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Original Article

Application value of bone metabolism and immune cell indicators in screening for tumor bone metastasis: a retrospective study

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Abstract: Objective: To evaluate the diagnostic value of bone metabolism and immune cell indexes in screening for tumor bone metastasis. Methods: A retrospective study was conducted on 247 patients with malignant tumors. conducted on 247 patients with malignant tumors. According the presence of tumor bone metastasis, patients were divided into a bone metastasis group (156 cases) and a non-bone metastasis group (91 cases). Bone metabolism markers [calcium ion (Ca^{2+}), β-Carbox-terminal telopeptide of type I collagen (β-CTX), type I procollagen N-terminal peptide (P1NP), osteocalcin (OC)] and immune cell indicators ($CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+CD25^+CD127^{low}$ Treg cells) were compared between groups. Correlations among these indices were analyzed using Pearson correlation, and interaction effects were evaluated using multiple linear regression with interaction terms. Receiver operating characteristic (ROC) curves were used to evaluate the screening efficacy of each index for tumor bone metastasis. Results: Compared with the non-bone metastasis group, the bone metastasis group showed significantly higher levels of Ca^{2+}, β-CTX, P1NP, $CD3^+CD4^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells ($P<0.05$), and lower levels of OC and $CD3^+CD8^+$ T cells ($P<0.05$). According to the Soloway classification, levels of Ca^{2+}, β-CTX, P1NP, $CD3^+CD4^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells increased progressively from grade I to grade III ($P<0.05$), whereas OC and $CD3^+CD8^+$ T cells decreased (grade I > grade II > grade III) ($P<0.05$). Ca^{2+}, β-CTX and P1NP were positively correlated with $CD3^+CD4^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells ($P<0.05$) but negatively correlated with $CD3^+CD8^+$ T cells ($P<0.05$). In contrast, OC was negatively correlated with $CD3^+CD4^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells ($P<0.05$) and positively correlated with $CD3^+CD8^+$ T cells ($P<0.05$). A significant interactive effect was observed between bone metabolism and immune indicators ($P<0.05$). The AUC the combined model (0.899) was higher than that of individual indicators - Ca^{2+} (0.835), β-CTX (0.843), P1NP (0.817), OC (0.750), $CD3^+CD4^+$ T cells (0.837), $CD3^+CD8^+$ T cells (0.771), $CD4^+CD25^+CD127^{low}$ Treg cells (0.848). Internal validation showed that the accuracy of the combined model in diagnosing tumor bone metastasis was 88.26%. Conclusions: The combined assessment of bone metabolism and immune indicators provides high clinical value for screening tumor bone metastasis.

Keywords: Bone metabolism, immune cells, malignant tumors, bone metastasis, application value

Introduction

Bone tissue is a common site of metastasis for malignant tumors. Patients with bone metastasis often develop skeletal complications, such as bone pain, pathological fractures, spinal cord compression, and hypercalcemia [1]. These manifestations not only seriously impairs patients' quality of life but also indicates a poor prognosis. At present, the diagnosis of bone metastases in malignant tumors mainly relies on imaging examinations. However, its high cost and involvement of radiation make them unsuitable for frequent monitoring [2]. Therefore, there is an urgent need for reliable biomarkers that can aid in the early diagnosis and treatment of tumor bone metastasis.

Bone metabolism markers, including β-type I collagen carboxy-terminal peptide (β-CTX), type I procollagen N-terminal peptide (P1NP), osteocalcin (OC), and serum calcium ions (Ca^{2+}), reflect bone remodeling and metabolic activity. These markers are widely used in the diagnosis of metabolic bone diseases such as osteoporo-

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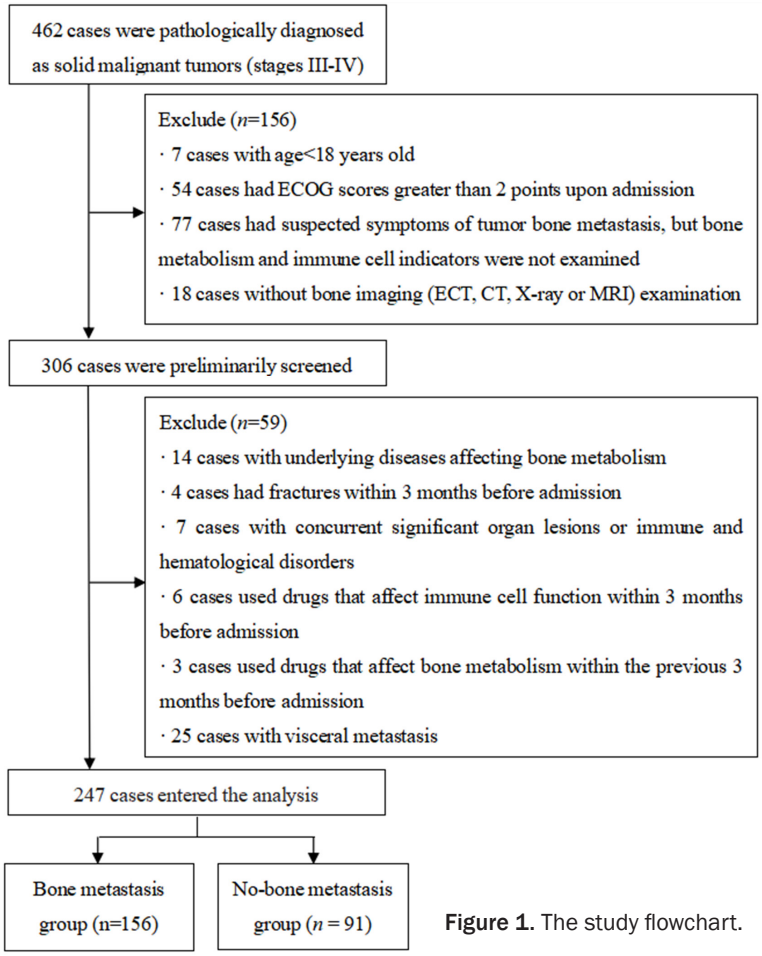

Figure 1. The study flowchart.

sis [3], and are also closely associated with bone metastasis in malignant tumors [4]. Tumor bone metastasis involves both the proliferation of tumor cells at the primary site and the alteration of the bone microenvironment through the secretion of cytokines, extracellular vesicles, and other bioactive molecules. The bone microenvironment contains a variety of immune cells [5], and dynamic interactions between immune cells and tumor cells play crucial roles during bone metastasis [6].

Osteoclasts release transforming growth factor-beta (TGF-β) stored in the bone matrix [7]. Activated TGF-β directly inhibits T-cell receptor signaling, impairing the function of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells [8]. It also induces the differentiation of naive T cells into regulatory T (Treg) cells and enhances Treg cell function [9], thereby altering the proportion of $CD4^+CD25^+CD127^{low}$ Treg cells. However, the immune system is limited in its ability to eliminate tumor cells. Tumor cells can suppress immune function by secreting and expressing various inhibitory molecules [10], facilitating tumor adaptation and immune escape within the bone microenvironment.

Current research primarily focuses on the relationship between bone metabolism and tumor bone metastasis, whereas the involvement of immune cell subsets such as $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells remains insufficiently explored. Therefore, this study evaluated the application value of bone metabolism markers (Ca^{2+}, β-CTX, P1NP, OC) and immune cell indicators ($CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+CD25^+CD127^{low}$ Treg cells) in screening for tumor bone metastasis, with the goal of providing new insights into the diagnosis and treatment of this condition.

Material and methods

Sample size estimation

The sample size was calculated using the formula for cross-sectional studies: $N = Z_\alpha^2 \times [P \times (1 - P)]/E^2$. The confidence level (Z) was set at 1.96, the expected proportion (P) was conservatively estimated at 0.7, and the allowable error (E) was set at 9.5%. Based on these parameters, the minimum required sample size was 175 cases. Assuming a 20% dropout rate of eligible medical records, the final sample size included was 247 cases.

Research subjects

A retrospective study was conducted using clinical data collected from patients diagnosed with malignant tumors at Suzhou Ninth People's Hospital between June 2023 to June 2025. As shown in **Figure 1**, a total of 247 patient were included. According to the presence or absence of tumor bone metastasis, patients were divided into a bone metastasis group (n = 156) and a non-bone metastasis group (n = 91). For patients with bone metastases, the Soloway classification [11] was app-

lied based on the number of metastatic lesions. This classification system is widely used in relevant literature and is applicable to all tumor types included in this study, such as prostate cancer [12], lung cancer [13] and breast cancer [14]. According to the Soloway grading criteria, grade I: 1-2 metastatic lesions; grade II: 3-5 metastatic lesions; grade III: >5 metastatic lesions.

