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ORIGINAL ARTICLE

Role Portrayed by Serum NGAL and Vitamin D in Patients with Bone Tumors

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SUMMARY

Background: **Bone tumors are responsible for considerable morbidity and mortality at an early age. Malignant bone tumors are quite aggressive in nature. Thus, an accurate and timely diagnosis is essential for bone tumors. Neutrophil gelatinase associated lipocalin (NGAL) and vitamin D have been found to be associated with cancer and may have potential to act as biomarkers for bone tumors also.**
Methods: **Serum levels of NGAL and 25-OH vitamin D were estimated in 14 patients with benign and 14 with malignant bone tumors and compared with 14 apparently healthy controls. The data collected was compared among different groups using appropriate statistical analysis. NGAL was estimated by enzyme linked immunosorbent assay (ELISA) and 25-OH vitamin D by radioimmunoassay (RIA) in the serum samples.**
Results: **Serum NGAL levels were found to be increased significantly and 25-OH vitamin D levels decreased significantly in patients with malignant bone tumors as compared to healthy controls ($p < 0.001$) while this difference was not statistically significant in patients with benign bone tumors ($p = 0.05$). The difference in serum levels of NGAL and 25-OH vitamin D in patients with malignant bone tumors was found to be statistically significant as compared to patients with benign bone tumors ($p < 0.05$). The correlation was not statistically significant between the levels of 25-OH vitamin D and NGAL in group I ($r = 0.067$, $p = 0.819$), group II ($r = 0.204$, $p = 0.483$), and group III ($r = -0.086$, $p = 0.772$).**
Conclusions: **Serum NGAL and 25-OH vitamin D may be used as important serological biomarkers in patients with bone tumors along with other standard investigative modalities.**
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INTRODUCTION

Neoplasms of bone may be primary neoplasms or metastatic deposits. Primary bone tumors are less common than metastatic neoplastic disease. Classification of bone neoplasms is done on the basis of cell of origin. Primary bone tumors may be benign or malignant. Benign bone tumors are more common than malignant tu-

mors. The most common primary benign tumor is osteochondroma, other types are giant cell tumor (GCT), osteoid osteoma, and osteoblastoma. The most common primary malignant neoplasm is osteosarcoma, others being Ewing's sarcoma and chondrosarcoma. Of the tumors originating from the adjacent soft tissue, rhabdomyosarcoma, neurofibrosarcoma, and angiosarcomas are the malignant ones, whereas lipoblastoma and neurofibroma are benign soft tissue tumors. The gold standard test for confirmation of malignancy and for planning of surgery is biopsy [1]. This disease is in need of early diagnostic biomarkers which may help in decreasing mortality and for better application of limb salvage strategies.

Neutrophil gelatinase associated lipocalin (NGAL) has emerged as a biomarker for cancer. It is also known as lipocalin-2 (LCN-2), belongs to lipocalin protein family and is encoded by LCN-2 gene on chromosome 9. Increased expression of NGAL has been found associated with several solid and hematological malignancies. NGAL is a potential valuable diagnostic and prognostic biomarker in a number of malignancies like breast, brain, ovarian, endometrial, pancreatic, colorectal, bladder, liver, and lung cancers [2,3]. At present, to the best of our knowledge, no study could be found to throw some light on the association of NGAL with bone tumors.

Vitamin D deficiency is the most common nutritional deficiency worldwide. Vitamin D is a steroid hormone crucial for attaining calcium and phosphate homeostasis in the body. Besides this, it exerts numerous extra skeletal functions including antitumorigenic and immunomodulatory effects. The vitamin D receptor (VDR) is essential to initiate various signaling pathways that are induced by 1,25 dihydroxycholecalciferol and have been shown to play a substantial role in its anti-cancer activity [4]. Vitamin D and its several analogues exhibit anti-proliferative effects, mediated by stalling the cell cycle at G1/S check point by increasing the inhibitors and reducing activators of cyclin dependent kinase complexes which prevent deoxyribose nucleic acid (DNA) synthesis and cell growth [4,5]. Vitamin D deficiency is reported to be prevalent in patients with bone tumors [6].

