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High Expression of Parathyroid Hormone-related Protein and Tumor Necrosis Factor-α in Cancer Cells as Risk Factors for Hypercalcemia in Bone Metastases Lytic Lesions

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Abstract

BACKGROUND: Cancer mortality is more commonly due to metastases of the tumor to other organs and the complications that accompany it than the tumor growth itself. Until recently, metastasis has been an insurmountable problem.

AIM: As the most frequent site of metastases, apart from the lungs and liver, tumor metastases to bone are associated with hypercalcemia which is fatal for the affected patient.

METHODS: This study used a case-control study design. The case group consisted of paraffin block samples derived from bone metastatic cancer cell biopsies of patients with hypercalcemic lytic lesions. The control group consisted of paraffin block samples derived from bone metastatic cancer cell biopsies of patients with non-hypercalcemic lytic lesions. Radiological examination was performed to examine the presence of lytic lesions, followed by data collection of serum calcium levels. The data obtained from the histopathological examination was confirmed along with the availability of paraffin blocks of bone metastasis cancer cell biopsy samples, and immunohistochemical analysis was performed to determine the expression of tumor necrosis factor-α (TNF-α) and parathyroid hormone-related protein (PTHrP). A Mann–Whitney test was performed to determine the expression of TNF-α and PTHrP between hypercalcemia and non-hypercalcemia groups. To identify the cut-off point, Youden index on receiver operating characteristic was used, then the optimal cut-off point was determined where the sensitivity and specificity curves intersect. Analysis of risk factor assessment was done by creating a 2 × 2 cross-tabulations and calculating the association size in the form of odds ratio (OR).

RESULTS: The expression of PTHrP and TNF-α in the case group was significantly different from the control group with p < 0.05. The cut-off point for PTHrP expression was 267.5 with an area under the curve of 0.93, indicating a high accuracy value. The cut-off point for TNF-α expression was 227.5 with an area under the curve of 0.68, indicating a moderate accuracy value. The OR between hypercalcemia and non-hypercalcemia to PTHrP expression was 110.3 (Fisher's exact statistical test obtained p < 0.05), while the OR between hypercalcemia and non-hypercalcemia to TNF-α expression was 7.27 (Fisher's exact test statistical obtained p = 0.01).

CONCLUSION: Significant differences in the expression of PTHrP and TNF-α were found between patients with bone metastases lytic lesions with hypercalcemia compared to those without hypercalcemia. We can conclude that either a high level of PTHrP expression and/or TNF-α expression in cancer cells can serve as risk factors for hypercalcemia in patients with bone metastatic lytic lesions.

Introduction

Cancer mortality is more commonly due to metastasis of the tumor to other organs and the complications that accompany it than the growth of the tumor itself. Each year 350,000 people die from bone metastases in America. Bone is the site of the most frequent occurrence of metastases other than lung and liver. In prostate and breast cancer patients, bone metastases have a prevalence of more than 70% [1]. Tumor metastases to bone are associated with hypercalcemia which is fatal for the patient.

Cancer-related hypercalcemia has a poor prognosis, in which 80% of patients will die within 1 year and the median survival time is 3–4 months [2].

Hypercalcemia leads to arrhythmias, which will lead to cardiac arrest and eventually to death. Thirty percent of cancer patients will have hypercalcemia. It is estimated that 50% of cancer patients with hypercalcemia will die within 30 days. With oncology and supportive therapy, patients with symptomatic hypercalcemia still have a poor prognosis with low survival rate [3], [4], [5], [6].

Hypercalcemia in bone metastases is associated with the local effect of the tumor where

bone resorption occurs. The humoral factor is secreted in the form of paracrine or endocrine or, very rarely, is the result of excessive activation of Vitamin D by tumor cells. However, not all cases of bone metastases with lytic lesions resulted in hypercalcemia. In the case of advanced bone metastases causing massive local osteolytic, it is documented that only 20% of patients with cancer patients are associated with hypercalcemia. The cause of this still cannot be explained until now [6], [7].