Inclusion criteria: (1) Age ≥ 18 years; (2) Histopathologically confirmed solid malignant tumor; (3) Baseline physical condition at admission assessed by the Eastern Cooperative Oncology Group (ECOG) performance status score ranging from 0 to 2; (4) Presence of mild bone pain, worsening nocturnal pain, or other symptoms suggestive of bone metastasis at admission, with active cooperation in completing bone metabolism and immune cell index detection; (5) Completion of whole-body bone scans using emission computed tomography (ECT), with further examination of suspected areas by X-rays, computed tomography (CT), or magnetic resonance imaging (MRI).

Exclusion criteria: (1) Presence of comorbidities affecting bone metabolism, such as diabetes, osteoporosis, or rheumatoid arthritis; (2) History of fracture within 3 months prior to admission; (3) Patients with immunodeficiency diseases or hematological disorders; (4) Use of immunosuppressive drugs or any medication known to influence immune cell function within the past 3 months; (5) Use of medications that may affect bone metabolism, including bisphosphonates, steroids, or calcium supplements, within the past 3 months; (6) Presence of visceral metastasis (e.g., liver, kidney, brain, spleen, or pancreas).

This study was approved by the Medical Ethics Committee of Suzhou Ninth People's Hospital.

Data collection

Baseline data: Data were collected by reviewing patients' electronic medical records, including age, body mass index (BMI), sex, personal history (smoking and alcohol consumption), tumor type, tumor stage, maximum tumor diameter, tumor differentiation and lymph node metastasis at the time of admission.

Bone metabolism indicators: Bone metabolism data were retrieved from the patients' bone metabolism test records. The testing procedures were as follows: Prior to confirming bone metastasis, 5 mL of fasting venous blood was collected from each patient into an EDTA anticoagulant tube. A 2 ml aliquot of peripheral blood was then allowed to stand at room temperature for 10 minutes and centrifuged at 3000 r/min for 10 minutes to isolate serum. Serum levels β-carboxy-terminal telopeptide of type I collagen (β-CTX), type I procollagen N-terminal peptide (P1NP), and osteocalcin (OC) were examined by enzyme-linked immunosorbent assay (ELISA) kits (β-CTX: Wenzhou Kemu Biotechnology Co., Ltd., item number: KMEHu012338; P1NP: Jiangxi Jianglan Pure Biological Reagent Co., Ltd., item number: JLC-A8374; OC: Wuhan Tiande Biotechnology Co., Ltd., item number: TD711187). Serum Ca^{2+} concentration was detected using the colorimetric method (Beijing Baolabio Technology Co., Ltd., item number: HR8229-CGV) with a fully automated biochemical analyzer (Cobas 8000 C702, Roche Diagnostics GmbH, Germany).

Immune cell detection: Immune cell index data were obtained by reviewing the patient's immune cell index test records. The testing procedures were as follows: Prior to determining bone metas-tasis status, 5 mL of fasting venous blood was collected from each patient using an EDTA anticoagulant tube.

(1) T-cell subsets analysis: A 100 μL aliquot of peripheral blood was taken from the anticoagulant tube and diluted mix an equal volume of phosphate buffered saline (PBS). The diluted blood was carefully layered onto 5 mL of lymphocyte separation solution in a centrifuge tube. The sample was centrifuged at 2000 r/min for 20 minutes to isolate peripheral blood mononuclear cells (PBMC). The PBMCs were collected, washed twice with PBS, and resuspended. For flow cytometry, 5×10^5 cells per tube were incubated with fluorescently labeled antibodies against surface molecules. After centrifugation at 1650 r/min for 5 minutes, the supernatant was discarded, and each sample was resuspended in 100 μL of staining buffer containing 10 μL each of anti-hCD3 PE-Cy7, anti-hCD4 FITC, and anti-hCD8 Pacific Blue antibodies. Samples were mixed gently and incubated at 4°C in the dark for 20 minutes. Cells were washed twice with cold FACS buffer, resuspended in 500 μL of wash solution, and

fixed. The proportions of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in peripheral blood were analyzed using a BD FACS Canto™ flow cytometer (BD Biosciences, USA).

(2) Regulatory T cell (Treg) analysis: 50 uL of whole blood was added to the bottom of a flow cytometry tube (avoiding contact with the tube wall). Then, 8 μL of anti-CD4, 8 μL of anti-CD25, 2 μl of anti-CD127 antibodies were added and vortexed for 5 seconds. The mixture was incubated at room temperature in the dark for 20 minutes. Subsequently, 450 μL of pre-diluted 1× FACS lysing solution was added, vortexed again for 5 seconds, and incubated for an additional 15 minutes under the same conditions. Afterward, 2 mL of saline was added, and the sample was centrifuged at 300 g for 5 minutes. The supernatant was discarded, and the pellet was vortexed for 5 seconds, resuspended in 300 μL of saline, and mixed thoroughly prior to acquisition. Data acquisition and analysis were performed using BD FACSDiva™ software with the Treg analysis template to determine the proportion of $CD4^+CD25^+CD127^{low}$ Treg cells among $CD4^+$ T cells.

Diagnosis of bone metastasis

Although biopsy remains the gold standard for diagnosing bone metastasis, pathological examination cannot be performed on all suspected lesions due to technical limitations and ethical constraints. Therefore, imaging findings (CT, MRI, or PET/CT) were used as the reference standard. Specifically, in the absence of pathological confirmation, bone metastasis was determined based on characteristic findings on PET/CT (e.g., extensive bone metastases) and corresponding morphological characteristics consistent with bone metastasis on CT. Lesions showing atypical manifestations on CT or MRI were considered benign. The diagnosis of bone metastasis was established when at least one of the following criteria was met [15, 16]: (1) Pathological confirmation of the lesion; (2) Unexplained bony changes not attributable to causes other than tumor bone metastasis, accompanied by abnormal tracer uptake in the same region on PET/CT images; (3) Consistent evidence of bone metastasis detected by two or more imaging modalities (CT, MRI, or PET/CT).

Observation indicators

Primary outcomes: ① To compare the levels of Ca^{2+}, β-CTX, P1NP, OC, as well as the percentages of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells between patients with tumor bone metastasis and those without bone metastasis. ② To compare the differences in Ca^{2+}, β-CTX, P1NP, OC, and the percentages of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells among patients with tumor bone metastasis according to Soloway classification subgroups. ③ To evaluate the diagnostic performance of the combined detection of Ca^{2+}, β-CTX, P1NP, OC, $CD3^+CD4^+$ T cell percentage, $CD3^+CD8^+$ T cell percentage and $CD4^+CD25^+CD127^{low}$ Treg cell percentage for identifying tumor bone metastasis.

Secondary outcome: To analyze the correlation and interaction effects between metabolism markers (Ca^{2+}, β-CTX, P1NP, OC) and immune cell indicators ($CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells) in patients with tumor bone metastasis.

Statistical analysis

Statistical analyses were performed using SPSS 27.0 software. Continuous variables were tested for normality and, when normally distributed, were expressed as Mean ± standard deviation (SD). Differences between two groups were compared using the independent-samples t-test. Categorical data were expressed as number of cases and percentage [n (%)] and compared using the χ^2 test. Analysis of variance (ANOVA) was used to compare differences in various indicators among subgroups with different degrees of bone metastasis. Correlations between continuous variables were analyzed using Pearson's correlation test (for bivariate normal distributions). Interaction effects between indicators were evaluated using multiple linear regression models incorporating interaction terms.

Receiver operating characteristic (ROC) curves were drawn to evaluate the diagnostic efficacy of each index for tumor bone metastasis. The Delong test was used to compare differences in the area under the curve (AUC) among indica-

Table 1. Comparison of baseline data between the two groups

Baseline information	Bone metastasis group (n = 156)	Non-bone metastasis group (n = 91)	t/χ^2 value	P value
Age (years)	56.34±15.95	55.49±14.34	0.419	0.676
Body Mass Index (kg/m^2)	21.69±3.15	22.08±3.46	0.905	0.366
Gender			1.732	0.188
Male	95 (60.90)	63 (69.23)		
Female	61 (39.10)	28 (30.77)		
Smoking history			1.120	0.290
Yes	76 (48.72)	38 (41.76)		
No	80 (51.28)	53 (58.24)		
Alcohol consumption history			0.271	0.602
Yes	67 (42.95)	36 (39.56)		
No	89 (57.05)	55 (60.44)		
Tumor type			1.219	0.748
Lung cancer	71 (45.51)	38 (41.76)		
Breast cancer	40 (25.64)	29 (31.87)		
Carcinoma of the prostate	34 (21.80)	19 (20.88)		
Other malignant tumors	11 (7.05)	5 (5.49)		
Tumor staging			7.521	0.006
Phase III	9 (5.77)	15 (16.48)		
Phase IV	147 (94.23)	76 (83.52)		
Maximum tumor diameter			0.969	0.325
>5 cm	94 (60.26)	49 (53.85)		
≤5 cm	62 (39.74)	42 (46.15)		
Degree of tumor differentiation			0.278	0.870
Tall	19 (12.18)	11 (12.09)		
Centre	65 (41.67)	35 (38.46)		
Low	72 (46.15)	45 (49.45)		
Lymph node metastasis			4.776	0.029
Yes	70 (44.87)	28 (30.77)		
No	86 (56.13)	63 (69.23)		

tors. Based on the optimal cutoff values of each indicator, a logistic regression model was constructed to combine indicators for joint prediction of tumor bone metastasis. A two-tailed P<0.05 indicated that the difference was statistically significant.