Evaluation of serum levels of NGAL and 25-OH vitamin D together may provide better information regarding their roles as potential biomarkers in bone tumors. Vitamin D and NGAL, in association, have been reported to play an important role in inhibition of cholangiocarcinoma in rats according to a few studies. These studies suggest that the growth inhibition of the cells of cholangiocarcinoma by vitamin D is mediated by decreased expression of NGAL and that anti-cancer effects of vitamin D supplementation are brought about by down regulation of NGAL [6,7].

Though the deficiency of vitamin D is found to be associated with bone tumors, there are no studies in literature to evaluate the association of vitamin D and NGAL in patients with bone tumors. Therefore, this research was planned to estimate the serum levels of 25-OH vitamin D and NGAL in patients with bone tumors, both benign and malignant, and to compare their levels with healthy controls.

MATERIALS AND METHODS

This study was carried out on 28 histopathologically proven patients with bone tumors, 14 patients with benign and 14 with malignant bone neoplasms, and on 14 apparently healthy age and gender matched controls irrespective of age and gender. Informed consent was obtained from all the participants and the study was approved by Institutional Ethical Committee vide no. IEC/18/637 dated 28.09.18. Besides plain X-ray and biopsy, all the patients underwent computerized tomography (CT) scan and magnetic resonance imaging (MRI), whenever necessary, for establishing the diagnosis and staging of the tumors. Patients with any other chronic disease or on dietary supplements were excluded from the study. The subjects were divided into 3 groups:

Group I: Healthy controls (n = 14)

Group II: Patients with benign bone tumors (n = 14)

Group III: Patients with malignant bone tumors (n = 14)

Venous blood sample was collected under all aseptic precautions from all the patients at the time of diagnosis. Similar samples were collected from healthy controls also. Serum sample was analyzed for routine biochemical investigations the same day and was stored at -20ºC in separate aliquots for subsequent estimation of NGAL and 25-OH vitamin D in batches. Serum NGAL was analyzed by enzyme linked immunosorbent assay (ELISA) method [8]. Serum 25-OH vitamin D was estimated by radioimmunoassay (RIA) method, on the fully automated RIA. Analyzer SR300 by Stratec Biomedical AG, Germany using Beckman Coulter 25-OH vitamin D total RIA kit [9]. Routine biochemistry parameters were estimated on autoanalyzer (RxSuzuka, United Kingdom) using kits by Randox.

The data was compiled and analyzed using chi-squared test, Fisher's exact test, Student's *t*-test and ANNOVA F-test. The relationship between variables was analyzed using Pearson's correlation coefficient. A p-value of < 0.05 was considered statistically significant.

RESULTS

Of 14 patients with benign tumors, 10 (71.4%) were males and 4 (28.6%) were females while there were 9 (64.3%) males and 5 (35.7%) females in malignant tumor group. Of 14 controls, 10 (71.4%) were males and 4 (28.6%) were females. The gender ratio was comparable in the three groups ($p > 0.05$). The mean age of the patients was found to be 22.5 ± 12.6 years ranging from 6 - 62 years while that of controls was 21.3 ± 10.8 years ranging from 9 - 62 years. Among 14 patients with benign tumors, 7 had giant cell tumor (GCT), 4 aneu-

Table 1. The comparison of serum levels of 25-OH vitamin D and NGAL in different groups.

