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Estimation of Serum Vitamin D Level in Carcinoma Breast Cases and its Relation to Grade, Stage and Hormone Receptor Status.

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Abstract: ***Introduction:-****In the cancer research field, vitamin D has emerged as the most prolific topic in the last decade with work connecting it with risk reduction in various epithelial cancers. The role of vitamin D with histological type and hormone receptor status of breast cancer is particularly controversial.*
Materials and Methods :- *58 diagnosed cases of carcinoma breast and 60 control groups were taken, grading and staging and hormone receptor status was done. Vitamin D total assay was done by 25OH vitamin D Total ELISA kit by Competition principle and correlated with the grade, stage and hormone receptor status. Statistical analysis was done by chi square test .*
Results :- *Vitamin D deficiency was found more in cancer cases (75.86%) than in control group (53.33%) and this was statistically significant (p=<0.01). In all the grades, stages and molecular types vitamin D deficiency was found to be highly prevalent. But there was no association between vitamin D values and histological grade, AJCC stage and molecular type.*
Conclusion :- *To our knowledge, limited number of studies were undertaken till now. Most of breast cancer patients are vitamin D deficient in comparison with the control groups.*
Keywords: *Breast carcinoma , Grade and Stage , Hormone receptor status, Vitamin D .*

I. Introduction

Cancer - The scourge of humanity. Breast cancer is a significant global public health issue. In 2012, approximately 144,000 new diagnosis and 70,000 died from breast carcinoma in India . [1] Breast cancer is unanimously considered a highly heterogeneous disease under several distinct viewpoints. Indeed, different types of this neoplasm exhibit variable histopathological and biological features, different clinical outcome and different response to systemic interventions. [2]

Vitamin D has been traditionally known as the antiricketic factor or the sunshine vitamin. It is considered unique due to its ability to be synthesized in the body and functioning as a hormone. Also, it plays a crucial role in calcium homeostasis and bone mineral metabolism. $25(OH)D_3$ is converted to $1,25\text{-}(OH)_2D_3$, the active hormonal form of vitamin D. This biologically active metabolite, $1,25(OH)_2D_3$, binds to nuclear vitamin D receptors (VDRs) in many cells, including those of the liver, pancreas , brain, lung, breast, skin, muscle and adipose tissue . [3]

Vitamin D deficiency is considered to be the most common nutritional deficiency [4] and also one of the most common undiagnosed medical conditions in the world. It has been estimated that 1 billion people worldwide have vitamin D deficiency or insufficiency. [5] The prevalence of vitamin D deficiency is 50-90% in the Indian subcontinent and is attributed to low dietary calcium along with skin colour and changing lifestyle. [6]

In vitro, $1,25(OH)_2D$ has been shown to inhibit cell proliferation and to promote apoptosis and cell differentiation in breast tumour tissue, providing a biologic basis for a study of the relationship between vitamin D concentration and breast cancer . [7] This effect is mediated by its active metabolite, $1,25(OH)_2$vitaminD_3, through binding to the Vitamin D receptor (VDR), that is present in almost all tissues including both normal and malignant breast cells. In vitro studies have shown vitamin D can inhibit the cell growth of other malignant cells beside breast cancer such as melanoma, prostate, colon, ovary and myeloid leukaemia. [8]

In recent years, there has been considerable interest whether vitamin D inhibits breast cancer development. According to the literature, vitamin D deficiency is a risk factor for development of breast cancer . The anticancer effect of vitamin D has been well demonstrated in different cells as well as normal and malignant breast cells. However, there is little evidence supporting the association of vitamin D and prognosis of breast cancer.

II. Material and methods

The present study was conducted in the department of Pathology, Kalinga Institute of Medical Sciences , Bhubaneswar, India from September 2013 to August 2015. This was approved by institutional ethical committee . It included 58 cases of carcinoma breast, confirmed by biopsy and 60 numbers of age-matched healthy females who are coming to the hospital as the "control group." Modified radical mastectomy and lumpectomy specimens with axillary clearance included in our study. Cases excluded were –Trucut biopsies, lumpectomy without axillary clearance, metastatic carcinoma, cell blocks, history of radiotherapy , chemotherapy ,intake of calcium or vitamin D supplements.

Immunohistochemical evaluation of ER, PR and HER2 was done on formalin fixed paraffin embedded tissue sections (4-5 micrometer thick) on poly-L lysine coated slides by using polymer two step indirect method.