The cause of the release of calcium from the bone microenvironment is due to increased osteoclast activity. Parathyroid hormone-related protein (PTHrP) and cytokines such as tumor necrosis factor-α (TNF-α) are mediators that are known to support bone resorption through osteoclast activity. PTHrP is a paraneoplastic protein that takes its name from its similarity between the amino-terminal portions and is related to the portion of the parathyroid hormone [8]. PTHrP acts on osteoblasts that secrete RANKL to activate osteoclasts [9]. PTHrP is secreted by tumor cells then binds to the PTHRP1 receptor on osteoblasts and perhaps even on osteocytes which further increases RANKL expression. PTHrP also decreases the production of Osteoprotegerin. RANKL then binds to RANK on the osteoclast precursor. This binding activates the transcription factor AP1 through JUN N-terminal kinase and NF-κB through κ-B kinase inhibitor which in turn stimulates osteoclastogenesis and causes osteoclastic resorption of the bone matrix [5], [10], [11], [12].

TNF-α stimulates bone resorption by osteoclasts in vitro and causes hypercalcemia *in vivo* [6]. TNF-α-mediated osteoclastogenesis consists of 2 different mechanisms, RANK dependent and RANK independent. During the early phase of osteoclastogenesis, TNF-α increases the proliferation and differentiation of osteoclast precursors in response to macrophage-colony stimulating factor and by stimulating c-Fms expression, this is referred to as the independent RANK pathway. The osteoclast precursor will differentiate into mature osteoclasts in the presence of RANKL; here, TNF-α acts on RANK, so it is called the RANK dependent pathway [13].

Recognizing risk factors is vital and if hypercalcemia is left untreated, it will quickly worsen and can be life-threatening. Recognizing the role of PTHrP and TNF-α as risk factors for hypercalcemia can aid in the investigation and facilitate appropriate management to prevent poor prognosis and extend the life expectancy of patients with bone metastases.

Materials and Methods

This research is an analytic observational study with a case-control study design. The case group consisted of paraffin block samples derived from bone metastatic cancer cell biopsies of patients with hypercalcemic lytic lesions. The control group consisted of paraffin block samples derived from bone metastatic cancer cell biopsies of patients with non-hypercalcemic lytic lesions. The research was conducted at Orthopaedic and Traumatology Polyclinic and the Department of Anatomical Pathology, Sanglah Hospital, from November to December 2020. The number of samples was determined using a two-way hypothesis test resulted in a total sample size of 46 patients per group. The samples were selected from the database of orthopedic patients at Sanglah Hospital from 2016 to 2019. Consecutive sampling technique was performed where samples that met the inclusion criteria were selected until the number of samples was met. Inclusion criteria include all bone metastatic cancers with lytic lesions on X-ray, good paraffin block preparations sufficient for examination, laboratory results showing hypercalcemia, and no hypercalcemia. The exclusion criteria are as follows: Patients with a history of or in treatment with drugs containing Thiazide, Bisphosphonate, or RANKL antibodies (Denosumab), a history of or in hyperparathyroidism conditions, patients with a history of or in conditions of intoxication of Vitamin A and Vitamin D, damaged paraffin block reparations, and unavailable laboratory data on calcium levels.

Research procedure

First, data collection of cancer patients with bone metastases from orthopedic patient recapitulation data was carried out, then the patients were sorted according to the inclusion and exclusion criteria. Patients with lytic lesions, as proven by the X-ray, were collected and serum calcium data were obtained. Following that, the histopathological examination data were confirmed and the availability of paraffin blocks of the bone metastasis cancer cell biopsy samples at the Anatomical Pathology Lab of Sanglah Hospital was examined. Immunohistochemical analysis was then carried out to determine the expression of TNF-α and PTHrP.

Calcium examination procedure

Blood samples from the patients were obtained using a tube without EDTA and then centrifuged for 15 min. Serum supernatant was taken and treated with COBAS 6000 machine.