		Group I (n = 14)	Group II (n = 14)	Group III (n = 14)	p-value between Group I & II	p-value between Group I & III	p-value between Group II & III
25-OH Vitamin D (ng/dL)	Mean ± S.D.	26.30 ± 5.19	22.70 ± 7.26	16.92 ± 6.95	0.1438	0.0004	0.0407
	Range	15.81 - 35.12	10.22 - 31.41	9.32 - 30.93			
NGAL (ng/mL)	Mean ± S.D.	2.97 ± 1.54	3.89 ± 0.78	5.27 ± 2.17	0.0569	0.0033	0.0341
	Range	1.12 - 6.64	2.31 - 9.49	1.94 - 10.21			

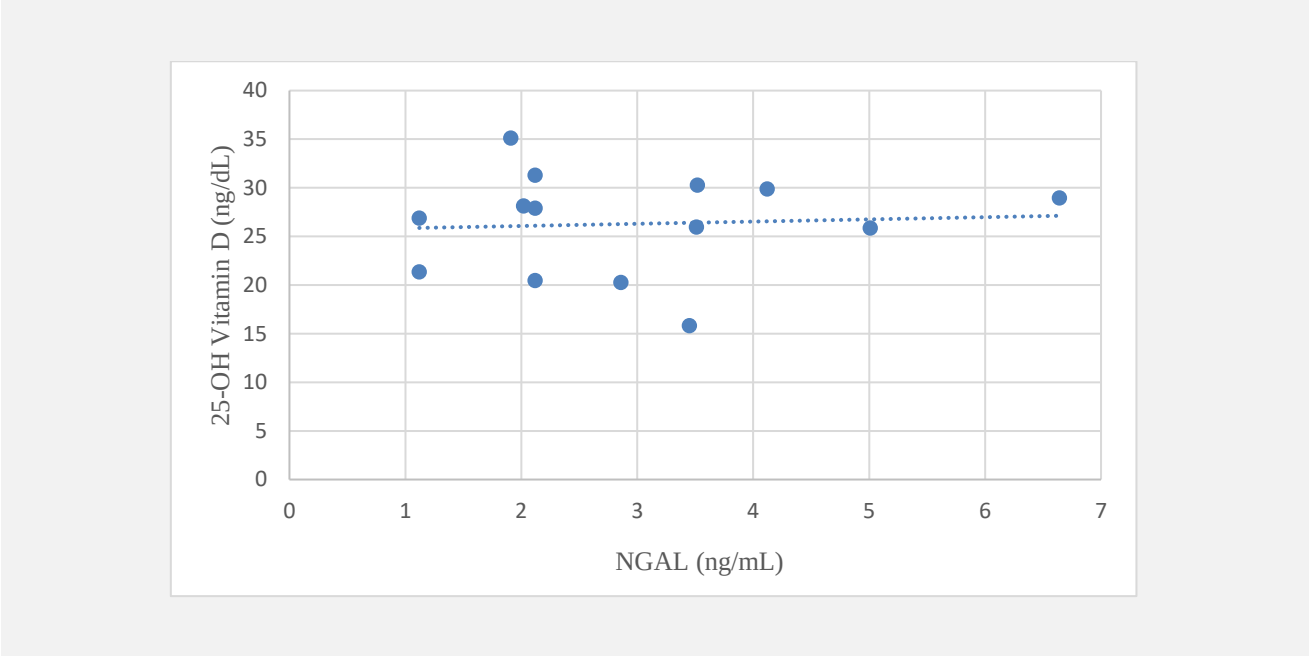

Figure 1. Scatter diagram showing correlation between Vitamin D and NGAL in group I.

rysmal bone cyst, 2 enchondroma, and 1 chondroblastoma. Of 14 patients with malignant bone tumors, 9 were suffering from osteosarcoma, 4 from Ewing's Sarcoma and 1 from chondrosarcoma. Based on tumor location, 19 patients had tumors of the lower limb whereas 9 had tumors of the upper limb and 16 had left sided lesions while 12 had lesions on the right side of the body. The levels of 25-OH vitamin D and NGAL in different groups are presented in Table 1. The difference in the serum levels of both the parameters was found to be highly significant in group III as compared to group I ($p < 0.001$) and significant as compared to group II ($p < 0.05$) while the difference between group I and group II was not statistically significant ($p > 0.05$). A negative non-significant correlation was found between 25-OH vitamin D and NGAL levels ($r = -0.086$, $p = 0.772$) in group III while the correlation in group I ($r = 0.067$, $p = 0.819$) and group II ($r = 0.204$, $p = 0.483$) was also not statistically significant.