Results

Comparison of baseline data between tumor bone metastasis and non-bone metastasis groups

There were no statistically significant differences between the bone metastasis group and non-bone metastasis group in terms of age, BMI, sex, smoking history, alcohol consumption history, tumor type, maximum tumor diameter, or tumor differentiation degree (all P>0.05). However, there were significant differences in tumor stage and lymph node metastasis between the two groups (P<0.05), as shown in **Table 1**.

Comparison of bone metabolism and immune cell indices between the two groups

The levels of Ca^{2+}, β-CTX, P1NP,$CD3^+CD4^+$ T cells, $CD4^+CD25^+CD127^{low}$ Treg cells group were significantly higher in the bone metastasis group than in the non-bone metastasis group, whereas the levels of OC and $CD3^+CD8^+$ T cells were significantly lower (**Table 2**).

Representative flow cytometry analysis scatter plots further illustrate these differences. As shown in **Figure 2**, the percentage of $CD3^+CD4^+$ T cells was significantly increased in patients

Table 2. Comparison of bone metabolism markers and immune cell indices between the two groups

	Bone metastasis group (n = 156)	Non-bone metastasis group (n = 91)	t value	P value
Ca^{2+} (mmol/L)	2.89±0.41	2.16±0.32	14.590	<0.001
β-CTX (pg/mL)	822.45±94.62	428.16±68.35	34.791	<0.001
P1NP (ng/mL)	95.68±20.54	55.93±10.87	17.110	<0.001
OC (ng/mL)	10.53±2.98	23.85±4.19	29.072	<0.001
$CD3^+CD4^+$ T cell (%)	52.68±6.93	31.62±3.21	27.310	<0.001
$CD3^+CD8^+$ T cell (%)	12.37±1.65	25.39±4.06	29.960	<0.001
$CD4^+CD25^+CD127^{low}$ Treg cell (%)	10.38±1.52	4.47±1.29	31.120	<0.001

Notes: β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin.

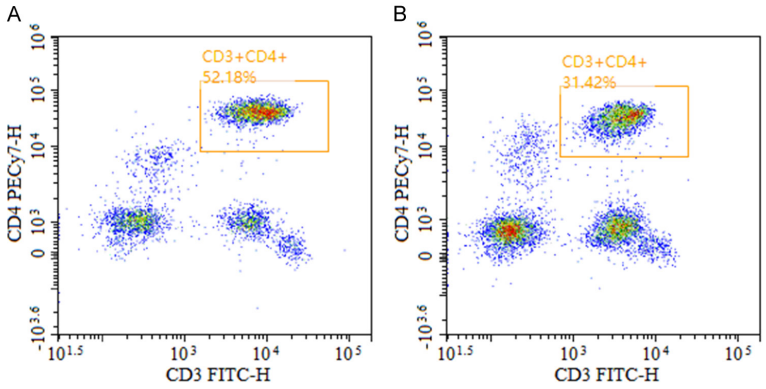

Figure 2. The percentage of $CD3^+CD4^+$ T cell in the two groups. A: The percentage of $CD3^+CD4^+$ T cells was 52.18% in the bone metastasis group; B: The percentage of $CD3^+CD4^+$ T cells was 31.42% in the non-bone metastasis group.

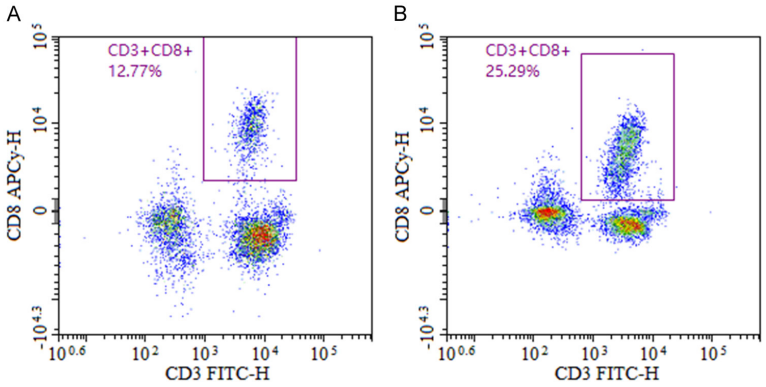

Figure 3. The percentage of $CD3^+CD8^+$ T cells in the two groups. A: The percentage of $CD3^+CD8^+$ T cells was 12.77% in the bone metastasis group; B: The percentage of $CD3^+CD8^+$ T cells was 25.29% in the non-bone metastasis group.

with bone metastasis compared with those without bone metastasis. In contrast, **Figure 3** demonstrates that the percentage of $CD3^+CD8^+$ T cells was significantly lower in the bone metastasis group. Moreover, **Figure 4** shows that the proportion of $CD4^+CD25^+CD127^{low}$ Treg cells was notably higher in patients with tumor bone metastasis compared with those without bone metastasis.

Comparison of bone metabolism and immune cell indexes among patients with different numbers of bone metastases

According to the Soloway classification, the 156 patients with malignant tumor bone metastasis were divided into grade I (n = 50), grade II (n = 54) and grade III (n = 52). The peripheral blood Ca^{2+}, β-CTX, P1NP, $CD3^+CD4^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells increased progressively with the severity of bone metastasis (grade I < grade II < grade III) (P<0.05). Conversely, the expression levels of OC and $CD3^+CD8^+$ T cells decreased progressively with the increasing of disease severity (grade I > grade II > grade III) (P<0.05), as shown in **Table 3**.

Correlation analysis between bone metabolism indicators and immune cell indicators in patients with tumor bone metastasis

All variables were tested for normality using the Shapiro-Wilk test and were normally distributed, meeting the assumptions for Pearson correlation analysis. As shown in **Figure 5**, serum Ca^{2+} levels in patients with malignant tumor bone metastasis were positively

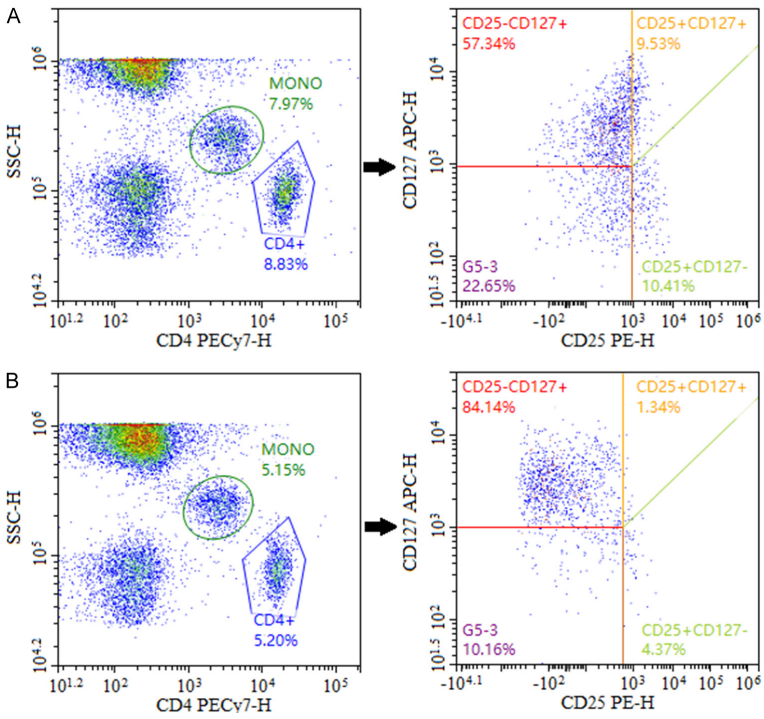

Figure 4. The percentage of $CD4^+CD25^+CD127^{low}$ Treg cells in the two groups. A: The percentage of $CD4^+CD25^+CD127^{low}$ Treg cells in the bone metastasis group was 10.41%; B: The percentage of $CD4^+CD25^+CD127^{low}$ Treg cells in the non-bone metastasis group was 4.37%.

correlated with the percentages of $CD3^+$ $CD4^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells (r = 0.409, 0.393, P<0.001), and negatively correlated with $CD3^+CD8^+$ T cells (r = -0.314, P<0.001). As shown in **Figure 6**, the serum β-CTX levels were positively correlated with the percentages of $CD3^+CD4^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells (r = 0.368, 0.410, P<0.001), but negatively correlated with $CD3^+CD8^+$ T cells (r = -0.341, P<0.001). Similarly, the serum level of P1NP in patients with malignant tumor bone metastasis was positively correlated with the percentages of $CD3^+CD4^+$ T cells (r = 0.299, P<0.001) and $CD4^+CD25^+CD127^{low}$ Treg cells (r = 0.451, P<0.001), but negatively correlated with $CD3^+CD8^+$ T cells (r = -0.209, P = 0.009), see **Figure 7**. The serum level of OC in patients with malignant tumor bone metastasis was negatively correlated with the percentages of $CD3^+CD4^+$ T cells (r = -0.298, P<0.001) and $CD4^+CD25^+CD127^{low}$ Treg cells (r = -0.309, P<0.001), but positively correlated with the percentage of $CD3^+CD8^+$ T cells (r = 0.201, P = 0.012), see **Figure 8**.