PTHrP and TNF-α immunohistochemical examination procedure

To get an image of PTHrP expression in the tissue, immunohistochemical staining was performed using the Avidin-Biotin Complex (ABC) method. Paraffin blocks were cut using Leica RM2235 microtome with a thickness of 3 μm, then dried and affixed to a poly-L-lysine

coated slide. The slides were heated on a slide warmer at 60° for 30 min. Furthermore, deparaffinization with xylol was performed gradually (xylol I, II, III) for 5 min each, followed by rehydration in decreasing concentrated alcohol (absolute alcohol, 96%, and 80%) gradually for 4 min each. Then, the endogenous peroxidase was neutralized with hydrogen peroxide/methanol 0.5% v/v for 10 min. The slides were washed in running water, then heat-induced retrieval antigen was carried out using Tris EDTA buffer solution (pH 9.0) for 3 min. The slides were washed with Phosphate-buffered Saline (PBS) pH 7.4 for 5 min, then the slides were coated with normal serum for 10 min. Slides were incubated with primary antibody (polyclonal antibody PTHrP, Elabscience with 1:100 dilution; TNF-α monoclonal antibody, and Elabscience with 1:100 dilution) for 1 h. The slides were washed with PBS for 5 min and then incubated with biotinylated secondary antibodies for 15 min. Slides were washed with PBS for 5 minutes then incubated with ABC-horseradish peroxidase (ABC-HRP) for 15 minutes. Slides were then washed with PBS for 5 min and incubated with diaminobenzidine tetrahydrochloride for 1 min. The slides were rinsed under running water and counterstained with hematoxylin. Slides were rinsed under running water then immersed in saturated lithium carbonate for 1 min. Dehydration and clearing processes were performed, then the slides were closed with a mounting solution and coverslip [14], [15].

The PTHrP and TNF-a review was conducted blindly by researchers who did not know the histopathological diagnosis of the cases they were evaluating. Slides were examined at 400× magnification, evaluation was performed on the cytoplasm of tumor cells colored brown when positive [16]. Breast cancer tissue was used for PTHrP positive and negative control. Tonsil tissue was used for TNF-a positive and negative control. The PTHrP and TNF-a expression score was carried out using the Histo-Score (H-Score) with a total range of 0–300 which is the sum of the percentage of positive tumors (range 0–100) and the intensity score (0 = negative, 1 = weak, 2 = intermediate, 3 = strong) [17].

Data analysis

Descriptive analysis was used to determine the standard deviation, mean, and median, while frequency distribution was used to describe general characteristics and variations between groups. The normality of data was performed using Shapiro–Wilk test.

To determine if there is a difference in the TNF-α and PTHrP expression between the hypercalcemia and non-hypercalcemia groups, a Mann–Whitney test was performed. To identify the cut-off point for TNF-α and PTHrP expressions, a Youden Index on receiver operating characteristic (ROC) is used, the optimal cut-off point was determined where the sensitivity and specificity curves intersect. Youden's Index Formula = Sensitivity + Specificity -1.

The index with the highest value was selected as the intersection point on the ROC curve [18], [19]. Risk Factor Assessment was carried out by creating a 2 × 2 cross-tabulations and calculating the association size in the form of odds ratio (OR). All data analysis was performed with a significance level of α = 0.05 or 95% confidence interval (CI) value.

Results

The study samples consisted of 23 paraffin blocks from the biopsy results of MBD patients with hypercalcemia and 23 paraffin blocks from those who were not hypercalcemic. Each group was stained with PTHrP antibody and TNF-α antibody.

Normality test

Table 1. Normality test for PTHrP expression, TNF-α expression, and calcium levels with Shapiro–Wilk test.

Table 1: Normality test for PTHrP expression, TNF-α expression, and calcium levels with Shapiro–Wilk test

Variable	Group	p	n	Note
PTHrP expression	Case	0.00	23	Not normal
	Control	0.00	23	Not normal
TNF-α expression	Case	0.00	23	Not normal
	Control	0.46	23	Normal
Calcium level	Case	0.117	23	Normal
	Control	0.140	23	Normal

TNF: Tumor necrosis factor, PTHrP: Parathyroid hormone-related protein.

