INTERNATIONAL JOURNAL OF
MEDICAL BIOCHEMISTRY

DOI: 10.14744/ijmb.2023.36002
Int J Med Biochem 2024;7(1):33–40

Research Article

Exploratory role of serum FGF-8 as a marker of bone metastasis in tumor progression

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Abstract

Objectives: FGF-8, a member of the FGF family, plays a crucial role in cellular processes and has been implicated in cancer progression. The study aims to comprehend FGF-8's involvement in bone metastasis, emphasizing its potential as a diagnostic marker and focusing on its association with Bone-Alkaline Phosphatase (B-ALP) and other biochemical parameters.

Methods: The case-control study spans 12 months, involving 60 participants, including 30 with secondary bone metastases and an equal number without metastasis. FGF-8 levels were quantified using ELISA, and B-ALP, serum ALP, and various biochemical parameters were assessed. The study employed standardized procedures to minimize bias, including matching cases and controls, and obtaining ethical approval.

Results: In patients with bone metastasis, serum ALP levels, particularly B-ALP, were significantly higher. The metastatic group exhibited elevated FGF-8 concentrations, showcasing a positive correlation with B-ALP and serum calcium levels. The study successfully differentiated ALP isoenzymes through heat inactivation and L-phenylalanine inhibition. Additionally, serum calcium levels were markedly elevated in the metastatic group.

Conclusion: The findings suggest that FGF-8 is a potential diagnostic marker for bone metastasis, particularly in breast and prostate cancers. Elevated FGF-8 levels correlate with increased B-ALP and serum calcium, indicating its role in osteoblastic differentiation in metastasis. The study proposes the utility of ELISA-based kits for FGF-8 in serum as a practical and efficient method for assessing bone tumor progression.

Keywords: B-ALP, bone metastasis, diagnostic marker, FGF-8, osteogenesis

How to cite this article: Singh Gill G, Kaushik M, Kharb S, Goyal G, Malik U. Exploratory role of serum FGF-8 as a marker of bone metastasis in tumor progression. Int J Med Biochem 2024;7(1):33–40.

Human FGF is a class of cytokines that significantly impacts tissue disease, metabolism, cell growth, and development. The FGF family comprises 22 members and can be further broken down into paracrine and endocrine subgroups [1]. The FGF family is divided into seven subfamilies, including the FGF-1, FGF-4, FGF-7, FGF-8, FGF-9, FGF-11, and FGF-19 subfamilies. Each subfamily is distinguished by the similarity and specificity of its protein structure [2]. FGF signaling controls the actions of articular chondrocytes, peripheral synoviocytes, and osteoblasts, essential for maintaining joint health and functional balance [3]. The FGF-8 subfamily consists of three proteins: FGF-8, FGF-17, and FGF-18 [4]. FGF-17 is essential for brain development, while FGF-18 and FGF-8 are crucial for chondrogenesis and osteogenesis [5]. Members of the FGF-8 subfamily exhibit distinct tissue distribution patterns and binding affinities for FGF receptors. FGF-8, also known as androgen-induced growth factor (AIGF) [6], plays a role in stimulating physiological cellular processes such as cell proliferation, differentiation, and migration [7]. FGF signaling pathways may be significant in cancer pathophysiology since they have been linked to tumor growth and progression. Cell lines from breast and prostate cancers express FGF-8 [8]. Bone develop-

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Submitted: January 05, 2024 **Accepted:** January 19, 2024 **Available Online:** January 24, 2024

ment is controlled by a network of signaling molecules and pathways, with FGF and its receptors shown to be important in bone formation. It has been found that several FGFRs perform complementary roles and show some functional redundancy during osteogenesis [9]. During the metastatic phase, cancer cells subvert these pathways and alter cell-to-cell interactions in bone. Depending on the precise interaction between cancer cells and bone cells, bone metastases can be osteoblastic, like those from prostate cancer, or osteolytic, like those from multiple myeloma. However, both components, osteolytic and osteoblastic, are present in most bone metastases at various levels [10]. In particular, people with lung, breast, and prostate cancer are more likely to develop metastatic bone disease than other cancer patients [11]. Rising evidence suggests that the FGF axis contributes to bone metastases and has been linked to the development of numerous malignancies.