Analysis of interaction effects between bone metabolism indices and immune cell indices in tumor bone metastasis

After adjusting for age, smoking history, alcohol consumption history, tumor type, tumor stage, maximum tumor diameter, tumor differentiation, and lymph node metastasis, multiple linear regression analysis revealed significant interaction effects between bone metabolism and immune cell indicators on tumor bone metastasis. Specifically, the interaction terms between serum Ca^{2+} and $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells were all statistically significant (β = 0.212, -1.543, 0.195; P< 0.05); Similarly, interaction terms between β-CTX and these three immune cell subsets were significant (β = 0.231, -0.168, 0.226, P<0.05); For P1NP, its interaction with $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells were all significant (β = 0.167, -0.143, 0.278, P<0.05); In contrast, OC demonstrated inverse interaction patterns with $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells (β = -0.185, 0.137, -0.187, P<0.05), see **Table 4**.

Efficacy analysis of bone metabolism and immune cell indicators in screening for tumor bone metastasis

ROC curve analysis showed that bone metabolism indicators, including Ca^{2+}, β-CTX, P1NP, and OC, have potential clinical value in screening for bone metastasis of tumors. Among them, the *AUC* values for Ca^{2+}, β-CTX, P1NP, and OC were 0.835, 0.843, 0.817, and 0.750, respectively (**Figure 9**). Based on the Youden index, the optimal cut-off values, sensitivities, and specificities for Ca^{2+}, β-CTX, P1NP, and OC were calculated (**Table 5**). The Delong test was applied to compare the differences in AUCs among these bone metabolism indicators. AUC

Table 3. Comparison of bone metabolism markers and immune cell indices among patients with various metastasis lesions

	Grade I (*n* = 50)	Grade II (*n* = 54)	Grade III (*n* = 52)	*F* value	*P* value
Ca^{2+} (mmol/L)	2.47±0.21	2.87±0.23*	3.31±0.29*,#	148.901	<0.001
β-CTX (pg/mL)	760.34±80.22	815.45±89.26*	889.44±99.72*,#	26.360	<0.001
P1NP (ng/mL)	87.52±8.43	96.84±9.29*	102.36±9.94*,#	33.430	<0.001
OC (ng/mL)	13.28±2.05	10.56±1.95*	7.85±1.62*,#	106.204	<0.001
$CD3^+CD4^+$ T cells (%)	40.50±4.17	51.73±5.48*	63.38±7.35*,#	196.502	<0.001
$CD3^+CD8^+$ T cells (%)	15.83±4.25	12.54±2.19*	8.87±1.86*,#	71.930	<0.001
$CD4^+CD25^+CD127^{low}$ Treg cells (%)	9.25±1.32	10.27±1.48*	11.77±1.69*,#	36.221	<0.001

Notes: β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin. Compared with Level I, *P<0.05; Compared with level II, #P<0.05.

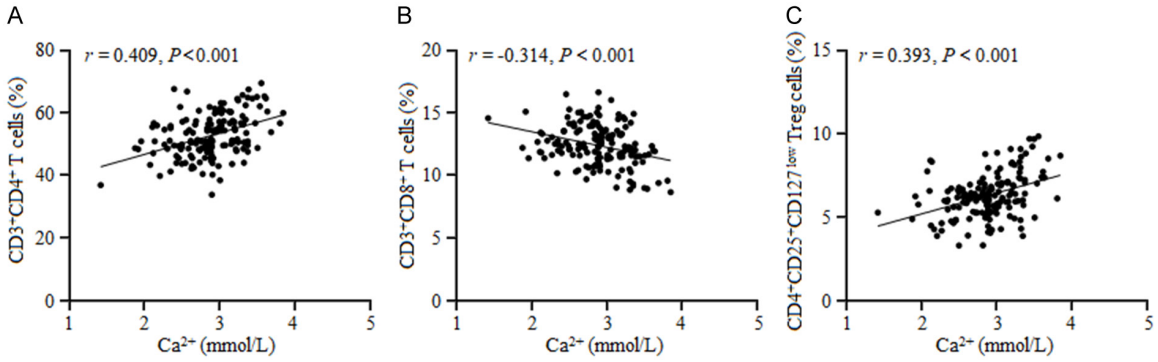

Figure 5. Correlation analysis of serum Ca^{2+} levels with immune cell indices in patients with malignant tumor bone metastasis. A: Correlation between Ca^{2+} and the percentage of $CD3^+CD4^+$ T cells; B: Correlation between Ca^{2+} and the percentage of $CD3^+CD8^+$ T cells; C: Correlation between Ca^{2+} and the percentage of $CD4^+CD25^+CD127^{low}$ Treg cells.

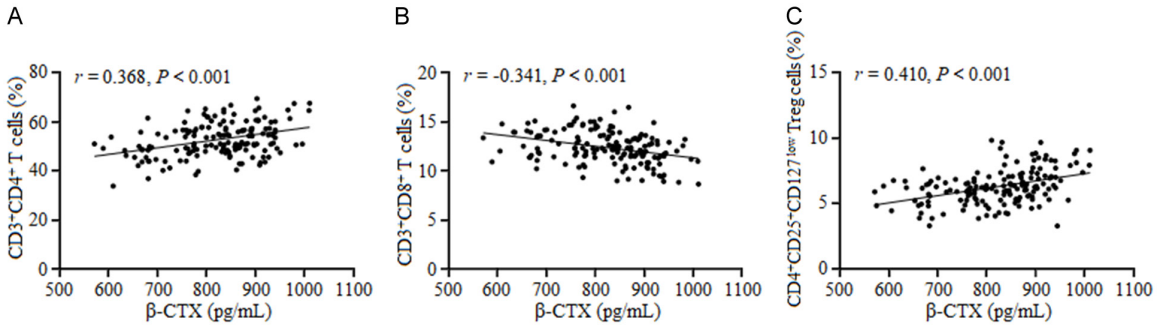

Figure 6. Correlation analysis of serum β-CTX levels with immune cell indices in patients with malignant tumor bone metastasis. A: Correlation between β-CTX and the percentage of $CD3^+CD4^+$ T cells; B: Correlation between β-CTX and the percentage of $CD3^+CD8^+$ T cells; C: Correlation between β-CTX and the percentage of $CD4^+CD25^+CD127^{low}$ Treg cells. Notes: β-CTX, β-carboxy-terminal telopeptide of type I collagen.

of Ca^{2+} (0.835) was significantly higher than that of OC (0.750; Z = 2.318, P = 0.020), and the *AUC* of β-CTX (0.843) was also significantly higher than that of OC (0.750; Z = 2.467, P = 0.014) (**Table 6**).

ROC curve analysis showed that, percentages of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells, exhibited potential discriminative power for screening bone metastasis in malignant tumors. The *AUC* values for $CD3^+CD4^+$ T cell, $CD3^+CD8^+$ T cell, and $CD4^+CD25^+CD127^{low}$ Treg cell were 0.850, 0.826, and 0.899, respectively (**Figure 10**). The optimal cutoff values and the corresponding sensitivity and specificity for each immune

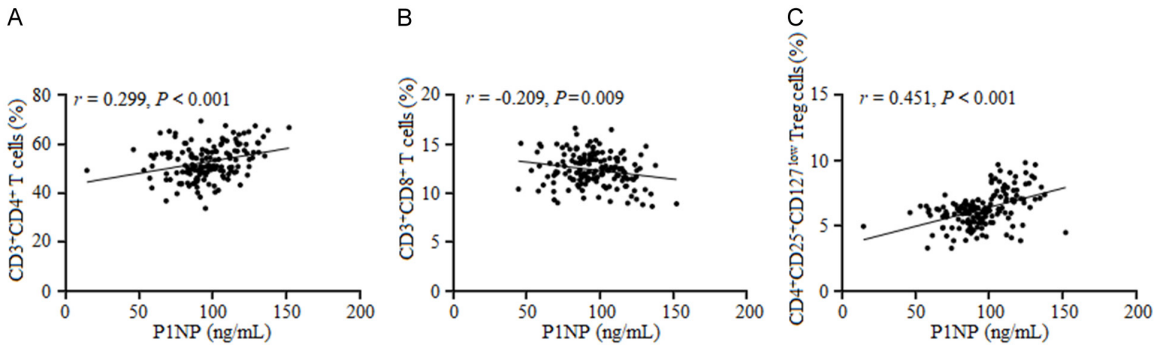

Figure 7. Correlation analysis of serum P1NP levels with immune cell indices in patients with malignant tumor bone metastasis. A: Correlation between P1NP and the percentage of $CD3^+CD4^+$ T cells; B: Correlation between P1NP and the percentage of $CD3^+CD8^+$ T cells; C: Correlation between P1NP and the percentage of $CD4^+CD25^+CD127^{low}$ Treg cells. Notes: P1NP, Type I procollagen N-terminal peptide.