From the normality test, it was found that PTHrP expression in both the case and control group did not have a normal distribution, while TNF-α expression has variable normality distribution. The distribution of calcium levels has a normal distribution (Table 1).

Characteristics of research subjects

In general, the study sample consisted of 17 men (37%) and 29 women (63%). The mean age of the patients was 55.24 (±12.16) years. Age, sex, and tumor origin from solid organs did not differ statistically between case and control group (Table 2). Most types of tumors came from breast cancer (9 cases; 19.6%) (Table 3). Most MBD locations were in the proximal femur region (12 cases; 26.1%) and in the thoracic vertebrae region (16 cases; 34.8%) (Table 4).

Table 2: Characteristics of research subjects

Characteristics	Case (n = 23)	Control (n = 23)	p
Age (Mean ± SD) year	55.3 (± 11.8)	55.2 (± 12.,8)	0.326[a]
Sex			
Male	10	7	1.000[b]
Female	13	16	
Calcium level (Mean±SD) mg/dL	12.13 (± 1.41)	8.57 (± 0.76)	0.035[a]
PTHrP expression (median)	280	240	0.000[c]
TNF-α expression (median)	250	225	0.042[c]

[a]t-test; [b]Chi-square; [c]Mann–Whitney. TNF: Tumor necrosis factor, PTHrP: Parathyroid hormone-related protein.

Differences in expression of PTHrP and TNF-α in the case and control groups

Mann–Whitney test was performed to determine differences in the expression of PTHrP and TNF-α. PTHrP expression in the group case was significantly different from the control group with a p < 0.05 (p = 0.00) and the TNF-α expression in the case group was significantly different from the control group with a p < 0.05 (p = 0.04).

Table 3: Origin of bone metastatic cancer

Origin of cancer	Group		Total	%
	Case	Control		
Adeno Ca	5	3	8	17,4
Squamous Cell Ca	1	1	2	4,3
Adeno Ca thyroid	2	2	4	8,7
Not otherwise specified	5	3	8	17,4
Ca Cervix	2	0	2	4,3
Clear cell carcinoma	1	0	1	2,2
Adeno Ca Gaster	1	0	1	2,2
Ca Nasofaring	0	2	2	4,3
Ca Mammae	3	6	9	19,6
Adeno Ca lungs	3	4	7	15,2
Adeno Ca Prostate	1	1	2	4,3
Total	23	23	46	100

Hypercalcemia risk factors

Bivariate analysis was performed on the independent variables PTHrP expression and TNF-α expression as risk factors for hypercalcemia in bone metastatic lytic lesions by calculating the OR. The calculation of the OR was done by cross-tabulating the case and control pairs. The cut-off point for the PTHrP expression variable was obtained using Youden Index at the intersection of the ROC curve. From the analysis, it was found that the cut-off point for PTHrP expression was 267.5. The results of PTHrP area under curve obtained a value of 0.93 (p = 0.00; 95% CI; 0.83–1) which means it has a high accuracy value [19]. Thus, an H-Score below 267.5 belongs to the low PTHrP expression group and an H-Score equal to or more than 267.5 belongs to the high PTHrP expression group.

Table 4: Location of bone metastatic cancer

Metastases location	Group		Total	%
	Case	Control		
Proximal humerus	1	0	1	2,2
Shaft humerus	3	0	3	6,5
Distal humerus	0	1	1	2,2
Scapula	1	0	1	2,2
Proximal femur	6	6	12	26,1
Shaft femur	2	1	3	6,5
Distal femur	1	0	1	2,2
Vertebra cervical	0	1	1	2,2
Vertebra thoracal	7	9	16	34,8
Vertebra lumbar	2	2	4	8,7
Pelvic	1	2	3	6,5
Total	23	23	46	100

The cut-off point for TNF-α expression variable was obtained using Youden Index at the intersection point coordinates of ROC curve. From the analysis, it was found that the cut-off point for TNF-α expression was 227.5. The results of TNF-α under curve area obtained a value of 0.68 (p = 0.04; 95% CI; 0.52–0.84) which means that it has a moderate accuracy value [19]. Thus, an H-Score below 227.5 belongs to the low TNF-α expression group and an H-Score equal to or more than 227.5 belongs to the high TNF-α expression group.