The four isoenzymes of alkaline phosphatase, namely liver, bone, kidney (L/B/K) alkaline phosphatase, and intestinal alkaline phosphatase (I-ALP), placental alkaline phosphatase (P-ALP), and germ cell alkaline phosphatase, are expressed throughout various tissues. In bone diseases, increased osteoblastic activity and/or enhanced hepatocyte production are the main causes of high blood alkaline phosphatase [12]. Bone alkaline phosphatase (B-ALP) is a relatively specific marker for osteogenesis and an indicator of osteoblast metabolism [13]. Therefore, B-ALP measurements may be a helpful addition to identifying individuals with skeletal involvement. Preoperative estimates of predicted survival have a role in determining whether surgical management for bone metastasis patients is appropriate. Several prognostic indicators have been identified in the past to aid in decision-making, but there is currently no precise biomarker for bone metastasis.

To include the ideas of the current understanding of the involvement of the FGF axis in bone metastases, this study was designed to comprehensively characterize the role of Fibroblast Growth Factor-8 (FGF-8) in bone metastasis and to investigate the potential of FGF-8 as a diagnostic marker for bone metastasis. The secondary objective is to correlate FGF-8 levels with Bone-Alkaline Phosphatase (B-ALP) for a more comprehensive understanding of FGF-8's role in bone health and disease.

Materials and Methods

Study design

This study employed a case-control design to investigate the associations between FGF-8 levels and bone metastasis, with a specific study duration of 12 months. The study was conducted within the Department of Biochemistry, and approval for the study protocol was obtained from the Institutional Ethical Committee, ensuring strict adherence to ethical guidelines.

Participants

The participant cohort was meticulously selected based on specific criteria, comprising a total of 60 individuals. This group included 30 participants with secondary bone metastasis and an equal number without metastasis. The identification of bone metastasis was achieved through whole-body MRI, in females diagnosed with breast and males with prostate cancer. All cases of metastasis were recurrences of breast and prostate cancer in females and males, respectively, and enrolled before the commencement of chemotherapy.

Variables

The study incorporated distinct variables, both independent and dependent. The primary focus was on evaluating FGF-8 levels as the independent variable. Dependent variables encompassed Bone-Alkaline Phosphatase (B-ALP), serum ALP levels, and various biochemical parameters such as ALT, AST, total bilirubin, blood urea, creatinine, and calcium.

Data sources/measurement

Data acquisition and measurement were meticulously conducted through standardized procedures. Each participant underwent the aseptic collection of 5 mL venous blood, followed by centrifugation for serum separation. Biochemical analyses were performed on the obtained serum samples using the Beckman Coulter AU-480 analyzer (California, United States). The evaluation of B-ALP levels employed an inhibition method. The initial phase encompassed the quantification of serum ALP levels in individuals, thereby determining the overall ALP concentration. Subsequent heat inactivation at 56°C for 10 minutes resulted in a substantial reduction, yielding B-ALP levels. Further diminution ensued with the addition of L-phenylalanine (10 mmol/L), leading to the inhibition of the intestinal fraction of ALP and the concomitant yield of the L-ALP fraction. The residual portion was designated as the intestinal ALP fraction [14]. FGF-8 levels were quantified using Enzyme-Linked Immunosorbent Assay (ELISA) with a kit from Elabscience®.

Bias

Concerted efforts were made to minimize bias. Matching cases and controls from the same socioeconomic background and age group aimed to control potential selection bias. Standardized procedures for blood collection and analysis were implemented to minimize information bias. Selecting participants with breast and prostate cancer was strategic to control potential confounding factors.

Sample size

The study encompassed a total of 60 participants, with 30 in the metastatic group and 30 in the non-metastatic group.

Statistical methods

Primary statistical tools included Student's independent t-test and Pearson correlation coefficient. The MedCalc® platform from MedCalc Software Ltd in Belgium served as the

analytical tool. A predetermined significance level of $p<0.05$ indicated statistical significance. Calculating the Coefficient of Variation (CV%), interassay and intra-assay variability for FGF-8 measured by an ELISA Kit involved assessing the precision and reliability of the assay. The percentage of coefficient of variation, CV% of intra-assay variability was 2.63% (mean=18.27, SD=0.48), and the CV% of inter-assay variability was 3.39% (mean=18.20, SD=0.62).