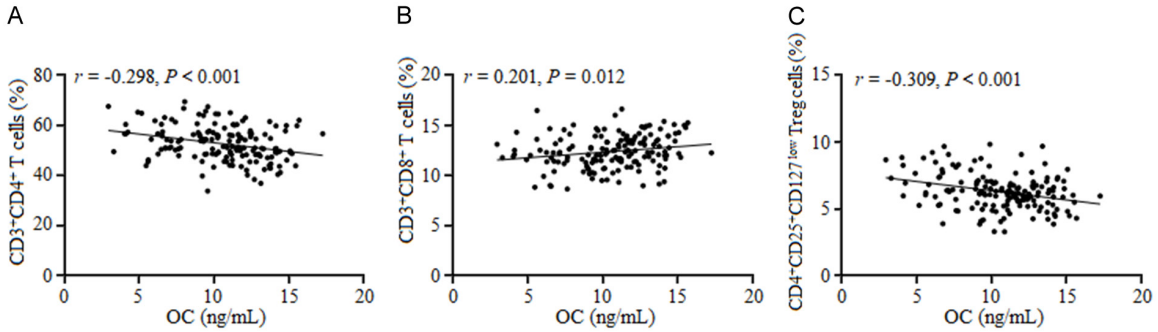

Figure 8. Correlation analysis of serum OC levels with immune cell indices in patients with malignant tumor bone metastasis. A: Correlation between OC and the percentage of $CD3^+CD4^+$ T cells; B: Correlation between OC and the percentage of $CD3^+CD8^+$ T cells; C: Correlation between OC and the percentage of $CD4^+CD25^+CD127^{low}$ Treg cells. Notes: OC, Osteocalcin.

Table 4. Analysis of the interaction effect of bone metabolism markers and immune cell indices on tumor bone metastasis

Item	Model 1			Model 2		
	β	95% *CI*	*P* value	*β*	95% *CI*	*P* value
Ca^{2+} - immune cell index						
Ca^{2+}	0.243	0.128-0.431	0.019	0.254	0.169-0.487	0.005
Ca^{2+} × $CD3^+CD4^+$ T cells	0.174	0.136-0.465	0.013	0.212	0.113-0.431	0.007
Ca^{2+} × $CD3^+CD8^+$ T cells	-0.198	-0.264–-0.059	0.024	-0.154	-0.225–-0.036	0.009
Ca^{2+} × $CD4^+CD25^+CD127^{low}$ Treg cells	0.163	0.072-0.394	0.009	0.195	0.054-0.354	0.004
β-CTX - immune cell index						
β-CTX	0.258	0.158-0.537	0.014	0.279	0.184-0.559	0.008
β-CTX × $CD3^+CD4^+$ T cells	0.205	0.114-0.403	0.004	0.231	0.120-0.419	0.002
β-CTX × $CD3^+CD8^+$ T cells	-0.187	-0.255–-0.062	0.017	-0.168	-0.246–-0.087	0.009
β-CTX × $CD4^+CD25^+CD127^{low}$ Treg cells	0.155	0.096-0.395	0.009	0.226	0.109-0.427	0.006
P1NP - immune cell index						
P1NP	0.328	0.148-0.678	0.016	0.394	0.182-0.729	0.014
P1NP × $CD3^+CD4^+$ T cells	0.258	0.058-0.487	0.024	0.167	0.025-0.346	0.009
P1NP × $CD3^+CD8^+$ T cells	-0.104	-0.237–-0.019	0.068	-0.143	-0.374–-0.021	0.014
P1NP × $CD4^+CD25^+CD127^{low}$ Treg cells	0.241	0.105-0.786	0.003	0.278	0.167-0.562	<0.001

OC - immune cell index						
OC	-0.408	-0.745--0.186	0.045	-0.398	-0.519--0.086	0.037
OC × $CD3^+CD4^+$ T cells	-0.295	-0.483--0.112	0.017	-0.185	-0.340--0.058	0.008
OC × $CD3^+CD8^+$ T cells	0.104	0.029-0.334	0.064	0.137	0.045-0.245	0.019
OC × $CD4^+CD25^+CD127^{low}$ Treg cells	-0.259	-0.394--0.128	0.023	-0.187	-0.269--0.093	0.008

Note: β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin. Model 1: Unadjusted skew variable model; model 2: Adjust the model after age, sex, smoking history, drinking history, tumor type, tumor stage, tumor maximum diameter, tumor differentiation degree and lymph node metastasis.

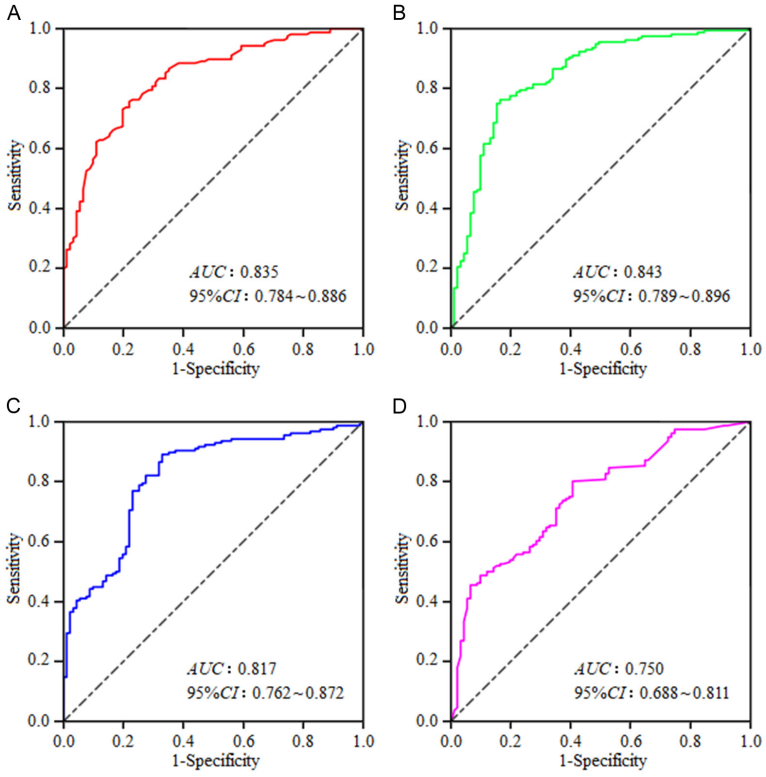

Figure 9. ROC curve analysis for bone metabolism markers in screening bone metastasis. A: Ca^{2+}; B: β-CTX; C: P1NP; D: OC. Notes: ROC, Receiver Operating Characteristic; AUC, Area Under the Curve; β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin.

index were determined using the maximum Youden index (**Table 7**). The Delong test further showed that the *AUC* value for $CD4^+CD25^+CD127^{low}$ Treg cells (0.848) was significantly higher than that of $CD3^+CD8^+$ T cells (0.771) (Z = -2.147, P = 0.032), as shown in **Table 8**.

Multivariate logistic regression analysis of tumor bone metastasis

Variables with P<0.05 in the univariate analysis (**Table 1**) were included as independent variables. Based on *ROC* curve analysis, the optimal cutoff values of bone metabolism and immune cell indices for predicting tumor bone metastasis were determined (**Tables 5** and **7**). The dependent variable was the occurrence of bone metastasis in patients with malignant tumors (0 = no, 1 = yes), and the variable assignments are shown in **Table 9**.

Multivariate Logistic regression analysis (**Table 10**) showed that elevated levels of Ca^{2+} (>2.75 mmol/L), β-CTX (>780.60 pg/mL), and P1NP (>88.95 ng/mL), as well as reduced OC (<13.50 ng/mL), were significantly associated with an increased risk of bone metastasis in tumor patients (P<0.05). In addition, immune parameters-including increased $CD3^+CD4^+$ T cells (>50.57%) and $CD4^+CD25^+CD127^{low}$ Treg cells (>10.35%), as well as $CD3^+CD8^+$ T cells (<13.78%) were also identified as independent risk factors for tumor bone metastasis (P<0.05).

