases and controls

Mean plasma 25-hydroxy vitamin D (25(OH)D) concentration was significantly lower in breast cancer patients than in matched controls (80.1 nM, SD 43.4 *versus* 97.8, SD 41, $P < 0.001$). Values were then separated into four quartiles; quartile 1 $\leqslant$ 50 nM, quartile 2 = 50–100 nM, quartile 3 = 100–150 nM, quartile 4 = 150–200 nM. Analysis shows that risk of breast cancer is greatest for women grouped in quartile 1, i.e., those with the lowest, insufficient levels of 25(OH)D. Compared with women with the highest levels of 25(OH)D (quartile 4), women in quartile 1 have more than five times the risk of breast cancer (OR = 5.83 (95% CI 2.31–14.7), $P < 0.001$). The increase in risk of breast cancer for women in quartiles 1–3 compared with women in quartile 4 can be seen in Table 1. Overall, subjects with 25(OH)D levels <50 nM have an odds ratio (OR) of 3.54 (95% CI 1.89–6.61, $P < 0.001$) for breast cancer risk compared with those with levels >50 nM. If this analysis is restricted to the 47 newly diagnosed cases and their controls, the above OR becomes 3.67, which is borderline significant (95% CI 0.97–13.9, $P = 0.06$).

Table 1
Number of controls and cases in each 25(OH)D quartile and odds ratio (OR) for breast cancer risk for each quartile

Quartile (nM)	Controls *n* (%)	Cases *n* (%)	OR (95% CI)[a]	*P*-value
1 (<50)	21 (12)	54 (30)	5.83 (2.31 – 14.7)	<0.001
2 (50–100)	79 (44)	69 (39)	1.83 (0.83 – 4.03)	0.13
3 (100–150)	54 (30)	43 (24)	1.61 (0.71 – 3.64)	0.25
4 (>150)	25 (14)	13 (7)	1.0	

CI, confidence interval.
[a] ORs are for an increase in breast cancer risk for women in each quartile compared with women in quartile 4 (>150 nM).

3.4. VDR polymorphisms and circulating 25-hydroxy vitamin D

We have previously reported that the *Bsm*I polymorphism (*B*/*b*) in the gene encoding the vitamin D receptor (*VDR*) is associated with breast cancer risk in a UK Caucasian population such that presence of two *b* alleles almost doubles risk of developing the disease compared with the *BB* genotype. In this study population, we found that the *bb* genotype was present in 84 (47%) patients and 52 (29%) controls. There was no deviation from the expected Hardy–Weinberg frequency in either the control ($P > 0.05$) or cancer population ($P > 0.1$). The odds ratio for breast cancer risk for women with the *bb* genotype was 2.02 (95% CI 1.03–3.97, $P = 0.04$) compared with the *BB* genotype (Table 2).

Subjects were subdivided into four groups; those who have (A) the *bb* genotype and insufficient (<50 nM) 25(OH)D levels, (B) the *bb* genotype and sufficient (>50 nM) 25(OH)D levels, (C) the *Bb* or *BB* genotype and insufficient 25(OH)D levels, and (D) the *Bb* or *BB* genotype and sufficient 25(OH)D levels (Table 3). Analysis showed that, after adjustment for age and menopausal status, subjects in group A are 6.82 (95% CI 2.57–17.1) times more likely to have breast cancer than subjects in group D ($P < 0.001$). Subjects in groups B and C, who had just one of the proposed risk factors (either low 25(OH)D levels or the *bb* genotype) also had a higher risk of breast cancer than subjects in group D ($P < 0.001$ and $P = 0.001$, respectively), although this risk was lower than for women in group A.

3.5. Low 25-hydroxy vitamin D levels do not correlate with clinical parameters

Breast cancer is categorised as grade 1, 2 or 3 depending on the state of differentiation of the cells, grade 3 being the least differentiated. Similar numbers of cancer patients in this study were grade 2 and 3, 65 (36%) and 70 (39%), respectively, while 38 (21%) were grade 1, and 6 (4%) were of unknown grade. Analysis showed that 25(OH)D status was not associated with high grade of tumour (i.e., grade 3) in these patients ($P = 0.23$). We also analysed whether 25(OH)D levels correlated with lymph node involvement. A total of 102 (57%) patients in this study had no lymph node involvement, while 58 (32%) did have lymph node involvement and 19 (11%) had no lymph nodes taken. We found no association between lymph node involvement and 25(OH)D status ($P = 0.69$).