Ethics approval and Helsinki declaration

The present study received ethical approval from the Institutional Ethical Committee. The study adheres to the principles outlined in the Declaration of Helsinki, as adopted by the World Medical Association, guiding ethical conduct in biomedical research involving human subjects. Participants were provided with detailed information about the research objectives, procedures, potential risks, and benefits. Informed consent was obtained from all participants, and their confidentiality and privacy were strictly maintained throughout the study.

Results

In the present study, 30 patients diagnosed with secondary bone metastasis were included. Out of these, 10 were females with breast cancer, while 20 were males with prostate cancer. The average age of the metastatic group was 60.86±12.14 years (ranging from 38 to 78 years). Females with breast cancer had an average age of 46.2±5.26 years, whereas males with prostate cancer had an average age of 66.7±8.24 years. Two-way ANOVA was used to analyze the association between age, sex, and metastatic/non-metastatic status, but no significant interaction was found. Out of 20 males with prostate cancer, 6 had a history of smoking, and all 10 females with breast cancer were non-smokers. Initially, the serum ALP level was measured in patients with bone metastasis, which averaged 471.13±135.23 U/L. Following heat inactivation at 56°C for 10 minutes, the mean serum ALP level dropped to 74.55±24.72 U/L. Additionally, after the addition of L-phenylalanine (10 mmol/L), the mean ALP level further decreased to 33.55±11.96 U/L. The inhibition percentage after heat inactivation was calculated to be 84.39%, which increased to 92.59% after the addition of L-phenylalanine. The inhibition study was utilized to calculate the fractions of Bone (B), Liver (L), and Intestinal (I) ALP. The mean B-ALP activity, which was inhibited by heat inactivation, was 396.07±111.84 U/L. The remaining 74.55±24.72 U/L of ALP level consisted of both liver and intestinal fractions. The addition of L-phenylalanine successfully inhibited the intestinal fraction of ALP, resulting in a mean ALP level of 33.66±11.99 U/L, representing the L-ALP fraction. The remaining 38.19±14.34 U/L was considered the intestinal ALP fraction (Fig. 1). In patients with bone metastasis, the percentages of bone, liver, and intestinal ALP in serum were 84.17%, 7.45%, and 8.31%, respectively, whereas in the non-metastatic group, the percentages were 18.42%, 41.26%, and 41.68%, respectively. The B-ALP/total ALP ratio was 0.87 in the metastatic and 0.19 in the non-metastatic. Other biochemical parameters, including serum ALT, AST, total bilirubin, blood urea, creatinine, and calcium, were measured in both groups (Table 1).

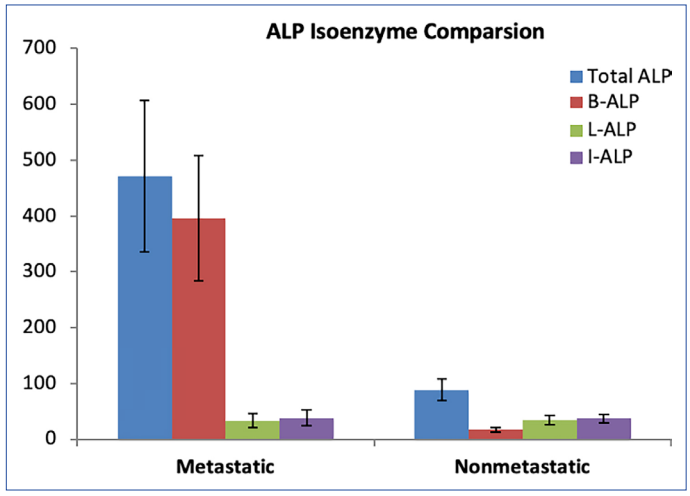

Figure 1. Mean±SD of fractions of ALP in controls and cases.
SD: Standard deviation; ALP: Alkaline phosphatase.