The efficacy and verification of the combination model of bone metabolism and immune cell indicators for screening for malignant tumor bone metastasis

According to the multivariate logistic regression analysis, the predictive model for tumor bone metastasis incorporating bone metabolism and immune cell indicators was established as follows: Logit (P) = 0.436 × Ca^{2+} + 0.548 × β-CTX

Table 5. *ROC* parameters of each bone metabolism marker in screening tumor bone metastasis

Index	*AUC*	Critical value	Sensitivity (%)	Specificity (%)	*P* value	95% *CI*
Ca^{2+}	0.835	2.75 mmol/L	0.847	0.769	<0.001	0.784-0.866
β-CTX	0.843	780.60 pg/mL	0.829	0.783	<0.001	0.789-0.896
P1NP	0.817	88.95 ng/mL	0.807	0.729	<0.001	0.762-0.872
OC	0.750	13.50 ng/mL	0.748	0.842	<0.001	0.688-0.811

Notes: β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin.

Table 6. Comparison of *AUC* values of each bone metabolism markers in screening malignant tumor bone metastasis

Item 1	Item 2	*AUC* difference value	Standard Error	95% *CI*	*Z* value	*P* value
Ca^{2+}	β-CTX	-0.008	0.229	-0.064-0.049	-0.261	0.794
Ca^{2+}	P1NP	0.018	0.232	-0.044-0.081	0.572	0.567
Ca^{2+}	OC	0.086	0.239	0.013-0.158	2.318	0.020
β-CTX	P1NP	0.026	0.234	-0.037-0.088	0.809	0.419
β-CTX	OC	0.093	0.242	0.019-0.167	2.467	0.014
P1NP	OC	0.067	0.244	-0.010-0.145	1.705	0.088

Notes: β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin.

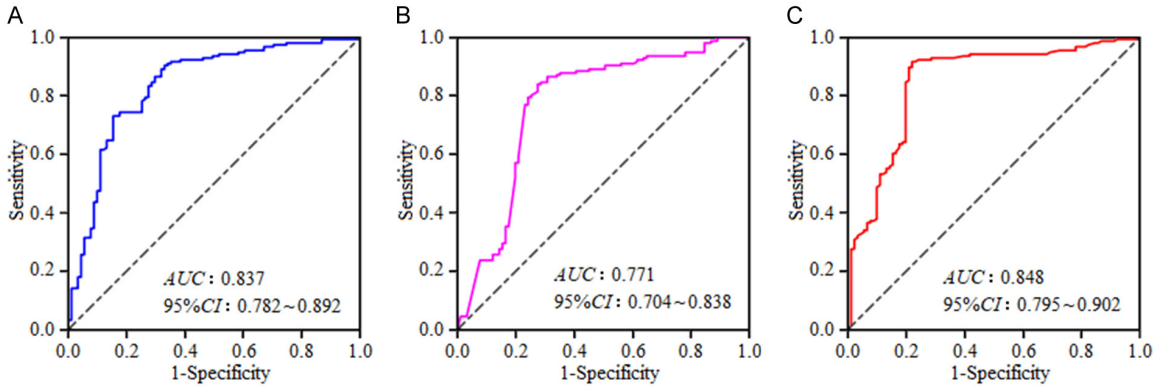

Figure 10. ROC curve analysis for immune cell indices in screening bone metastasis. A: $CD3^+CD4^+$ T cells; B: $CD3^+CD8^+$ T cells; C: $CD4^+CD25^+CD127^{low}$ Treg cells. Notes: ROC, Receiver Operating Characteristic; AUC, Area Under the Curve.

Table 7. *ROC* parameters of immune cell indices in screening tumor bone metastasis

Index	*AUC*	Critical value	Sensitivity (%)	Specificity (%)	*P* value	95% *CI*
$CD3^+CD4^+$ T cell	0.837	50.57%	0.835	0.782	<0.001	0.782-0.892
$CD3^+CD8^+$ T cell	0.771	13.78%	0.778	0.851	<0.001	0.704-0.838
$CD4^+CD25^+CD127^{low}$ Treg cell	0.848	10.35%	0.845	0.839	<0.001	0.795-0.902

Table 8. Comparison of AUC values of each immune cell index in screening malignant tumor bone metastasis

Item 1	Item 2	*AUC* difference value	Standard Error	95% *CI*	*Z* value	*P* value
$CD3^+CD4^+$ T cell	$CD3^+CD8^+$ T cell	0.066	0.249	-0.012-0.145	1.670	0.095
$CD3^+CD4^+$ T cell	$CD4^+CD25^+CD127^{low}$ Treg cell	-0.011	0.234	-0.073-0.051	-0.349	0.727
$CD3^+CD8^+$ T cell	$CD4^+CD25^+CD127^{low}$ Treg cell	-0.078	0.247	-0.148--0.007	-2.147	0.032

Table 9. Assignment table

Variable	Assignment explanation
Tumor staging	Phase III = 0; Phase IV = 1
Lymph node metastasis	Not have = 0; Have = 1
Ca^{2+} (mmol/L)	≤2.75 = 0; >2.75 = 1
β-CTX (pg/mL)	≤780.60 = 0; >780.60 = 1
P1NP (ng/mL)	≤88.95 = 0; >88.95 = 1
OC (ng/mL)	≥13.50 = 0; <13.50 = 1
$CD3^+CD4^+$ T cell (%)	≤50.57 = 0; >50.57 = 1
$CD3^+CD8^+$ T cell (%)	≥13.78 = 0; <13.78 = 1
$CD4^+CD25^+CD127^{low}$ Treg cell (%)	≤10.35 = 0; >10.35 = 1

Notes: β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin.

Table 10. Multivariate logistic regression analysis for tumor bone metastasis

Variable	*β*	SE	Wald χ^2	*P*	*OR* (95% CI)
Tumor staging	0.198	0.110	3.240	0.074	1.219 (0.982-1.513)
Lymph node metastasis	0.203	0.108	3.533	0.061	1.225 (0.991-1.514)
Ca^{2+} >2.75 mmol/L	0.436	0.174	6.279	0.012	1.547 (1.101-2.175)
β-CTX >780.60 pg/mL	0.548	0.190	8.319	0.004	1.729 (1.192-2.509)
P1NP >88.95 ng/mL	0.397	0.168	5.584	0.018	1.487 (1.070-2.067)
OC <13.50 ng/mL	0.304	0.150	4.107	0.043	1.355 (1.010-1.818)
$CD3^+CD4^+$ T cell >50.57%	0.479	0.183	6.851	0.009	1.614 (1.127-2.312)
$CD3^+CD8^+$ T cell <13.78%	0.325	0.147	4.888	0.027	1.384 (1.038-1.846)
$CD4^+CD25^+CD127^{low}$ Treg cell >10.35%	0.629	0.204	9.507	0.002	1.876 (1.259-2.795)
Constant term	-5.905	1.668	12.533	<0.001	-

Notes: β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin.

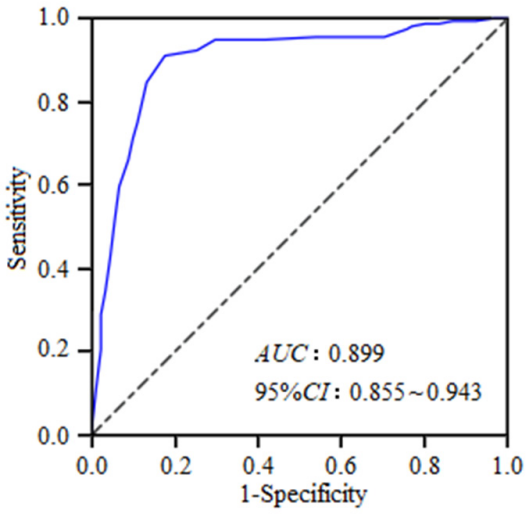

Figure 11. *ROC* curve analysis for combined detection in screening tumor bone metastasis using a combination of bone metabolism indicators (Ca^{2+}, β-CTX, P1NP, OC) and immune cell indicators ($CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+CD25^+CD127^{low}$ Treg cells). Notes: ROC, Receiver Operating Characteristic; AUC, Area Under the Curve.

+ 0.397 × P1NP + 0.304 × OC + 0.479 × $CD3^+CD4^+$ T cells + 0.325 × $CD3^+CD8^+$ T cells + 0.629 × $CD4^+CD25^+CD127^{low}$ Treg cells - 5.905. *ROC* curve analysis showed that, the combined use of Ca^{2+}, β-CTX, P1NP, OC, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells yielded an *AUC* value of 0.899 for screening tumor bone metastasis (**Figure 11**).

