Relationship of vitamin D deficiency with mammographic breast density and triple-negative breast cancer: A cross-sectional study

CHANDRA KUNWARI SINGH, SHAJI THOMAS, BINITA GOSWAMI, SHAILI TOMER, OM PRAKASH PATHANIA

ABSTRACT

Background. As breast epithelium is affected by vitamin D, it may have a direct effect on breast density and the risk of breast cancer. Our aim was to study the serum levels of vitamin D in patients with malignant and benign breast disease, and to study the association, if any, between vitamin D levels, mammographic breast density (MD) and molecular subtypes of breast cancer.

Methods. In this cross-sectional, observational study, we enrolled 162 consecutive adult women with benign and malignant breast masses subjected to mammography and core-needle biopsy. Serum levels of vitamin D were estimated and correlated with MD and with immunohistochemical subtyping of breast cancer.

Results. The mean vitamin D level in these 162 patients was 12.44 (5.88) ng/ml, with vitamin D deficiency seen in 98%. The mean (SD) vitamin D level in MD type 1 was 16.19 (4.62) ng/ml and it decreased to 7.54 (2.58) ng/ml in MD type 4. High MD was associated with significantly lower vitamin D levels. The mean vitamin D level in patients with benign breast disease ($n = 102$) was 13.73 (5.68) ng/ml, while it was significantly lower in patients with breast cancer ($n = 60$) at 10.26 (5.61) ng/ml. Among patients with breast cancer, the good prognosis luminal A molecular subtype had mean vitamin D level of 12.94 (6.16) ng/ml, whereas the poor prognosis triple-negative subtype had a significantly lower value of 7.68 (3.42) ng/ml.

Conclusion. Our study shows that vitamin D deficiency has a significant relationship with breast cancer (*v.* benign breast disease), high MD (showing increased breast cancer risk) and poor prognosis triple-negative breast cancer. Vitamin D deficiency could be an important, potentially modifiable, risk factor for the prevention of breast cancer in susceptible populations.

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Lady Hardinge Medical College, Shaheed Bhagat Singh Marg, New Delhi 110001, India
CHANDRA KUNWARI SINGH, SHAJI THOMAS, OM PRAKASH PATHANIA Department of Surgery
BINITA GOSWAMI Department of Biochemistry
SHAILI TOMER Department of Radiology

Correspondence to SHAJI THOMAS; *drshajithomas@yahoo.com*

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INTRODUCTION

Breast epithelium belongs to a group of tissues that are affected by vitamin D,[1,2] leading to the assumption that vitamin D may have a direct effect on breast density and on risk of breast cancer. A meta-analysis showed that women with mammographic breast density (MD) of 75% had a 4–5 times increased risk of breast cancer than those with low breast density.[3] MD is, therefore, a reliable intermediate biomarker for assessing risk of breast cancer.

Research shows an inverse relationship between serum vitamin D levels and the risk of breast cancer.[4] Some studies have reported an up to 3-fold increase in the risk of breast cancer associated with severe vitamin D deficiency,[5] while others have found no correlation.[6]

Only a few studies have been done on the effect of serum vitamin D levels on MD. Brisson *et al*. have shown that an increase in serum vitamin D levels led to a reduction in MD.[7]

A direct association with risk of breast cancer will make vitamin D deficiency an important, potentially modifiable risk factor for prevention of breast cancer in developing countries such as India where vitamin D deficiency is widespread.

Our aim was to study the serum vitamin D levels in patients with malignant and benign breast disease (BBD), and to study the association, if any, between serum vitamin D levels and MD, and between vitamin D levels and molecular subtypes of breast cancer.

METHODS

This cross-sectional study was conducted on 162 consecutive, newly symptomatic women above the age of 35 years, attending the surgical outpatient department of the hospital of Lady Hardinge Medical College, New Delhi, with complaints of breast lump or nodularity, who were subjected to mammography and core needle biopsy.