Table 2
Number and percentage of cases and controls with each *Bsm*I *VDR* genotype

*Bsm*I genotype	Controls *n* (%)	Cases *n* (%)	OR[a](95% CI)
B	52 (29.1)	84 (46.9)	2.02 (1.03–3.97)*
Bb	99 (55.3)	70 (39.1)	0.71 (0.37–1.36)
BB	28 (15.6)	25 (14)	1.0

CI, confidence interval.
[a] Odds ratios (OR) given are for breast cancer risk for women with each genotype, with the *BB* genotype set at 1.0.
* $P = 0.04$.

4. Discussion

The major action of vitamin D is to maintain bone mineralisation by promoting absorption of dietary calcium. Vitamin D status is dependent upon cutaneous synthesis initiated by solar radiation and also on dietary intake; a reduction in one or both sources will lead to vitamin D insufficiency. Circulating concentrations of 25(OH)D serve as a useful index of vitamin D status and in vitamin D sufficiency, serum levels range from 50 to 200 nM [14]. Previous studies have investigated the relationship between dietary/environmental intake of vitamin D and risk of breast cancer. An early study showed an inverse relationship between risk of fatal breast cancer and intensity of local sunlight [5]. This was supported when several measures of sunlight exposure and vitamin D intake were associated with decreased breast cancer risk [6]. Other studies have shown that low vitamin D levels or sunlight exposure are related to risk of colon [15,16], ovarian and prostate cancers [17].

We now report findings from a study of 179 patients and matched controls, which has shown a significant association of low 25(OH)D levels with breast cancer risk. Women with the lowest levels of 25(OH)D (<50 nM) had over five times the risk of breast cancer compared with women with the highest levels (>150 nM). Overall, women with 25(OH)D levels <50 nM had an OR of 3.54 (95% CI 1.89–6.61, $P < 0.001$) for breast cancer risk compared with those with levels >50 nM. This indicates that low levels of circulating 25(OH)D may contribute to breast cancer development. In our study, cases and controls were

Table 3
Number of controls and cases segregated by genotype and 25(OH)D levels and odds ratios (OR) for breast cancer risk for each group

Group	Controls *n* (%)	Cases *n* (%)	OR (95% CI)	*P*-values
bb and quartile 1	8 (4.5)	25 (14)	6.82 (2.57–17.1)	<0.001
Bb/*BB* and quartile 1	13 (7.3)	29 (16.2)	4.01 (1.89–8.58)	<0.001
bb and quartile 2–4	44 (24.6)	60 (33.5)	2.66 (1.48–4.79)	0.001
Bb/*BB* and quartile 2–4	114 (63.6)	65 (36.3)	1.00	

matched for age at sampling. For those cases not recruited at diagnosis ($n = 132$) it is possible that measured 25(OH)D levels may not reflect 25(OH)D status when these patients were diagnosed. For this reason statistical analysis was repeated on patients recruited at diagnosis ($n = 47$), and matched controls, only. In this group, the OR for breast cancer risk for women with 25(OH)D levels <50 nM compared with those with levels >50 nM (3.67) was comparable to that for the group as a whole (3.54), although of only borderline significance ($P = 0.06$) due to the small number of patients in this group. The OR calculated for the newly diagnosed group suggests that results for the whole group are valid even though not all patients were recruited at diagnosis.

It has been shown that breast tissue contains the 1α-hydroxylase enzyme required for producing $1,25(OH)_2D$ from circulating 25(OH)D [3], suggesting that paracrine production of $1,25(OH)_2D$ could be important in maintenance of normal breast cell function. Local production of $1,25(OH)_2D$ in breast will be dependent on dietary/environmental access to vitamin D and with vitamin D insufficiency, generation of significant amounts of $1,25(OH)_2D$ in breast tissue may be impaired leading to increased risk of tumour development. $1,25(OH)_2D$ mediates its anti-tumour effects via interaction with its nuclear receptor VDR. Several studies have investigated an association between polymorphisms in the gene encoding the VDR (*VDR*) and breast cancer risk. Some studies have reported positive findings [18,19], while others have reported no association [20,21]. Other types of cancer have also been associated with *VDR* polymorphisms including prostate [22,23] and renal carcinoma [11]. We have previously assessed the relationship between the *Bsm*I *VDR* polymorphism and breast cancer risk in a UK Caucasian population and reported an almost doubling of risk associated with the *bb* genotype [13]. The population investigated in the present study has been taken from the larger study population recruited for this previous report, in which 398 cases and 427 controls were involved. Due to the criteria used for matching cases to controls when measuring 25(OH)D levels, at a particular time of year, we were limited to 179 cases and 179 controls. However, the association between the *VDR bb* genotype and breast cancer risk is confirmed in our present study.