Delong test showed that the AUC value of the combined detection for screening malignant tumor bone metastasis was significantly higher than their single use (**Table 11**). Based on the Youden index, the optimal critical value (Prob) for the combined model was calculated to be 0.845, with a corresponding sensitivity of 0.895 and a specificity of 0.875. When Prob ≥0.845, tumor bone metastasis is predicted to be present, and when Prob <0.845, it indicates the absence of tumor bone metastasis.

Internal validation using a confusion matrix demonstrated a high level of agreement bet-

Table 11. Comparison of AUCs between combined detection and each index alone

Item 1	Item 2	*AUC* difference value	Standard Error	95% *CI*	*Z* value	*P* value
Ca^{2+}	Index combination	-0.063	0.219	-0.110–0.016	-2.644	0.008
β-CTX	Index combination	-0.056	0.221	-0.104–0.007	-2.258	0.024
P1NP	Index combination	-0.082	0.224	-0.137–0.026	-2.891	0.004
OC	Index combination	-0.149	0.232	-0.217–0.081	-4.280	<0.001
$CD3^+CD4^+$ T cell	Index combination	-0.061	0.223	-0.115–0.007	-2.221	0.026
$CD3^+CD8^+$ T cell	Index combination	-0.128	0.237	-0.191–0.064	-3.946	<0.001
$CD4^+CD25^+CD127^{low}$ Treg cell	Index combination	-0.050	0.222	-0.100–0.001	-1.982	0.047

Notes: β-CTX, β-carboxy-terminal telopeptide of type I collagen; P1NP, Type I procollagen N-terminal peptide; OC, Osteocalcin.

Table 12. Accuracy of combined detection for screening tumor bone metastasis

Screening results	Actual results		In total	Sensitivity	Specificity	Accuracy
	Tumor bone metastasis	No tumor bone metastasis				
Tumor bone metastasis	138	13	151			
No tumor bone metastasis	18	78	96			
In total	156	91	247	88.46%	85.71%	87.45%

ween predicted and actual outcomes. The concordance rate for identifying bone metastasis was 88.46% (138/156), and the concordance rate for excluding bone metastasis was 85.71% (78/91). The overall predictive accuracy of the combined model was 87.45% [(138+78)/247)] (**Table 12**).

Discussions

The incidence of malignant tumors has been increasing annually. When tumor cells invade the bones via the bloodstream or lymphatic system, bone metastasis occurs, which reduces overall survival and impairs the quality of life of patients [17]. Therefore, early and effective diagnosis and treatment of bone metastasis are crucial for improving the prognosis and quality of life in these patients.

The results of this study showed that the proportions of stage IV tumors and lymph node metastases in the bone metastasis group were significantly higher than those in the non-bone metastasis group, suggesting an association between tumor stage, lymph node metastasis, and tumor bone metastasis. Numerous studies have also confirmed the significant correlation between tumor stage and the occurrence of bone metastasis, such as in prostate cancer [18], breast cancer [19], and others. It is possible that as the tumor stage progresses, the scope of tumor invasion expands. Tumor cells actively divide, reproduce, and grow, which increases their involvement with more distant lymph nodes, making bone metastasis more likely to occur. When lymph nodes are involved, tumor cells can escape immune-mediated cytotoxic effects and induce the production of regulatory T cells (Treg), leading to immune tolerance and promoting tumor metastasis [20]. Once tumor cells enter the bone marrow via the bloodstream, they interact with osteoblasts, osteoclasts, and bone stromal cells, thereby destroying bone tissue and releasing various factors that promote continued tumor proliferation and metastases [21]. Metabolites from bone marrow cells exhibit a chemotactic effect on tumor cells, further facilitating the spread of metastases. In cases of tumor bone metastasis, the bone remodeling process is significantly accelerated, the bone remodeling process is significantly accelerated, leading to an increased bone metabolic rate, which results in abnormalities in bone metabolism [22]. Our study found that the expression levels of Ca^{2+}, β-CTX, and P1NP in the peripheral blood of patients with tumor bone metastasis were significantly higher than those in patients without bone metastasis. Furthermore, these markers exhibited an increasing trend with higher Soloway grade of tumor bone metastasis (grade I < grade II < grade III). In contrast, the expres-

sion level of OC in the peripheral blood of patients with tumor bone metastasis was significantly lower than in those without bone metastasis, and it decreased further with increasing Soloway grade (grade I > grade II > grade III). These findings suggest that Ca^{2+}, β-CTX, P1NP and OC in peripheral blood may serve as indicators for early screening and severity assessment of bone metastasis in malignant tumors. As a bone metabolism indicator, Ca^{2+} reflects conditions such as skeletal metabolic disorders, abnormal parathyroid function, and vitamin D deficiency or excess. The primary cause of elevated Ca^{2+} levels in tumor bone metastasis is the disruption of skeletal integrity, which leads to the release of large amount of calcium from bone into the bloodstream, thereby increasing serum calcium ion concentration [23]. Additionally, during bone metastasis, tumor-stimulated osteolytic activity results in abnormal expression of parathyroid hormone-related protein (PTHrP), further mediating an increase in serum Ca^{2+} levels [24]. Consequently, patients with bone metastasis are prone to developing hypercalcemia. β-CTX, a cross-linked carboxy-terminal telopeptide of type I collagen, is released during bone resorption and serves as a specific biomarker of bone metabolism, reflecting the extent of bone destruction [25]. The destruction of bone cells during tumor bone metastasis can lead to osteoclast-mediated bone resorption, resulting in degradation of type I collagen (such as β-CTX) and release of β-CTX into the bloodstream. This results in increased expression levels of β-CTX in peripheral blood. Zuo *et al.* [26] found that the concentration of β-CTX in peripheral blood of patients with tumor bone metastasis is positively correlated with the concentration of P1NP. The increase in β-CTX also leads to an increase in P1NP. P1NP is a specific marker of type I collagen deposition and directly reflects osteoblast activity and bone formation rate. Lumachi *et al.* [27] confirmed that serum P1NP level in patients with tumor bone metastasis was significantly higher than in those without bone metastasis, which is consistent with the results of this study. OC, synthesized by osteoblasts, odontoblasts, and proliferating chondrocytes, regulates bone metabolism and serves as a specific and sensitive biochemical marker of bone turnover. In cases of tumor bone metastasis, the bone matrix enhances its reuptake of OC, leading to a relative decrease in OC released into the bloodstream [28]. Therefore, the expression level of OC in the peripheral blood of patients with tumor bone metastasis is reduced.

During the early stages of tumor development, both the innate and adaptive immune systems become activated, contributing to the recognition and elimination of tumor cells, thereby inhibiting tumor initiation and progression [29]. The bone marrow microenvironment contains a diverse array of immune cells, including myeloid-derived suppressor cells (MDSCs), Treg, helper T cell, and others, all of which play critical roles throughout the process of tumor bone metastasis [30]. $CD3^+CD4^+$ T cells are a subset of T lymphocytes that express both CD3 and CD4 antigens. Miao *et al.* [31] found that $CD3^+CD4^+$ T cell levels correlate with the efficacy of immune checkpoint inhibitor treatment in lung cancer patients. CD3 is a component of the T-cell receptor complex, expressed on the surface of T cells and internalized upon stimulation by certain lymphokines. CD4 is a glycoprotein expressed primarily on helper T cells, where it functions to recognize major histocompatibility complex (MHC) class II molecules on antigen-presenting cells, playing a central role in initiating immune response. Therefore, $CD3^+CD4^+$ T cells are important for immune regulation. $CD3^+CD8^+$ T cells are T cell subsets that express CD3 and CD8 antigens. $CD8^+$ T cells, also known as cytotoxic T cells lymphocytes (CTLs), are essential for adaptive immune responses due to their ability to specifically recognize and eliminate tumor and virus-infected cells [32]. Kraemer *et al.* [33] found that $CD3^+CD8^+$ T cells exhibit a more sensitive response in tumor immune monitoring, suggesting that tumor progression is subject to immune surveillance. Treg cells are a subset of T lymphocytes with immunosuppressive properties and play a significant role in regulating peripheral immune responses [34]. $CD4^+CD25^+CD127^{low}$ Treg cells represent a specific subset of Treg cells. A study of liver cancer [35] revealed that the expression level of $CD4^+CD25^+CD127^{low}$ Treg cells in peripheral blood can serve as an important predictor of biopsy outcomes. These cells inhibit effector T cells by regulating cytokines such as interleukin 10 (IL-10) and transforming growth factor-beta (TGF-β). Sun *et al.* [36] found that a higher percentage of $CD4^+CD25^+CD127^{low}$ Treg cells in