We excluded patients below 35 years of age, or with infective/inflammatory BBD, women using any vitamin D supplementation in the past 2 years, or those being treated for osteopenia/osteomalacia, those with a history of kidney failure, taking exogenous hormonal treatments, pregnant and lactating women, and patients on long-term medications that alter hormonal

status/mimic symptoms of BBD such as antipsychotics, antidepressants, thiazides, methyldopa and digoxin.

Our study was approved by the Ethics Committee of our institution, and an informed written consent was taken from all the patients. This study was conducted from November 2015 to February 2017.

All patients had a detailed history taken, followed by clinical evaluation and assessment by mammography and ultrasound, and a core needle biopsy.

ACR-BIRADS 2003[8] was used to classify MD into the following categories:

1. Type 1 (<25% fibroglandular tissue)
2. Type 2 (25%–50% fibroglandular tissue)
3. Type 3 (50%–75% fibroglandular tissue)
4. Type 4 (>75% fibroglandular tissue).

While volumetric assessment of MD with software is more objective than visual assessment, we did not have access to this software and we have been routinely using the well accepted ACR-BIRADS 2003 for classifying MD.

Patients with breast cancer were further evaluated for oestrogen receptor (ER), progesterone receptor (PR) and Her-2 neu receptor status. ER, PR and Her-2 expression in primary tumours were analysed by immunohistochemistry (IHC) staining of formalin-fixed and paraffin-embedded core needle biopsy specimens. Primary antibodies for ER, PR and Her-2 (DAKO REAL TM EnVision, DAKO, Denmark) were used. ER and PR were considered positive if tumours had more than 1% nuclear-stained cells. Her-2 staining was scored on a scale of 0 to 3+, according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) 2007 guidelines.[9] A positive Her-2 result was IHC staining of 3+ (uniform, intense membrane staining of >30% of invasive tumour cells), a fluorescent *in situ* hybridization (FISH) result of more than six Her-2 gene copies per nucleus or a FISH ratio (Her-2 gene signals to chromosome 17 signals) of more than 2.2; a negative result was an IHC staining of 0 or 1+, a FISH result of less than 4.0 Her-2 gene copies per nucleus, or FISH ratio of <1.8.

Each patient was designated into one of four distinct molecular subtypes according to their hormonal status: Luminal A (ER/PR +ve, Her-2 –ve), luminal B (ER/PR +ve, Her-2 +ve), Her-2-enriched (ER/PR –ve, Her-2 +ve), and triple-negative (ER/PR –ve, Her-2 –ve).

After the diagnosis of malignant or BBD was established, the serum vitamin D level was assessed by the chemiluminescence method. This automated immunoassay was done using the Cobas e immunoassay analyser (Roche Diagnostics, USA) based on electrochemiluminescence technology.

Serum vitamin D levels >30 ng/ml were considered normal. Levels between 20 and 30 ng/ml were considered as mild deficiency, between 10 and 20 ng/ml as moderate deficiency, and <10 ng/ml were considered as severe deficiency of vitamin D.

The serum vitamin D levels were correlated with the MD and also with the molecular subtyping of breast cancer.

Data were collected and recorded on a Microsoft Excel spreadsheet and analysed using the SPSS software 16.0 version. Descriptive statistics such as mean, median and standard deviation (SD) were used for quantitative variables and percentages for qualitative variables. Association between quantitative variables with the two subgroups of patients, benign and malignant breast disease, were analysed using Student *t*-test. Analysis of variance was used when more than two groups were present—between four groups of MD and the four molecular subtypes of breast cancer. A value of $p<0.01$ was considered statistically significant. Analysis was done to compare the levels of serum vitamin D in patients with malignant and BBD, and to study the association, if any, between MD and serum vitamin D levels, and between molecular subtypes of breast cancer and vitamin D levels.

In this cross-sectional study, where all the study patients consented to participate, there were no missing data.

All patients diagnosed with vitamin D deficiency were given vitamin D and calcium supplementation as per their requirements.

Patients with BBDs were treated as per the standard protocols. Patients with malignant breast lesions were staged and managed as per the National Comprehensive Cancer Network (NCCN) guidelines.