Blood was obtained for vitamin D assay from the patients in standard serum separator BD vacutainer tubes with gel and clot activator and allowed to clot at room temperature . After 15-30 minutes the tubes were centrifuged in 3000rpm for 15 mins and serum was separated .Samples can be stored frozen at -20^0C for less than 2 weeks. Vitamin D total assay was done by the 25OH Vitamin D Total ELISA kit by competition principle. The results were calculated by computer assisted calibration curve ELISA reader.

Patients were arbitrarily classified in three categories, according to the frequently used 25OHD cut-off values of 20 ng/ml and 30 ng/ml.

US Endocrine Society classification [9]	
Deficiency	<20 ng/ml (50 nmol/L)
Insufficiency	21-29 ng/ml (52.5–72.5 nmol/L)
Sufficiency	>30 ng/ml
Toxicity	>150 ng/ml

The statistical analysis was done to estimate relation between vitamin D values and histological grade, stage and hormone receptor status of breast carcinoma. All data described by mean, frequency and percentage. Relationships between qualitative parameters were determined by chi square test. Probability values of less than 0.05 were considered significant.

III. Results and Discussion

The study included 58 cases of carcinoma breast, confirmed by biopsy and 60 numbers of healthy age matched controls. The mean age of patients in our study group was 48.03years. Mean age of control group was 45.2 years. Out of 58 cases studied, IDC,NOS was the largest histological type accounting for 96.55% of cases. Highest number of cases 62.07% were in stage II tumours , followed by stage III (31.03%) and stage I only in 6.9% of cases. There were no stage IV tumours in the current study. In the present study HER2-neu positive were 58.62% , PR in 41.38% and ER in 34.48% of tumours . In the current study HER2 positive was found in maximum number of cases i.e. 58.62%, while basal like and luminal type accounted for 24.14% and 17.24% respectively.

Mean vitamin D value in breast carcinoma cases was 14.28ng/ml. Mean vitamin D value of control group was 22.39 ng/ml. In the insufficient category there was a little difference in proportion of case and control group . In deficient and sufficient category, 75.86% and 6.90% of cases was found against 53.33% and 26.67% of control groups respectively. These findings also strongly correlate statistically by chi square test (p= <0.01) .[TABLE-1]

Table-1 Comparison between cases and control group by Vitamin D values

	Cases	Control	
Vitamin D	%	%	p value
Deficient	75.86	53.33	
Insufficient	17.24	10	p = <0.01 .
Sufficient	6.90	26.67	

In all three grades , Vitamin D deficiency was found to be highly prevalent . Therefore , the association between histological grade and Vitamin-D Values cannot be predicted (p= >0.5) . In all the three stages vitamin D deficiency was highly prevalent . There was no cases of vitamin D insufficient and deficient in stage I . While prevalence of insufficient cases were 16.67% and 22.22% in stage II and stage III respectively and sufficient cases were almost in same ranges in stage II and III .There were no correlation between AJCC stage and Vitamin D values.(p= >0.5) In ER positive and ER negative cases deficient and insufficient patients were in equal proportion . But, sufficient category was more in number in ER positive cases than in negative cases . There were no correlation

between ER status and vitamin D values .(p=>0.1) In all three molecular types , Vitamin D deficiency was found to be highly prevalent . But insufficiency in all types were almost of equal prevalence . While in Her2 positive type , there was no cases in sufficient category but in luminal type and basal like 20% and 14.29% of tumours were in sufficient category. There was no association between Vitamin D level and molecular type (p= >0.05). [TABLE-2]

Table-2 Correlation of Vitamin D with other variants

Vitamin D values	Deficient	Insufficient	Sufficient	P values
Histological grade				
Grade -I	100%	0%	0%	P=>0.5
Grade-II	80%	10%	10%	
Grade-III	72.22%	22.22%	5.56%	
AJCC stage				
Stage- I	100%	0%	0%	P=>0.5
Stage-II	77.77%	16.67%	5.56%	
Stage-III	66.67%	22.22%	11.11%	
ER status				
ER positive	80%	20%	20%	P=>0.1
ER negative	73.68%	21.05%	7.27%	
Molecular type				
Luminal type	60%	20%	20%	P=>0.05%
HER2 positive	82.35%	17.65%	0%	
Basal like	71.42%	14.29%	14.29%	

The primary objective of present study was to estimate the serum vitamin D level in carcinoma breast cases, by comparing with the control groups. Current study is sufficient to support that most of breast cancer patients are vitamin D deficient in comparison with the control groups. However ,serum vitamin D level did not show any correlation with histological grade, tumour size , lymph node status, AJCC stage, and molecular type .