peripheral blood is associated a poorer prognosis in patients with liver cancer. Shen *et al* [37] showed that, in the tumor microenvironment, high expression of $CD4^+CD25^+CD127^{low}$ Treg cells in peripheral blood serves as a marker of Treg cell activity. However, the roles of $CD3^+CD4^+$ T cells, $CD3^+CD4^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells in tumor bone metastasis remain unclear. This study found that the percentages of both $CD3^+CD4^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells in the tumor bone metastasis group were significantly higher than those in non-bone metastasis group, with an increasing trend across the Soloway grades of bone metastasis (grade I < grade II < grade III). In contrast, the percentage of $CD3^+CD8^+$ T cells in the tumor bone metastasis group was significantly lower than that in the non-bone metastasis group and showed a decreasing trend with the increasing Soloway grade of tumor bone metastasis (grade I > grade II > grade III). These findings suggest that the expression levels of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD4^+CD25^+CD127^{low}$ Treg cells in peripheral blood could serve as indicators for early screening and evaluation of bone metastasis in malignant tumors. The possible reasons are as follows: (1) In the early stages of tumor bone metastasis, the immune response initially promotes the activity of $CD3^+CD8^+$ T cells. However, as tumor progression occurs, $CD3^+CD8^+$ T cells become suppressed. The ratio of $CD3^+CD4^+/CD3^+CD8^+$ T cells maintains cellular immune balance, and disruption of this balance promotes tumor bone metastasis [38]. (2) The inhibitory effect of $CD4^+CD25^+CD127^{low}$ Treg cells on the proliferation of effector T cells and tumor infiltration is correlated [39]. In tumor bone metastasis, the enrichment of Treg cells is often associated with bone metastasis. For example, through the release of cytokines and activation of signaling pathways, Treg cells inhibit the anti-tumor immune response and promote the growth of tumor cells in bone tissue [40]. For instance, Treg cells have been shown to promote bone resorption at the bone metastasis site by affecting the activity of osteoclasts or regulating the RANKL-RANK signaling pathway [41]. These findings suggests that the increased proportion of Treg cells in tumor bone metastasis is not only associated with immune suppression but may also contribute to bone destruction by regulating the bone metabolic microenvironment.

A complex interaction exists between the immune system and the skeletal system. Immune cells and bone cells coexist in the bone marrow, where immune cells contribute to the regulation of bone homeostasis through the secretion of inflammatory factors and related ligands [42]. Conversely, bone metabolism can influence the proliferation and differentiation of immune cells [43]. The results of this study indicate a significant correlation between peripheral blood bone metabolism markers (Ca^{2+}, β-CTX, P1NP, OC) and immunity parameters ($CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+CD25^+CD127^{low}$ Treg cells) in patients with tumor bone metastasis. Among them, the expression levels of Ca^{2+}, β-CTX and P1NP in peripheral blood of patients with tumor bone metastasis were positively correlated with the percentage of $CD3^+CD4^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells, and negatively correlated with $CD3^+CD8^+$ T cells percentage. In contrast, the expression level of OC was negatively correlated with the percentage of $CD3^+CD4^+$ T cells and $CD4^+CD25^+CD127^{low}$ Treg cells but positively with $CD3^+CD8^+$ T cells percentage. Potential mechanisms underlying these correlations include: (1) Active osteoclast-mediated bone resorption in tumor bone metastases not only releases Ca^{2+} and increases β-CTX levels but also releases TGF-β stored within the bone matrix [44]. Active TGF-β directly inhibits T cells receptor signaling and immune synapse formation, impairing the function of both $CD4^+$ and $CD8^+$ T cells. This subsequently affects immune cell migration and weakening anti-tumor immunity. (2) TGF-β is a key inducer of naïve T cells differentiation into Treg cells and enhances the immunosuppressive function of existing Treg cells, leading to an increased proportion of $CD4^+CD25^+CD127^{low}$ Treg cells. Zhao *et al.* [45] found that an increase in Treg cells promotes the formation of an immunosuppressive microenvironment, thereby facilitating tumor bone metastasis. Therefore, as bone destruction progresses (higher β-CTX), more TGF-β is released, potentially increasing the proportion of Treg cells. (3) The increase in P1NP and decrease in OC levels indicate osteoblast function dysregulated or an imbalance in bone formation and resorption [46, 47]. This imbalance reflects the disruption of bone microenvironment homeostasis due to tumor infiltration, which correlates with immune suppression within the metastatic niche [48]. The interaction between bone metabolism and

immune cells during tumor bone metastasis suggests a bidirectional relationship. We further analyzed the interaction effects between bone metabolism markers and immune cell indices, and the results revealed significant effects of these interactions on tumor bone metastasis. The underlying mechanism involves the release of receptor activator of nuclear factor-κB ligand (RANKL) by tumor cells upon bone infiltration. RANKL binds to RANK receptors in osteoclast precursors, leading to osteoclasts differentiation and maturation, which induces bone destruction (i.e., increased β-CTX and Ca^{2+}) and the release of TGF-β during bone resorption. This process promotes the expansion and activation of Treg cells, inhibiting T cell function (i.e., increased $CD3^+CD4^+$ T cells and decreased $CD3^+$ $CD8^+$ T cells), and facilitating tumor immune escape. On the contrary, the increase in Treg cells inhibits the effector T cells, destroys the homeostasis of bone microenvironment, leads to the imbalance of bone formation and absorption (i.e., the increase of P1 NP and the decrease of OC), and promotes osteoclast activation and bone resorption (i.e., the increase of β-CTX and Ca^{2+}). Therefore, elevated levels of β-CTX and Ca^{2+} may reflect not only the severity of bone metastasis but also a state of local and systemic immunosuppressive state (increased Treg, inhibited $CD8^+$ T cell function). The increased Treg cells contribute to the process of bone destruction, exacerbating the pathological cycle. Similarly, the rise in P1NP, accompanied by reduced or dysregulated OC levels, suggests impaired bone formation and repair mechanisms. This is closely associated with a dysregulated pathological microenvironment, including enhanced immune suppression.

The high affinity of tumor cells for bone is attributed to the rich blood supply in bone marrow and the frequent high expression of adhesion molecules on tumor cells, which facilitates their adherence to bone marrow stromal cells [49]. This interaction significantly increases the likelihood of bone metastasis. Numerous studies have demonstrated the utility of bone metabolism markers in monitoring and screening for tumor bone metastasis [50, 51]. However, these studies often overlook the regulation of the bone microenvironment by the immune system. This study applied *ROC* curve analysis and found that, the *AUC* for evaluating tumor bone metastasis by combining bone metabolism markers (Ca^{2+}, β-CTX, P1NP, OC) and immune cell indicators ($CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+CD25^+CD127^{low}$ Treg cells) was 0.899, which was higher than that of individual bone metabolism indicators [Ca^{2+} (0.835), β-CTX (0.843), P1NP (0.817), OC (0.750)] and individual immune cell indicators [$CD3^+CD4^+$ T cells (0.837), $CD3^+CD8^+$ T cells (0.771), $CD4^+CD25^+CD127^{low}$ Treg cells (0.848)]. At the same time, the overall accuracy of the combined bone metabolism and immune indicators for determining whether a tumor had bone metastasis was 88.26%. This suggests that the combination of bone metabolism markers and immune cell indicators in peripheral blood provides high diagnostic accuracy for evaluating bone metastasis in malignant tumors. This combined approach offers a promising tool for initial screening and risk stratification of patients suspected of having tumor bone metastasis, reducing unnecessary imaging procedures in certain patients. Moreover, integrating peripheral blood bone metabolism markers with immune cell markers enhances the monitoring of changes associated with tumor bone metastasis, thereby improving both diagnostic specificity and sensitivity. Thus, it is recommended that patients with malignant tumors undergo regular testing for both bone metabolism and immune markers. This strategy enables early detection and intervention of bone metastasis, ultimately improving patients' quality of life and reducing healthcare costs.

This study has several limitations: (1) It is a single-center, retrospective study with a relatively small sample size, which limits the representativeness of the results. (2) Due to the lack of external validation, the efficacy of combined screening using bone metabolism and immune cell indicators for tumor bone metastasis has not been externally validated. Given these limitations, future research should include multicenter, prospective studies with larger sample size and additional external validation (e.g., at least 500 cases from an independent cohort) to further elucidate the pathological mechanism of tumor bone metastasis.

Conclusion

The peripheral blood levels Ca^{2+}, β-CTX, P1NP, OC, $CD3^+CD4^+$ T cell percentage, $CD3^+CD8^+$ T

cell percentage, and $CD4^+CD25^+CD127^{low}$ Treg cell percentage have certain screening value for bone metastasis in malignant tumors. The combined monitoring of these indicators provides a more comprehensive assessment of the bone metastasis burden, activity, and immune microenvironment status.

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Disclosure of conflict of interest

None.

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