DISCUSSION

The mean serum levels of NGAL were found to be increased in both benign and malignant bone tumors as compared to healthy controls though the increase was not statistically significant in benign disease ($p > 0.05$). NGAL plays key roles in the regulation of cell growth and adhesion in normal and neoplastic tissues [2]. In addition, NGAL activates the Raf/MEK/ERK pathway and increases matrix metalloproteinase-9 (MMP-9) activity in cancer cells. It has been reported that when NGAL is present in a complex with MMP-9, there is less degradation of MMP-9 resulting in a higher gelati-

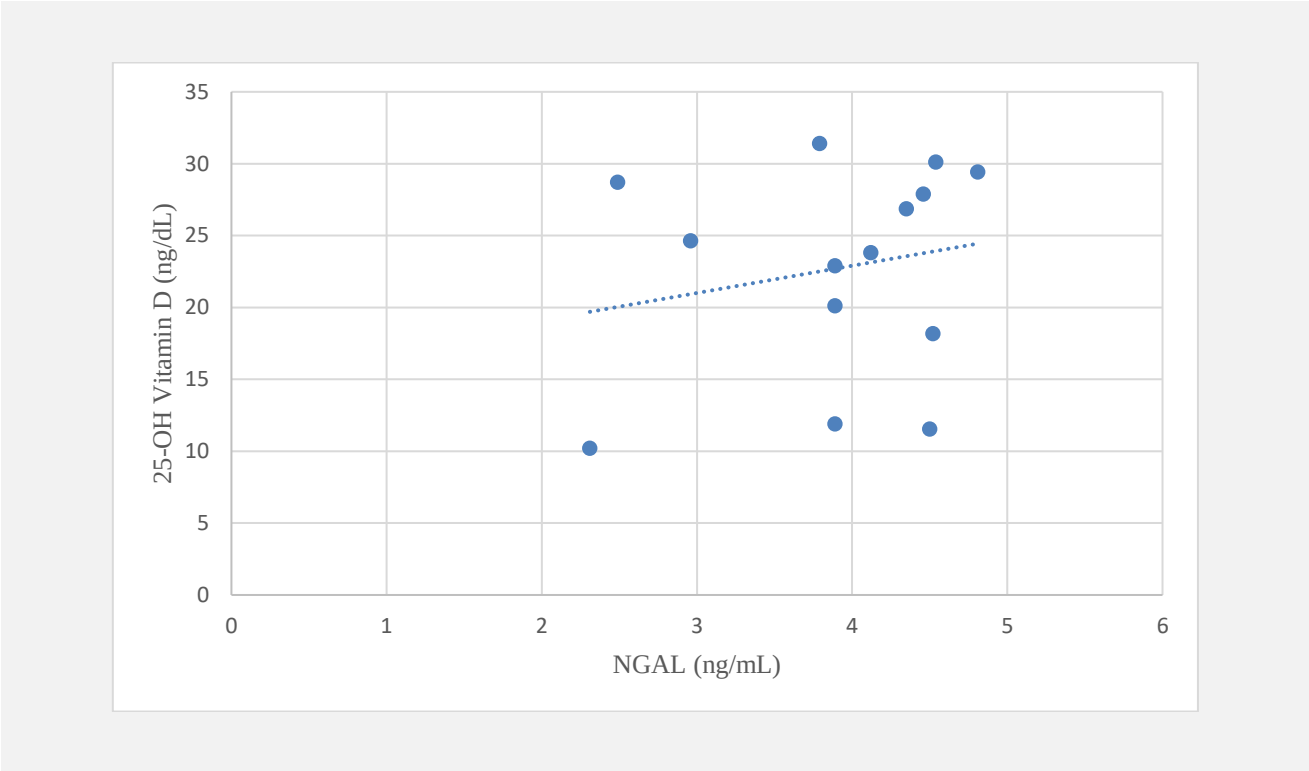

Figure 2. Scatter diagram showing correlation between Vitamin D and NGAL in group II.