Table 5: Cross-tabulation of PTHrP expressions between case and control

Group	Case	Control	Total
High PTHrP expression	21	2	23
Low PTHrP expression	2	21	23
Total	23	23	46

OR=110.3 (p = 0.000, CI 95%; 14.176–857.42). OR: Odds ratio, PTHrP: Parathyroid hormone-related protein.

Expression of PTHrP with hypercalcemia in bone metastases

Using the PTHrP cut-off point 267.5, the OR between hypercalcemia and non-hypercalcemia to PTHrP expression was 110.3. Statistically, this OR was declared significant because with the Fisher's Exact statistical test, the p < 0.05 (p = 0.000) (Table 5), this means that higher PTHrP expression is a risk factor for hypercalcemia in bone metastatic lytic lesions. Immunohistochemical staining for PTHrP antibody can be seen in Figures 1 and 2.
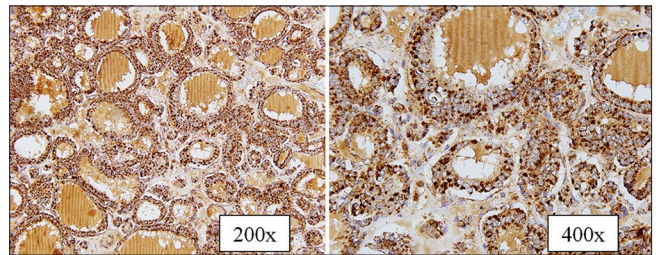

Figure 1: Immunohistochemical staining sample for parathyroid hormone-related protein (PTHrP) antibody in the case group. Histo-Score 280, high PTHrP expression
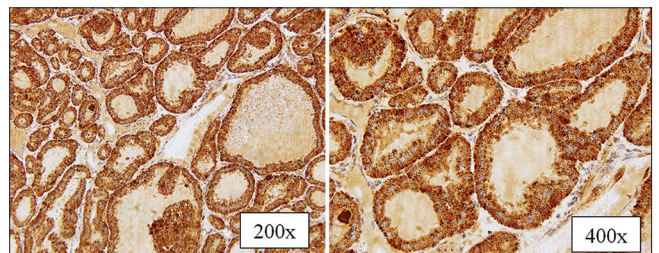

Figure 2: Immunohistochemical staining sample for parathyroid hormone-related protein (PTHrP) antibody in the control group. Histo-Score 250, low PTHrP expression

TNF-α Expression with hypercalcemia in bone metastases

Using TNF-α cut-off point of 227.5, OR obtained between hypercalcemia and non-hypercalcemia to TNF-α expression was 7.27.
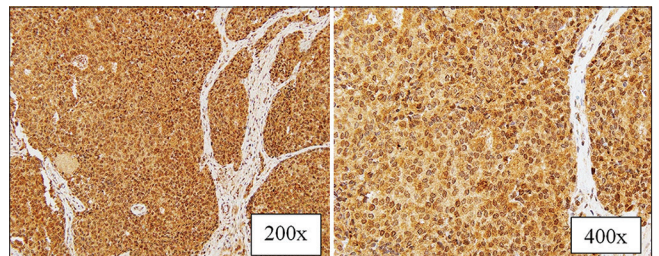

Figure 3: Immunohistochemical staining sample for tumor necrosis factor (TNF)-α antibody in the case group. Histo-Score 290, high TNF-α expression

Statistically, the OR was significant because with the Fisher's Exact statistical test, the p < 0.05 (p = 0.01) (Table 6), this means that a higher TNF-α expression is a risk factor for hypercalcemia in bone metastases lytic lesions. Immunohistochemical staining sample for TNF-α antibody can be seen in Figures 3 and 4.