Furthermore, FGF-8 protein levels were measured in the serum of both metastatic and non-metastatic groups. The mean concentration of FGF-8 in the metastatic group was 21.51±3.41 pg/ml (ranging from 15.13 to 28.35 pg/ml), while in the non-metastatic group, it was 5.36±1.79 pg/ml (ranging from 2.52 to 9.08 pg/ml). The metastatic and non-metastatic cases were stratified by gender. In females with metastasis, the mean FGF-8 concentration was 19.39±2.73 pg/ml compared to 6.15±1.90 pg/ml in females without metastasis. For males, the corresponding values were 22.56±3.26 pg/ml for metastatic cases and 4.46±1.16 pg/ml for non-metastatic cases. These higher mean values in metastatic cases for both genders indicated a significant difference compared to the non-metastatic group ($p<0.05$) (Fig. 2). The concentration of FGF-8 in metastatic and non-metastatic groups, comprising two categories, i.e., males and females, was analyzed using two-way ANOVA. The difference between males and females was not significant ($p=0.36$), but there was a significant difference between the metastatic and non-metastatic groups (independent variable) in relation to the dependent variable FGF-8 ($p<0.05$). It was also found that there was an interaction between the two variables, sex and metastatic/non-metastatic status, in relation to the dependent variable FGF-8 ($p<0.05$). A non-significant result between males and females cannot prove that there is no difference between sexes. Since the interaction between sex and metastatic/non-metastatic status was significant, it indicates that sex was important in influencing the dependent variable, i.e., FGF-8 (Fig. 3). In the metastatic group, FGF-8 protein showed a significant positive correlation with B-ALP isoenzyme ($r=0.58$) and serum calcium ($r=0.62$). Serum calcium also displayed a very high positive correlation ($r=0.59$) with B-ALP isoenzyme activity (Fig. 4).

Table 1. Mean±SD of various parameters in non-metastatic and metastatic groups

Parameters	Non-metastatic	Metastatic	p
Age (years)	55.63±11.26	60.86±12.14	0.19
Total serum ALP (U/L)	88.80±18.78	471.13±135.23	≤0.05
Bone-ALP (U/L)	17.05±4.69	396.07±111.84	≤0.05
Liver-ALP (U/L)	34.41±8.33	33.66±11.99	0.59
Intestinal-ALP (U/L)	37.33±7.90	38.19±14.34	0.93
Serum ALT (U/L)	31.16±8.69	49.36±19.94	0.07
Serum AST (U/L)	42.36±8.69	42.07±13.73	0.86
Total bilirubin (µmol/L)	13.17±2.73	12.65±3.93	1.00
Blood urea (mmol/L)	11.44±3.63	17.53±9.54	0.09
Serum creatinine (µmol/L)	68.92±23.86	99.89±54.80	0.16
Serum calcium (mmol/L)	2.02±0.23	2.65±0.17	≤0.05
Serum FGF-8 (pg/ml)	5.36±1.79	21.51±3.41	≤0.05

Data are expressed as Mean±SD and were compared using the Welch's T-test. P<0.05 was considered statistically significant. SD: Standard deviation; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FGF-8: Fibroblast Growth Factor-8.

Discussion

Secondary bone cancer, also known as bone secondaries or bone metastases, occurs when cancer spreads to the bones from its original site. In this study, 30 patients with secondary bone metastasis were enrolled. The research revealed that the age of men with prostate cancer with bone metastasis concurred with the findings of Plym et al. [15]. Similarly, Shoemaker et al. [16] reported that the majority of breast cancer cases occurred in women aged 40–44 and 45–49 years, which aligns with the results of this study. A case-control study by Rao et al. [17] conducted in India found that the mean age of breast cancer patients was 46.2 years.