RESULTS

During the study period, 232 newly symptomatic women above the age of 35 years presented to the outpatient department with complaints of breast lump or nodularity. Of these, 48 patients had inflammatory breast conditions (acute mastitis or abscess), and another 22 were on vitamin D or calcium supplementation and were thus excluded. The remaining 162 patients satisfied the inclusion and exclusion criteria and were included after informed consent.

Of the 162 patients, 102 had non-inflammatory BBD, whereas the remaining 60 women had malignant breast disease. Both these groups were comparable with respect to their clinical profile including body mass index (BMI), past history of any breast disease/biopsy, family history of any breast/ovarian cancer, age at menarche, menstrual cycles, menopausal status, age of attaining menopause, duration of breastfeeding, age of first child birth and their parity.

The mean (SD) age of patients was 43.69 (8.10) years. Patients with BBD were younger while those with malignant breast disease were older (BBD 40 [5] years; malignant 49 [10] years). Of the patients with cancer (n=60), 3 (5%) had stage 1, 15 (25%) had stage 2, 38 (63.3%) had stage 3, and 3 (5%) had stage 4 disease, while 1 (1.7%) had carcinoma *in situ* (stage 0). The final diagnosis was infiltrating ductal carcinoma in 53 (88.3%) patients, lobular carcinoma in 2 (3.3%), papillary carcinoma in 2 (3.3%), and medullary carcinoma, metaplastic carcinoma and carcinoma *in situ* in 1 each (1.7% each). On IHC, 25 patients (41.7%) were triple-negative (ER/PR –ve, Her-2 –ve) type, 12 (20%) were luminal A (ER/PR +ve, Her-2 –ve), 11 (18.3%) were luminal B (ER/PR +ve, Her-2 +ve) and 12 (20%) were Her-2 neu type (ER/PR –ve, Her-2 +ve). Overall, only 23 (38.3%) patients were ER-positive.

Mammographic breast density (MD)

The distribution of MD was as follows: 19 patients (11.7%) type 1; 55 patients (34%) type 2; 66 patients (40.7%) type 3 and 22 patients (13.6%) type 4.

Serum vitamin D levels

The serum vitamin D levels of all the patients ranged from 3.1 ng/ml to 40.2 ng/ml. Vitamin D deficiency was present in almost 98% of the study group, with about 92% having moderate-to-severe deficiency (Table I). In those with BBD, the serum vitamin D level ranged from 5.6 ng/ml to 40.2 ng/ml and in those with malignancy it ranged from 3.1 ng/ml to 28.4 ng/ml.

TABLE I. Serum 25(OH) vitamin D levels in patients with benign and malignant breast disease

Serum 25(OH) vitamin D levels	Total (*n*=162), *n* (%)	Benign breast disease (*n*=102), *n* (%)	Malignant breast disease (*n*=60), *n* (%)
Mean (SD); range (ng/ml)	12.45 (5.88); 3.1–40.2	13.73 (5.68); 5.6–40.2	10.26 (5.61); 3.1–28.4
Normal values (>30 ng/ml)	3 (1.9)	3 (2.9)	0
Mild deficiency (20–30 ng/ml)	10 (6.2)	6 (5.9)	4 (6.7)
Moderate deficiency (10–20 ng/ml)	89 (54.9)	67 (65.7)	22 (36.7)
Severe deficiency (<10 ng/ml)	60 (37.03)	26 (25.5)	34 (56.7)

The mean serum vitamin D level was significantly lower in those with malignancy (10.26 [5.61] ng/ml) compared with those who had BBD (13.73 [5.68] ng/ml; p<0.01). Furthermore, severe vitamin D deficiency was more common in those with a malignancy (56.7%) compared with those who had BBD (25.5%).

There is a difference of 9 years in the mean age between the breast cancer group and the BBD group. This as well as BMI could be confounding factors in the determination of MD.