3.1 Vitamin - D

Edward Mellan , first identified vitamin D in 1919 as a lipid soluble substance with anti-rachitic properties and is classically associated with its physiological role of calcium and phosphate regulation in bone metabolism. Humans can obtain vitamin D from two main sources: from the diet and sunlight exposure. Few natural foods contain vitamin D in significant amounts; among these, fatty fish such as salmon , fish liver oil best sources. Small amounts of vitamin D also found in eggs , sun-dried mushrooms ,cheese, beef liver . Still, the majority (90 to 95%) of the required vitamin D is produced by the skin when exposed to sunlight (ultraviolet B radiation) , which has caused vitamin D to be nicknamed 'the sunshine vitamin'.

Vitamin D is first converted to $25(OH)D_3$, the major circulating metabolite, by 25-hydroxylases(CYP24A) in the liver .[(5)] $25(OH)D_3$ then undergoes a second hydroxylation in the kidney into 1,25 dihydroxy vitamin D ($1,25(OH)_2D_3$), by 1-hydroxylase (CYP27B).[5] $1,25(OH)_2D_3$, also known as calcitriol is the biologically active form of vitamin D . Calcitriol acts by binding to an intracellular receptor, the vitamin D receptor (VDR) . [10] VDR, first identified in a breast cancer cell line in 1979, belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-activated transcription factor. [10] VDRs are present in many cells, including those of the liver, pancreas, brain, lung, breast, skin, muscle and adipose tissue . [11] In addition to its main function of maintaining extracellular calcium levels, the activation of VDR influences up to 200 genes that mediate cellular growth, differentiation, and apoptosis . [12] The best indicator of total body vitamin D stores is $25(OH)D_3$ because its long half-life of 15days which is far greater than that of vitamin D or $1,25(OH)_2D_3$ having short half-life of 15hrs .[13]

3.2 Vitamin D and breast tissue

Breast tissues, contain the 1-α-hydroxylase enzyme required for generation of the active vitamin D metabolite $1,25(OH)_2D_3$, from circulating $25(OH)D_3$. [14] The concentration of circulating $25(OH)D_3$ appears to be the key factor regulating tissue-specific synthesis of the active form of vitamin D . [14,15] The locally synthesized $1,25(OH)_2D_3$ bind to VDRs present in the breast epithelium and further binding to specific DNA sequences , namely vitamin D response elements , to regulate the expression of many genes . [15,16]

Two distinct pathways of vitamin D biosynthesis and action have been proposed in mammary carcinogenesis, one involving $1,25(OH)_2D_3$ and the other involving $25(OH)D_3$. [17,18] In the endocrine pathway, circulating $1,25(OH)_2D_3$ reaches the breast tissue to exert its anticarcinogenic effect. The other pathway is the autocrine/paracrine pathway, in which circulating $25(OH)D_3$ reaches the breast tissue and is further catalyzed to

$1,25(OH)_2D_3$ by the 1-α-hydroxylase in the breasts. The locally produced $1,25(OH)_2D_3$ may bind to VDR and therefore regulate cell proliferation, differentiation, and apoptosis . [18]

3.3 Vitamin D status in circulation

Vitamin D status in the circulation depends on exogenous vitamin D sources (from dietary and supplemental intake), endogenous production (through synthesis in the skin), and activities of vitamin D metabolic enzymes. In human plasma, the concentration of $25(OH)D_3$ (>20 ng/mL) is ≃1,000 times higher than that of $1,25(OH)_2D_3$ (20-60 pg/mL) . [19] Circulating 25(OH)D concentration varies with dietary intake and exposure to sunlight and is considered to be the best indicator of vitamin D status . [20]

3.4 Vitamin D deficiency

Vitamin D deficiency is considered to be the most common nutritional deficiency [4] and also one of the most common undiagnosed medical conditions in the world. It has been estimated that 1 billion people worldwide have vitamin D deficiency or insufficiency . [5] Though majority of population in India lives in areas receiving ample sunlight throughout the year, vitamin D deficiency is very common in all age groups and both sexes across the country. [6.21.22] The prevalence of vitamin D deficiency is 50-90 % in the Indian subcontinent and is attributed to low dietary calcium along with skin colour and changing lifestyle . [6]

3.5 Role of vitamin D in breast carcinoma

Vitamin D plays many role breast cancer tissue as to inhibit cell proliferation and to promote apoptosis and cell differentiation .