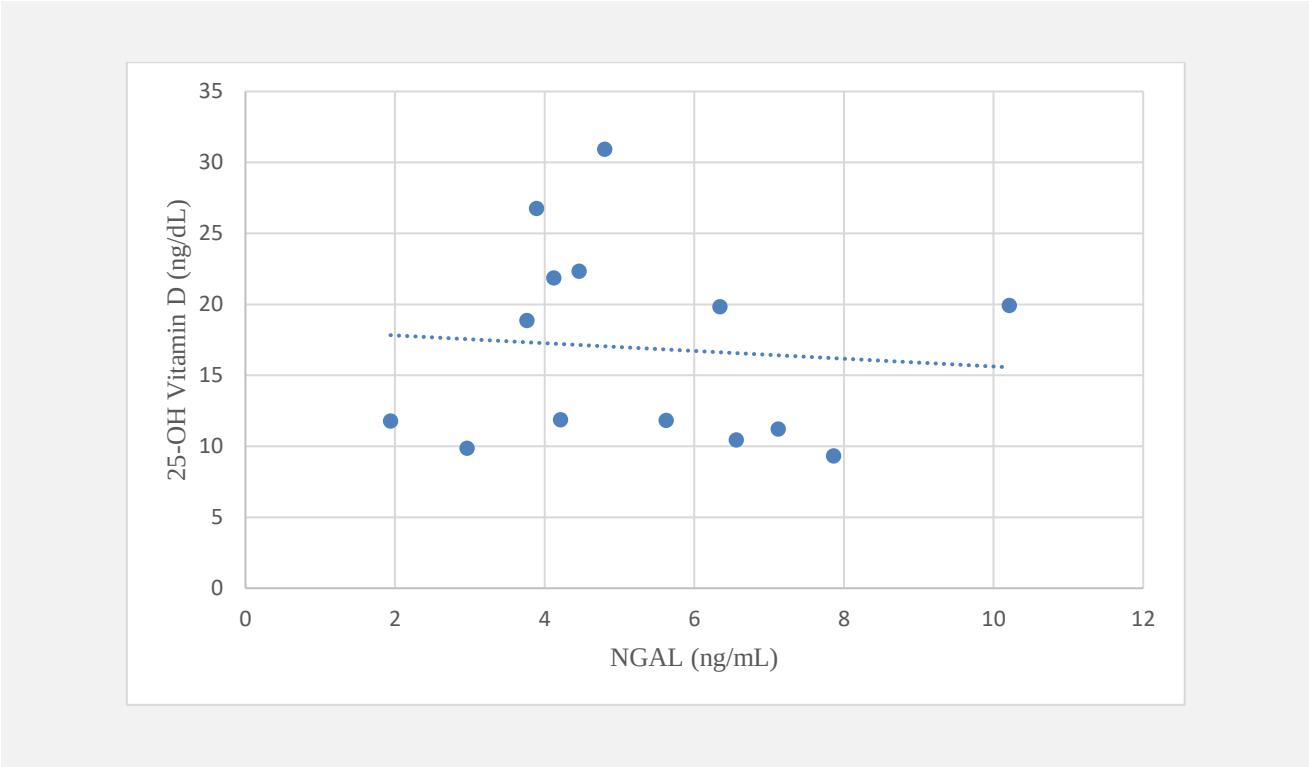

Figure 3. Scatter diagram showing correlation between Vitamin D and NGAL in group III.

nolytic activity of MMP-9 on the extracellular matrix. This may result in increased tissue invasiveness [10]. Over expression of NGAL in cancerous tissues can increase the migration, invasion, and metastasis while inhibition of NGAL expression decreases the invasiveness and metastasis of cancer cells. Increased expression of NGAL is associated with poor prognosis and enhanced invasion in a number of cancers like lung, breast, prostate, and renal cell etc. [2,10-13]. NGAL overexpression in tumors may result from stimuli present in the tumor microenvironment like hypoxia and inflammatory cytokines. The NF-κB signaling pathway, activated in most cancers, regulates the transcription of NGAL and the MAPK pathway and may cooperate with NF-κB to up-regulate the expression of NGAL. Furthermore, epigenetic modifications may also play an important role in promoting NGAL expression in the tumor cells [2,14]. This may be the reason for the increased levels of NGAL in patients with bone tumors and more so with the malignant ones.