Serum vitamin D levels and MD

The mean serum vitamin D level in MD type 1 was 16.19 ng/ml, in type 2 it was 16.03 ng/ml, in type 3 it was 10.01 ng/ml and in MD type 4 it was 7.54 ng/ml. Statistical analysis of MD with serum vitamin D levels revealed a significant difference between serum vitamin D levels in mammographic density types 1 and 3, 1 and 4, 2 and 3, and 2 and 4, indicating that high mammographic density was associated with significantly lower serum vitamin D levels (Table II).

This direct and significant correlation between vitamin D deficiency and increased mammographic breast density (a known breast cancer risk factor) suggests vitamin D deficiency to be a risk factor for the development of carcinoma of the breast.

Serum vitamin D levels and molecular subtypes of breast cancer

Serum vitamin D levels were analysed for the various molecular (IHC) subtypes of breast cancer. This showed a statistically significant lower mean level of serum vitamin D in TNBC (triple-negative breast cancer) when compared with luminal type A, luminal type B or Her-2 type (Table III).

TABLE II. Analysis of mammographic density type with mean serum 25(OH) vitamin D (ANOVA)

Mammographic density type	*n*	Mean (SD) vitamin D levels (ng/ml)	Standard error	p value
1	19	16.19 (4.62)	1.06	<0.01
2	55	16.03 (6.82)	0.92	
3	66	10.01 (3.15)	0.39	
4	22	7.54 (2.58)	0.55	
Total	162	12.44 (5.88)	0.46	

TABLE III. Analysis of immunohistochemistry subtype of breast cancer with mean serum 25(OH) vitamin D levels

Immunohisto-chemistry subtype	*n*	Mean (SD) vitamin D levels (ng/ml)	Standard error	p value
Luminal A (ER/PR +ve, Her-2 –ve)	11	12.94 (6.16)	1.86	0.008
Luminal B (ER/PR +ve, Her-2 +ve)	12	13.18 (4.53)	1.31	
Triple-negative breast cancer (ER/PR –ve, Her-2 –ve)	25	7.68 (3.42)	0.68	
Her-2 type (ER/PR –ve, Her-2 +ve)	12	10.27 (7.52)	2.17	
Total	60	10.26 (5.61)	0.72	

ER oestrogen receptor PR progesterone receptor Her-2 human epidermal growth factor receptor 2

DISCUSSION

Breast cancer: Current scenario and Indian perspective

A noticeable characteristic of the epidemiology of breast cancer in current years is its rapidly increasing rates of incidence in developing countries.[10,11] In India, it is now the most common cancer in women.[12,13]

Patients in developing countries are also about one decade younger than their counterparts in developed nations.[14,15] The mean and median age of our breast cancer patients was 49 and 47 years, respectively, and the largest proportion (36.7%) of patients was in the age range of 45–54 years. This was similar to the previous study on the Indian population by the authors.[13] The distribution of age at the diagnosis in many Asian countries is in the range of 45–50 years, whereas in most western countries, it is 55–60 years.[14] Younger age has been directly associated with a larger tumour size and higher stage, a higher number of metastatic lymph nodes, higher tumour grade, lower rates of positive hormone receptor status, more frequent and earlier locoregional recurrences and with a poorer overall survival.[16]

In developing countries, a majority of patients with breast cancer are still diagnosed at a relatively late stage, with locally advanced cancers comprising over 50% of all the patients diagnosed.[13,14] In our study, breast cancer was most commonly stage 3 at presentation (63.3%). This is in contrast to the US data where stage 3 disease forms only 8% of the disease-load and 59% of cases are *in situ* disease (20%) or stage 1 (39%) at presentation.[13]

Of note is the relatively higher percentage of TNBC, which has been reported in Indian studies.[13,17] 41.7% of our patients had a poor prognosis triple negative molecular type, compared to the USA where it constitutes only 25% of the disease subtypes.[13] Similar results have been reported by other Indian authors such as Shet *et al*.[13,17] This is significant because TNBC is known to be biologically aggressive, usually resistant to the conventional cytotoxic chemotherapy treatment regimens, and is associated with a worse prognosis with reduced overall survival compared to the other breast cancer subtypes.[18]