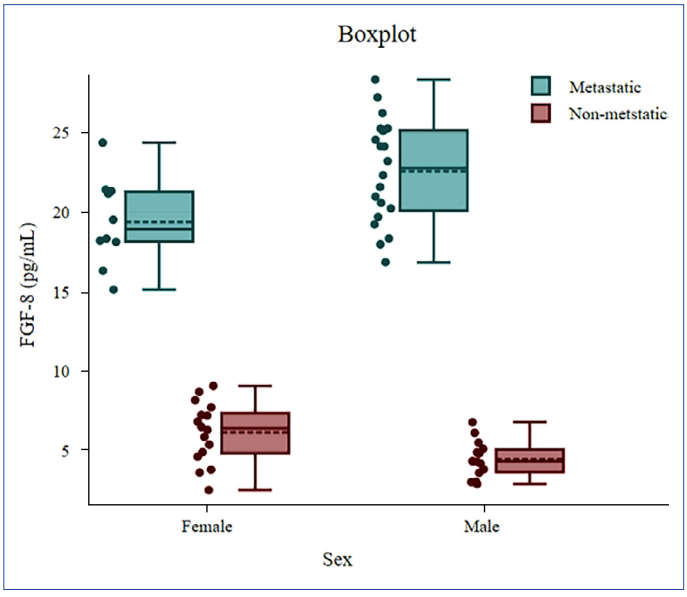

Figure 2. FGF-8 (pg/mL) Concentration in metastatic and non-metastatic stratified by gender.
FGF-8: Fibroblast Growth Factor-8.

The present study indicated that ALP levels in the metastatic group were significantly higher than those in the non-metastatic group. Singh et al. [18] concluded that women with breast cancer generally exhibit higher ALP activities than normal healthy women. The progressive increase in serum ALP activities with breast cancer serves as an indication of metastasis. Measuring this parameter could be a valuable diagnostic tool for monitoring the disease's progression and treatment, especially in areas lacking sophisticated studies. A meta-analysis by Jiang et al. [19] summarized that breast cancer patients had higher ALP levels compared to healthy controls, and elevated levels of both ALP and B-ALP were risk factors for bone metastasis. Another study by Akimoto et al. [20] found that ALP levels showed significant differences concerning the extent of bone metastasis. Researchers have employed heat stability and L-phenylalanine inhibition studies on human serum alkaline phosphatase to

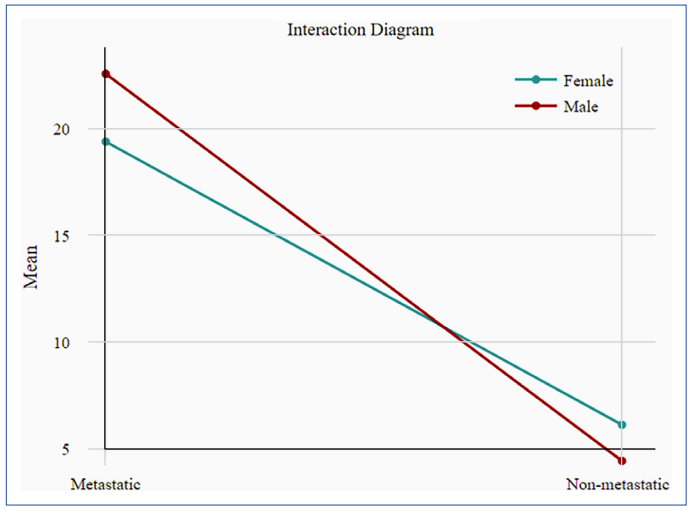

Figure 3. Interaction between the two variables sex and metastatic/non-metastatic in relation to the dependent variable FGF-8.