Low vitamin D status and *VDR* polymorphisms have been associated with risk of prostate cancer. One study, however, investigated vitamin D status combined with *VDR* genotype and risk of prostate cancer [9]. This group found that *Bsm*I *VDR* genotype was not associated with prostate cancer risk overall, but when men with low levels of 25(OH)D were analysed separately there was an increased risk of the disease associated with the *bb* genotype. This risk was increased further when only men over 61 years of age were analysed. To our knowledge, no previous study has analysed vitamin D status in combination with *VDR* genotype and risk of breast cancer. We found women with the *bb* genotype in combination with low/insufficient 25(OH)D levels had an even greater risk of breast cancer than low 25(OH)D levels alone. After adjustment for age and menopausal status, the OR for breast cancer risk for women with insufficient levels of 25(OH)D *and* the *bb* genotype was 6.82 while women with insufficient 25(OH)D levels alone had an OR of 3.54 for breast cancer risk. It can also be seen from this combination analysis that presence of one of the proposed risk factors (either the *bb* genotype or low 25(OH)D levels) also increases risk of breast cancer compared with women with neither factor, as would be expected, although women with both risk factors had the greatest risk.

The *Bsm*I SNP is intronic and so does not produce any alteration in the translated protein. However, a number of studies [12,24,25] have found strong linkage disequilibrium in Caucasian populations between *Bsm*I and a variable length polymorphism in the 3′ untranslated region (UTR), known as poly (A). This is present in either a long (L, A = 18–24) or short (S, A = 13–17) form, with the L form linked to the *b* form of *Bsm*I in up to 98% of Caucasian populations [12]. It has been speculated that associations found between *Bsm*I genotype and disease are in fact associations between poly (A) and that disease, and that *Bsm*I merely acts as a marker for the poly (A) variant. However, the functional significance of the poly (A) polymorphism is still unknown and so this relationship remains controversial. It is also possible that *Bsm*I genotype acts as a marker for an association between the disease and another gene situated near to *VDR* [26]. Further research is required to determine what exactly *Bsm*I is acting as a marker for, whether it is part of the *VDR* gene or some other gene.

Both *Bsm*I genotype [27] and low circulating vitamin D levels [28] have previously been associated with increased risk of metastatic breast cancer (MBC). In the present study group, we found 29 patients with MBC had lower levels of 25(OH)D than the 150 non-MBC patients (70.3 nM, SD 41.4 *versus* 82 nM, SD 43.7), although the number of patients in the MBC group was too small for this difference to reach statistical significance ($P = 0.18$). Women with MBC did have significantly lower 25(OH)D levels compared with their controls (70.2 nM, SD 41.4 *versus* 107.9 nM, SD 32, $P < 0.001$). However, since 25(OH)D levels did not correlate with clinical parameters that are known to increase the risk of developing MBC (lymph node involvement and high grade of tumour) and the majority of the MBC patients had metastatic disease at sampling ($n = 18$), it cannot be ruled out that this difference is due to a change in lifestyle and nutritional status in MBC patients and/or due to treatment for MBC.

In conclusion, this study provides evidence that low levels of 25(OH)D alone and in combination with the *bb VDR* genotype may be involved in breast cancer development. Maintaining sufficient circulating 25(OH)D concentrations may enable optimal generation of $1,25(OH)_2D$ locally in breast tissue. We suggest that women displaying the *bb* genotype may be less able to benefit from any intrinsic anti-tumour effects of the hormone. These two factors play a role in a complex system whose efficiency depends on many other proteins and genes. Any associated risk may be modified by other factors, such as expression of the *CYP24* gene (which encodes a $1,25(OH)_2D$ deactivating enzyme) or *VDR* co-activators/repressors. However, our present findings provide support to previous studies suggesting an inverse association between vitamin D status and breast cancer risk.

Conflict of interest statement

None declared.

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