Table 6: Cross-tabulation of TNF-α expressions between case and control

Group	Case	Control	Total
High PTHrP expression	20	11	31
Low PTHrP expression	3	12	15
Total	23	23	46

OR=7.27 (p = 0.01, CI 95%; 1.68–31.43). TNF: Tumor necrosis factor, OR: Odds ratio, PTHrP: Parathyroid hormone-related protein.

Discussion

Characteristics of gender, age, location of metastases, and cancer origin

Research by Silva *et al*. (2019) reported that out of 1283 patients with lung cancer in its development, 11.9% of patients had bone metastases with an average age of 60 years old, with male 52.7%. Based on age demographics, most bone metastases occur in adults over 50 years of age, while in contrast to most sarcomas which occur in adolescents or young adults (aged less than 30 years old). Therefore, bone-occupying mass in adults is more likely to be metastatic carcinoma than primary sarcomas of bone [20]. The gender in this study was different from other studies due to differences in the population where the study was conducted, different type of cancer, and the smaller number of samples.
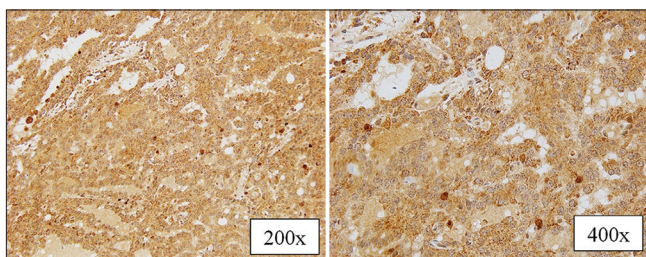

Figure 4: Immunohistochemical staining sample for tumor necrosis factor (TNF)-α antibody in the control group. Histo-Score 220, low TNF-α expression

Coleman *et al*. (2006) stated that primary cancer in the majority of bone metastases was breast cancer as much as 73% with vertebral region being the most metastases site by 20% followed by other places as much as 13% [21]. The primary sites of cancer metastases in this study were the same as other studies, mostly from breast cancer. This is due to several factors: Breast cancer forms multiple cell adhesion molecules, including integrins, cadherins, osteopontin, bone sialoprotein, laminin, and type IV collagen which play a role in breast cancer cells, so they prefer to metastasize to bone. Breast cancer cells also produce PTHrP which activates stromal and osteoblast

cells in the production of RANKL, eventually activating osteoclasts so that bone resorption occurs and growth factors are released from the bone matrix [22].

The location of most metastases is in the vertebral region because the types of cancer originating from breast, thyroid, prostate, lung, kidney, and hematopoietic are more often metastasizes there. Thoracic vertebrae are the most frequently involved region in this study because it contains the largest volume of bone marrow to accept metastatic deposits [23]. Lung and breast cancers mostly metastasize to the thoracal region due to venous flow from the breast through the azygos connected to Batson plexus in the thoracal region, while lung cancer spreads through pulmonary veins and is distributed to the bone. Prostate cancer usually spreads to the lumbosacral region because prostate cancer flows through pelvic plexus in lumbar region [24].

Expression of PTHrP in bone metastatic hypercalcemia

PTHrP is a protein encoded by a single gene on the short arm of chromosome 12. At approximately 130 amino acid sequences, PTHrP has 10% the same structure as PTH. With a similar but not identical structure, PTHrP has similar activities like PTH such as agonist action on PTH receptors in the kidneys and bones, resulting in increased bone resorption and calcium reclamation in the kidneys. Specifically in bone, PTHrP causes bone resorption by increasing osteoclast activation and suppressing osteoblasts, without being followed by bone formation which causes an increase in the amount of calcium in the circulation [25], [26].