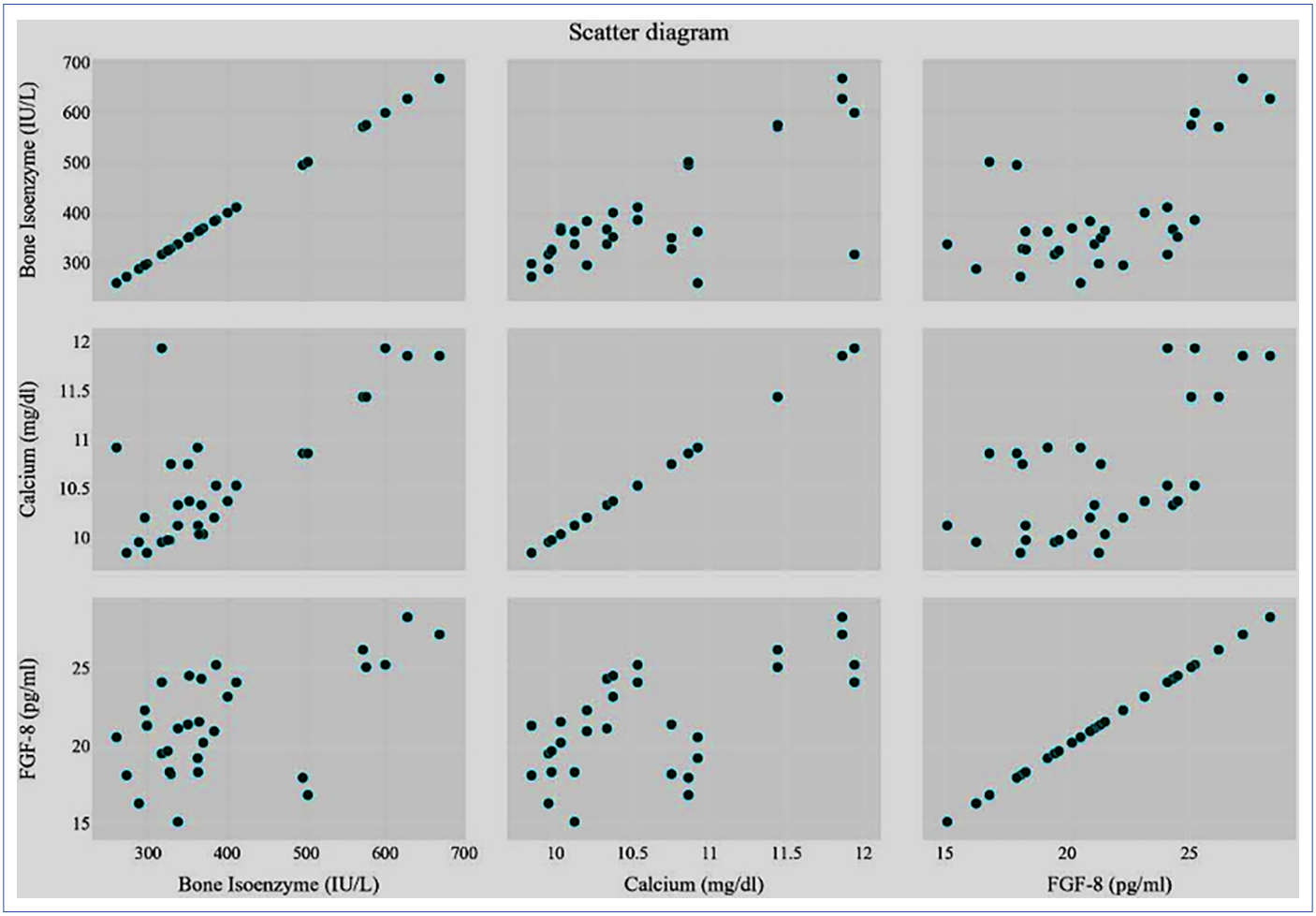

Figure 4. Scatter graph of person`s correlation between FGF-8, serum calcium and bone-ALP isoenzyme.

identify the isoenzyme contributing to the total enzyme activity in the serum. Bone alkaline phosphatase was observed to be more heat-labile than liver and intestinal alkaline phosphatase, allowing for differentiation through pre-incubation of the serum at 56°C for 10 minutes before enzyme assay. After heat treatment, serum alkaline phosphatase levels decreased to less than 14% of the original activity in the metastatic group. The bone ALP fraction was significantly higher in the metastatic compared to the non-metastatic group. In a study by Lorente et al. [21] analyzing bone alkaline phosphatase enzyme concentrations in patients with prostate cancer, it was concluded that the clinical use of bone alkaline phosphatase enzyme measurement is valuable for diagnosing bone metastasis and assessing the progression of prostate cancer due to its good sensitivity and specificity. Other biochemical parameters, including serum ALT, AST, total bilirubin, blood urea, and serum creatinine, did not show significant differences between the metastatic and non-metastatic groups, indicating that there was no invasion of cancer into other tissues except bone.

The serum calcium level was markedly elevated in the metastatic group compared to the non-metastatic group. According to a study by Joeckel et al. [22], the highly expressed calcium-sensing receptor (CaSR) and its downstream signaling pathways promote bone metastasizing cell migration and proliferation. The primary mechanism responsible for approximately 80% of malignancy-related hypercalcemia is PTHrP production. PTHrP's biochemical structure is very similar to that of PTH [23]. PTHrP acts on osteoblasts to increase the production of RANKL, which in turn activates osteoclasts and causes bone resorption, releasing calcium into the circulation. Another pathway through which PTHrP causes hypercalcemia is by increased renal calcium reabsorption. Osteolytic metastases and excessive calcium release from bone account for about 20% of all cases of hypercalcemia. The majority of cases of osteolytic hypercalcemia are caused by breast and prostate cancer [24].