The protective role of vitamin D against cancer has been well documented. Vitamin D receptor (VDR) is essential to initiate various signaling pathways that are induced by vitamin D and has been shown to play a substantial role in its anticancer activity. It has been proven that VDR expression is gradually reduced when the stage of malignancy advances and certain VDR polymorphisms are associated with increased cancer risk [15].

A decrease in 25-OH vitamin D levels was observed in bone tumors, both benign and malignant as compared to healthy controls. The decrease was found to be statistically significant between group I and III ($p = 0.000413$) and between groups II and III ($p = 0.0407$) while the difference was not significant between groups I and II ($p > 0.05$). Thus, a decrease in 25-OH vitamin D levels may be associated with malignancy. The role of vitamin D in cancer is because of its anti-proliferative properties and role in apoptosis. It can trigger the intrinsic mitochondria dependent pathway that induces cell death. It also reduces the metastatic potential of malignant cells by inhibiting angiogenesis. Lower circulating vitamin D is a reversible risk factor for cancer [4,5]. Not only vitamin D deficiency has a role to play in the causation, growth and spread of tumor, even its supplement has been reported to increase the efficiency of different treatment modalities used for cancer. It increases the radiosensitization of tumor cells. It has been reported that cancer vaccine alone may not be potent enough to reduce the tumor volume and its anti-metastatic effect improves considerably in combination with vitamin D [16]. Vitamin D deficiency has been reported to be prevalent in patients with bone tumors, levels being highly significantly decreased in patients with malignant bone tumors as compared to benign ones ($p < 0.0008$) [17].

Evaluation of serum NGAL levels in combination with vitamin D levels might help in understanding the underlying mechanism in a better way as it has been reported that vitamin D exerts its protective effect against cancer by decreasing the expression of NGAL. In a study on cholangiocarcinoma, repression of cholangiocarcinoma by MART-10, a newly synthesized 1,25 dihydroxycholecalciferol analogue was studied by analyzing the expression of VDR and NGAL. It was found that MART-10 repressed cholangiocarcinoma cell NGAL expression in a VDR dependent manner. A less pronounced effect on NGAL was seen in VDR silenced cells SNU308-VDR cells [6]. The effect of vitamin D supplementation also was studied by the same authors in cholangiocarcinoma in rats, and a total of 21 and 16 genes were found to be significantly upregulated and downregulated, respectively, after a series of bioinformatical analyses. Among the downregulated genes, NGAL was found to be the most suppressed by vitamin D supplementation. Vitamin D deficiency was found prevalent in cholangiocarcinoma patients along with greater NGAL expression and decreased cell growth. It was also suggested by the authors that *in vivo* anti-intrahepatic cholangiocarcinoma tumorigenesis by vitamin D supplementation is brought about by down regulation of NGAL [7]. In the present study, a negative correlation between serum NGAL and 25-OH vitamin D levels was observed in patients with malignant bone tumors ($r = -0.085576$), supporting the findings of the above research. But the correlation was not statistically significant ($p > 0.05$) which may be due to the small sample size of the study.

Thus, it may be concluded that both 25-OH vitamin D and NGAL bear potential to act as biomarkers in patients with bone tumors, especially malignant bone tumors, and can help in understanding the mechanism of the anti-cancer property of vitamin D in these patients. But as both these parameters are quite non-specific in nature, the claim regarding their role as diagnostic biomarkers for bone malignancy needs further supporting studies with larger sample size.

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None.

Declaration of Interest:
No conflict of interests exists.

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