PTHrP has been shown to have a role in bone metastases. Iddon *et al*. (2000) in their study of bone metastases samples from patients with prostate cancer found 10 out of 14 samples expressed significant PTHrP (p = 0.001) when compared with primary prostate cancer cells [27]. Previously Hayman *et al*. (1991) obtained similar results on immunohistochemical staining on 13 samples of bone metastases biopsy of patients with breast cancer, 12 of 13 samples of PTHrP positive expression (92.3%) with p < 0.0001 compared with those without bone metastases [27], [28].

Until now, there has been scarce publication on the expression of PTHrP in cases of bone metastases with hypercalcemia in humans. Miki *et al*. (2004) in their *in vivo* study on male S EB-17/lcr-scid mice aged 6–8 weeks that were injected with human lung cancer cells then given anti-PTHrP antibody intravenously, found an improvement in calcium levels and a lower amount of metastases compared to control group that was not given anti-PTHrP antibody [29].

One of the causes of hypercalcemia in cancer is bone metastases causing lytic lesions from osteoclast activity and calcium release. This process usually occurs

in patients with multiple myeloma and metastatic solid organ tumors such as breast cancer. Breast cancer cells, for example, produce PTHrP locally and increase RANKL activity which in turn activates osteoclasts and causes lytic processes and calcium release [30], [31].

From this explanation, it is evident that PTHrP has an important role in hypercalcemia occurring in bone metastases. The results of this study indicate that PTHrP has a role in the incidence of hypercalcemia in bone metastatic patients, where cancer with bone metastatic and high PTHrP expression will increase the risk of hypercalcemia, causing higher morbidity and even mortality to patients.

TNF-α expression in bone metastatic hypercalcemia

Several studies have shown that TNF-α plays a role in the inflammatory process associated with carcinogenesis. TNF-α is produced by many types of cells, such as macrophages, neutrophils, fibroblasts, T cells, B cells, and tumor cells themselves. TNF-α has an important role in tumor progression and microenvironment, where this cytokine enhances angiogenesis and increases the invasion and migration of cancer cells, including metastases [32]. Hamaguchi *et al*. (2011) found in an *in vivo* study that administering infliximab, a TNF-α antibody caused a decrease of lytic lesions in mice with bone metastatic breast cancer cells [33].

Yu, *et al*. (2019) in a study of patients with nasopharyngeal cancer proved that the mean TNF-α expression was higher in cancer cells of patients with bone metastases compared to those without, although it was not statistically significant due to the small number of case samples. Correspondingly, serum TNF-α levels were proven to have a positive correlation with high invasion of cancer cells to bone and poor patient prognosis [34].

Mazaki *et al*. (2019) performed a real-time PCR test to samples taken from mice vertebrae with breast cancer metastases, proving an increase in TNF-α gene expression compared to IL-6, and this was in line with the results of immunohistochemical expression. It is evident that TNF-α affects lytic lesions in bone metastases [35]. Takahashi *et al*. (2003) studied 29 cancer patients with hypercalcemia and found that TNF-α levels in serum increased, though not significantly [36].

From the explanation of several studies above, it can be concluded that the role of TNF-α has not been proven statistically, although the results did show an increase in TNF-α level. This is different from the results of this study, which proved that TNF-α expression was significantly different between case and control group, with a sample size of 23 patients each. This difference may be due to differences in the number of cases and the design of the study. It can be concluded that TNF-α has a role in hypercalcemia in bone metastatic lytic lesions.

Weaknesses of research

This study was conducted on 46 cases of bone metastatic cancer, it is necessary to consider increasing the number of cases so that variations in cancer types can be reproduced. The only cytokine tested was TNF-α due to limited resources in this study. In the future, other cytokines supporting hypercalcemia process in bone metastases should be further investigated.

Conclusion

There were significant differences in the expression of PTHrP and TNF-α between patients with bone metastases lytic lesions with hypercalcemia compared to those without hypercalcemia. Higher PTHrP and TNF-α expression in cancer cells is a risk factor for hypercalcemia in patients with bone metastatic lytic lesions.

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