FGF-8 concentration was measured in the serum of both the metastatic group (breast cancer or prostate cancer with bone metastasis) and the non-metastatic group, and it was significantly higher in the metastatic group compared to the non-metastatic group. The difference was statistically significant ($p<0.05$). FGF-8 mRNA expression was found in the cancerous prostatic epithelium by Dorkin et al. [25] using in situ hybridization. FGF-8 expression levels were substantially linked

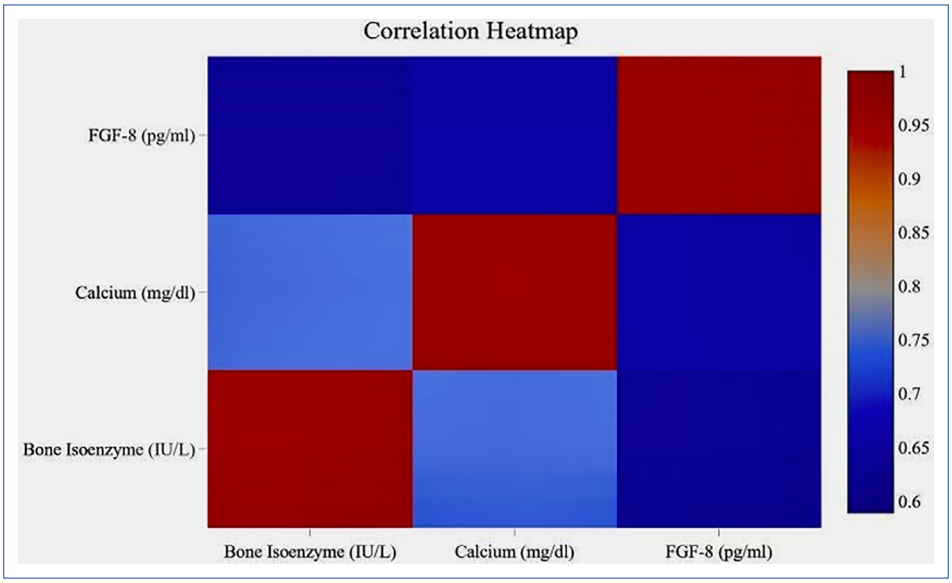

Figure 5. Heatmap diagram of correlation between FGF-8, serum calcium and bone-ALP isoenzyme.

with low survival rates, advanced tumor stages, and higher Gleason scores. Darby et al. [26] studied the expression of hSef, a crucial inhibitory regulator of FGF signaling, in prostate cancer and concluded that siRNA-induced forced downregulation of hSef promoted FGF-8-induced cell migration and invasion. Valta et al. [27] conducted a tissue microarray in patients with prostate cancer with bone metastasis and reported that 76% of samples tested positive for FGF-8. Similar observations were made by Gnanapragasam et al. [28]. Nilsson et al. [29] conducted a cDNA microarray analysis to identify target genes associated with FGF-8b-mediated breast cancer cell proliferation. The study revealed the involvement of several essential regulators of early cell cycle progression, such as Btg2 and cyclin D1, as well as mitosis-related regulators, including cyclin B, Plk1, survivin, and aurora kinase A. These findings suggest that FGF-8 plays a role not only in promoting cell cycle progression through the G1 restriction point but also in regulating critical proteins involved in chromosomal segregation during mitosis and cytokinesis in breast cancer cells. FGF-8 efficiently anticipates the differentiation of bone marrow cells into osteoblasts and boosts bone production *in vitro*. It is conceivable that FGF-8 also increases bone growth *in vivo*. The findings suggest that FGF-8, which is produced in significant amounts by malignant tumors of the breast and prostate, may play a role in the development of osteosclerotic bone metastases [30].