Mammographic breast density

The risk of developing breast cancer is directly proportional to and increases steadily with increasing breast density and is

4–6 times in women with an MD ≥75% when compared with same age group women with an MD of <10%. Increase in breast density is a strong and independent risk factor for developing breast cancer.[19,20]

Higher MD was also found to be directly associated with tumour characteristics that are related to poor prognosis, including higher tumour grade, larger tumour size and lymph node metastasis,[21] and conferred a greater risk of locoregional recurrence.[22]

Vitamin D deficiency

Deficiency of vitamin D is prevalent all across the Indian subcontinent, with a prevalence ranging from 70% to 100% in the general population.[23] Widely consumed food items, including dairy products, are not usually fortified with vitamin D and common cultural practices (such as clothing habits and vegetarianism) hamper adequate exposure to sun and may limit vitamin D-rich dietary options. Deficiency of vitamin D remains the most under-diagnosed and under-treated of the nutritional deficiencies in the world.[24]

Vitamin D and mammographic breast density

Sprague *et al*. conducted a study on 238 postmenopausal women with a mean age of 60.7 years, and with a mean serum 25-hydroxy vitamin D (25[OH]D) level above 34 ng/ml (normal range). Two-thirds of them were overweight or obese. Their results suggest no strong independent associations between the circulating molecules of the vitamin D pathway and mammographic breast density in post-menopausal women.[25]

Yaghjyan *et al*.[26] conducted a systematic review of 15 eligible studies on associations between vitamin D and mammographic breast density. They concluded that the cross-sectional nature of the studies limits conclusions about causal relationship between vitamin D and breast density, and that further studies are warranted to investigate long-term effects of vitamin D on breast density.[26]

Brisson *et al*.[7] in a cross-sectional study of 741 premenopausal women from Canada, recruited at screening mammography, showed that changes seen in serum vitamin D levels were inversely related to the changes in breast density with a lag time of about 4 months. He concluded that this finding encourages further investigation of the possibility that vitamin D could reduce breast density and the risk of breast cancer.[7]

Some studies have proposed an inverse association between vitamin D levels and breast density, and as breast density is already considered an established risk factor for breast cancer, Straub *et al*.[27] recruited for a cross-sectional study a total of 412 pre- and 572 post-menopausal women for whom mammography was indicated. When the analysis took menopausal status into account, the breast density of pre-menopausal women was lower following regular vitamin D intake; this lower breast density of pre-menopausal women was statistically highly significant ($p<0.001$ for ACR 1 and ACR 2 *v.* ACR 4, respectively). This effect was not found in post-menopausal women. Frequent intake of vitamin D-containing nutrition had no significant impact on ACR in either of the groups. They concluded that these results reinforce the assumption made earlier by several authors that higher levels of 25(OH)D pre-menopause and vitamin D substitution are associated with lower breast density and could reduce the risk of breast cancer. The findings did not confirm any post-menopausal association between vitamin D and mammographic breast density. They also showed an inverse relationship between serum vitamin D and breast density and determined that low vitamin D levels made the probability of high breast density significantly more likely.

To test the hypothesis that plasma concentration of 25(OH)D is associated with mammographic density, Bertrand *et al*.[28] conducted a cross-sectional study among 835 premenopausal women in the Nurses' Health Studies. Plasma 25(OH)D concentration was significantly inversely associated with breast cancer risk among women with high mammographic density (p-trend <0.01) but not among women in lower tertiles of mammographic density (p-interaction <0.01). There was evidence that the association between premenopausal 25(OH)D and breast cancer risk varies by mammographic density, with an inverse association apparent only among women with high mammographic density.

Our study indicates a statistically significant inverse relationship between serum vitamin D levels and mammographic breast density.