3.5.1 Growth Arrest

Vitamin D induce increased expression of the CDK inhibitors p21 and p27, thus blocking cell cycle progression . [23-25] The active vitamin D metabolite also can down regulate the expression of oncogenes such as c-myc and c-fos and the actions of several growth factors, including epidermal growth factor , transforming growth factor, and insulin-like growth factor (IGF-1) , further contributing to the inhibition of cell proliferation .[26]

TCF-4, a breast cancer tumour suppressor was a transcriptional regulator, and another molecular target of vitamin D action . Its level was lower in Vdr knockout mice, suggests a role for TCF-4 in antiproliferative effects induced by vitamin D. [27] Vitamin D induction of the BRCA1 gene had also been correlated with cell proliferation. [28]

3.5.2 Apoptosis

$1,25(OH)_2D_3$ induce morphological changes associated with apoptosis in breast cancer cells . [29] These changes could be related to regulation of the Bcl-2 family of genes that leads to a relatively lower expression level of anti-apoptotic proteins such as Bcl-2 and Bcl-XL versus pro-apoptotic proteins such as Bax and Bak . [26,29]

Vitamin D was still described as a prooxidant in breast cancer cells, causing an increase in the overall cellular redox potential , [30] which may also be an important mechanism underlying the pro-apoptotic effects of this hormone.

3.5.3 Invasion and metastasis

In some breast cancer cell lines, $1,25(OH)_2D_3$ increases the expression of E-cadherin, which prevents invasion and metastasis . [31] In addition, $1,25(OH)_2D_3$ has potent antiangiogenic activity that likely contributes to its inhibition of tumour invasion. [26] $1,25(OH)_2D_3$ also has been shown to decrease the activity of matrix metalloproteinases (MMP-9), urokinase-type plasminogen activator, and tissue-type plasminogen activator and to increase the expression of plasminogen activator inhibitor and MMP inhibitor 1, which are all important mediators of invasion and metastasis .[32] Vitamin D decreased the activity and expression of metalloproteinases and serine proteases, at the same time induces their inhibitors .Vitamin D was able to down regulate the expression of P-cadherin, [33] an invasion promoter molecule in breast cancer cells . [34]

3.5.4. Angiogenesis

Vitamin D can modulate angiogenesis. Vitamin D was able to inhibit angiogenesis at low concentration in vivo . [35] Using xenografted mice with vascular endothelial growth factor (VEGF)-over expressing MCF-7 breast cancer cells, it was demonstrated that the administration of vitamin D results in reduced vascularisation of tumours .

[36] Vitamin D may also inhibit vessel growth and maturation. Additional evidence for angiogenesis inhibition by vitamin D occurred through a decrease in expression of VEGF and tenascin-C . [37,38]

3.5.5 Anti-Inflammation

$1,25(OH)_2D_3$ has been shown to down regulate the expression of cyclooxygenase-2 (COX-2), which plays a critical role in prostaglandin synthesis in several human breast cancer cell lines .[39] It also increases the expression of 15-hydroxyprostaglandin dehydrogenase, which catalyzes the conversion of prostaglandins to biologically inactive ketoderivatives . [39] Prostaglandins have been suggested to play a role in the development and progression of breast cancer . [40]

3.5.6 Estrogen Pathway Inhibition

$1,25(OH)_2D_3$ suppresses the estrogen pathway by reducing the expression of the gene coding aromatase, the enzyme that converts androgens to estrogens. [39] $1,25(OH)_2D_3$ also down regulates estrogen receptor (ER)-α, the nuclear receptor that mediates the actions of estrogen . [41,42] The combined actions of $1,25(OH)_2D_3$ can decrease the levels of estrogens and the receptor that mediates their signalling .

IV. Conclusion

The present study had several limitations. Vitamin D concentration was measured only before surgery and not during follow up . Therefore, the prognostic significance of changes in vitamin D status, especially in vitamin D deficient patients remains to be determined . To conclude , the current study suggests vitamin D value was lower in breast cancer patients compare to healthy volunteers. The evidence suggests that efforts to improve vitamin D status, by vitamin D supplementation could reduce cancer incidence and mortality at low cost, with no or few adverse effects .

To our knowledge, limited number of studies are undertaken till now . Further studies are warranted to investigate possible relationships of vitamin D with the breast cancer phenotypes, pathological grades, clinicopathological stages and overall cancer specific survival .To confirm the potential protective effects of vitamin D on breast cancer, well designed cohort studies and clinical trials are warranted .So, new trials specifically tailored on the vitamin D cancer - biology are in progress and should provide additional information in a few years time.

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