Fibroblast growth factor 8 (FGF-8) emerges as a pivotal player in the insidious choreography of cancer metastasis, wielding its influence through diverse mechanisms. One key act involves triggering epithelial-mesenchymal transition (EMT), a phenotypic metamorphosis where tumor cells shed their epithelial adherence and don mesenchymal traits, granting them enhanced motility and invasiveness [31]. FGF-8 accomplishes this feat by activating signaling pathways like Wnt and MAPK, dismantling epithelial cell junctions, and promoting the expression of mesenchymal markers [32]. Moreover, FGF-8 fosters a pro-metastatic microenvironment by inducing angiogenesis, the construction of new blood vessels that nourish and oxygenate tumors and facilitate their dissemination [33]. Additionally, FGF-8 bolsters the resilience of cancer cells against the hostile conditions encountered during metastasis, such as hypoxia and anoikis (detachment-induced cell death) [34]. This multifaceted orchestration by FGF-8 underscores its critical role in propelling cancer cells along the metastatic odyssey, highlighting its potential as a therapeutic target for curbing cancer's spread.

FGF-8, bone ALP isoenzyme, and serum calcium level all exhibited strong positive correlations. These associations indicate that the FGF-8 protein plays a role in the initial bone metastasis of breast or prostate cancer (Fig. 5). Several lines of evidence point towards a positive correlation between FGF-8 protein levels and bone ALP activity. *In vitro* studies by Mansukhani et al. [35] demonstrated that FGF-8 directly stimulates ALP expression in osteoblast precursors, suggesting a direct regulatory role. In an *in vivo* model of prostate cancer with bone metastasis, FGF-8 expression enhances PC-3 prostate cancer cells' proliferation as intratibial tumors and regulates the occurrence of bone lesions [27]. These observations suggest that FGF-8 acts as a potent stimulator of osteoblast differentiation and maturation, as evidenced by elevated ALP levels. However, the relationship is not unidirectional. Bone ALP may influence FGF-8 signaling through a feedback loop. Recent studies suggest that ALP can dephosphorylate FGF-8, potentially modulating its activity and downstream effects [36]. This intricate interplay highlights the delicate balance between FGF-8 and B-ALP.

The present study suggests that measuring FGF-8 in serum holds promise as a superior marker for bone metastasis compared to B-ALP, due to several key advantages. First, FGF-8 levels are significantly higher in patients with bone metastasis compared to those without, while B-ALP levels can be elevated

due to other bone diseases. This specificity makes FGF-8 a more reliable indicator of bone metastasis. Secondly, FGF-8 may be detectable earlier in the course of bone metastasis compared to B-ALP and may correlate with the extent and severity of bone metastasis, potentially providing valuable prognostic information for treatment planning. FGF-8 plays a direct role in promoting bone metastasis through mechanisms like EMT and angiogenesis, while B-ALP is a downstream marker of bone activity. Understanding the underlying mechanisms of FGF-8 involvement provides more insight into disease progression.

Conclusion

FGF-8 is expressed at a high frequency in bone metastases of hormonal cancers as it is associated with the induction and facilitation of prostate tumorigenesis and increases the growth and angiogenesis of breast and prostate cancer. FGF-8 has demonstrated a robust autocrine growth factor role, fostering osteoblastic differentiation in metastasis, leading to increased B-ALP levels. Previous expression studies have assayed total FGF-8 levels in tissue samples, not particularly in serum. However, affordable, quicker, and more accurate assays like ELISA-based kits that measure FGF-8 in serum might be helpful to assess the progression of bone tumors in patients with breast or prostate cancer for better treatment plans.

Acknowledgements: We would like to express our deepest gratitude to all the individuals and institutions whose valuable contributions and support made this research possible.

Conflict of Interest: The authors declare that there is no conflict of interest.

Ethics Committee Approval: The study was approved by The Pt. B.D. Sharma Post Graduate Institute of Medical Sciences Rohtak, Department of Biochemistry Ethics Committee (No: ECI/21/26, Date: 18/07/2021).

Financial Disclosure: The authors declared that this study has received no financial support.

Peer-review: Externally peer-reviewed.

Authorship Contributions: Concept – G.S.G.; Design – G.S.G.; Supervision – S.K.; Funding – G.S.G.; Materials – M.K.; Data collection &/or processing – G.S.G.; Analysis and/or interpretation – G.G.; Literature search – U.M.; Writing – G.S.G.; Critical review – M.K.

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