Vitamin D and breast cancer

Low levels of 25(OH)D and polymorphisms in the vitamin D receptor gene (VDR) have been found separately to increase risk of breast cancer. Lowe *et al*.[29] aimed to determine whether low 25(OH)D levels, alone and in combination with BsmI 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. VDR genotype was determined by the polymerase chain reaction and restriction enzyme digest. Analysis showed that participants with 25(OH)D levels <50 nm and the bb BsmI 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 2.31–14.7, $p<0.001$). This study indicates that low levels of circulating 25(OH)D, both alone and in combination with BsmI VDR genotype, may increase the risk of breast cancer in a UK Caucasian population. Serum vitamin D levels were found to be significantly lower in those patients with breast cancer when compared to age-matched controls.[29]

A literature search for all studies that reported risk of breast cancer by quantiles of 25(OH)D identified two studies with 1760 individuals.[30] Data were pooled to assess the dose–response association between serum 25(OH)D and risk of breast cancer. Results from the pooled analysis of these studies showed that participants with serum vitamin-D levels of >50 ng/ml had a significant 50% lower risk of developing breast cancer, when compared with women having serum values of <13 ng/ml.[30]

No study has evaluated serum vitamin D levels in malignant and benign breast patients in the Indian population.

Vitamin D and triple-negative breast cancer (TNBC)

Vitamin D deficiency is not only a risk factor for breast cancer but it is also associated with worse breast cancer outcomes. Low vitamin D levels were shown to be associated with ER/PR-negative phenotypes and with positive lymphovascular invasion.[27]

Analysis of our data revealed serum vitamin D levels to be significantly lower in the TNBC subtype (7.68 [3.42] ng/ml) compared to hormone-positive IHC subtypes (12.9 [6.16] ng/ml in luminal A and 13.1 [4.52] ng/ml in luminal B).

In our study, lower levels of vitamin D were seen to have a significant association with poor prognosis TNBC subtype.

Since TNBC has been shown to be far more common in the Indian population, this could have important preventive and therapeutic implications.

Our study is a cross-sectional study in which a causal relationship is difficult to establish. One can at best show a strong association between two factors. A case–control study, which retrospectively compares exposure to risk factors between a patient group and a control group, is more likely to establish the relationship between risk factors and disease. However, in vitamin D deficiency, such a case–control study is not possible because when a person is found to be vitamin D deficient, it would be unethical not to treat her. Hence, one cannot determine the duration of vitamin D deficiency in either group as they would have been treated when diagnosed. This is the reason why almost all such studies on vitamin D (including ours) are cross-sectional studies.

Key results

Our study highlights the dissimilarities in the clinico-epidemiological profile of breast cancer in our population compared to that in the developed countries, such as: (i) earlier age at presentation, (ii) late stage at diagnosis, and (iii) higher proportion of triple-negative subtype.

Most importantly, our study shows that vitamin D deficiency (widely prevalent in India) has a significant relationship with: (i) breast cancer (*v.* BBDs), (ii) high mammographic breast density (which is a surrogate marker of breast cancer risk), and (iii) TNBC (which is associated with much poorer prognosis).

The rapidly increasing incidence of breast cancer in India and many developing countries, presentation of the disease more than a decade earlier than their western counterparts, the high mortality rates, absence of known risk factors, late stage of presentation, high percentage of poor prognosis TNBC is an increasing source of worry and suggests that factors other than genetics must be contributing to this rising incidence of breast cancer.

Our study has important implications for developing countries such as India, with widely prevalent vitamin D deficiency.

Conclusion

Our study identifies vitamin D deficiency as a possible important (and easily modifiable) risk factor for breast cancer and could improve our understanding of the aetiopathogenesis of breast cancer and help define effective preventive strategies. However, more multicentre studies with larger number of patients will be required to overcome the limitations of our study and confirm these results.

The results of our study could have important public health implications for India and other developing countries with endemic vitamin D deficiency and call for urgent remedial measures such as mandatory food fortification with vitamin D and increasing public health awareness regarding vitamin D deficiency, and its likely role in the alarming increase in incidence of poor prognosis TNBC (especially at a young age).

Conflicts of interest. None declared

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