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Association of Tartrate-Resistant Acid Phosphatase-Expressed Macrophages and Metastatic Breast Cancer Progression

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Abstract: Infiltrating neutrophils, lymphocytes, macrophages, and cytokines constitute a state of chronic inflammation within the tumor microenvironment. Tartrate-resistant acid phosphatase 5a (TRACP5a) protein, a novel product of activated macrophage, is postulated to be a biomarker for systemic inflammatory burden in states of chronic inflammation. We aimed to investigate the clinical significance of TRACP5a expression in tumor-infiltrating macrophages and serum TRACP5a in patients with metastatic breast cancer (BC).

We retrospectively analyzed the clinical data from 34 BC patients with confirmed skeletal/visceral metastasis upon or during first-line palliative treatment. Patients were stratified into 3 groups based on the therapeutic responses and follow-up disease course. The association of TRACP5a protein with other inflammatory and cancer biomarkers was assessed among the clinically distinct group of patients. Higher TRACP5a protein was significantly correlated with earlier disease progression and survival ($P = 0.0045$) in comparison to other inflammatory markers, CRP or IL-6. Patients with higher serum TRACP5a level and shorter survival and treatment refractoriness also had more TRACP+ tumor-infiltrating macrophages.

Our data support a hypothesis that serum TRACP5a protein can potentially be a predictive and prognostic marker to evaluate disease progression and therapeutic response in BC patients with bone/visceral metastasis. The associations between overall survival and TRACP expression by macrophages require further prospective investigation.

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Abbreviations: ANOVA = analysis of variance, BC = breast cancer, CEA = carcinoembryonic antigen, CRP = C-reactive protein, IL = interleukin, PET = positron emission tomography, TAM = tumor-associated macrophage, TIM = stumor-infiltrating macrophages, TNF = tumor necrosis factor, TRACP = tartrate-resistant acid phosphatase.

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INTRODUCTION

Unresolved chronic inflammation is a risk factor for cancer development; a link already perceived in the nineteenth century.[1,2] In addition, tumor derived factors present in the microenvironment can amplify the inflammatory state, increasing cytokine release and attracting more inflammatory cells. These processes promote angiogenesis, and tumor cell invasion, migration, and distant metastasis.[3] Clinical and experimental data suggest that chronic inflammation promotes mammary tumor development through mechanisms involving chronic activation of humoral immunity and infiltration of type 2 helper T (Th2) cells and polarization of innate inflammatory cells toward a tumor-supporting phenotype.[4] Among these, the tumor-associated macrophage (TAM) is a cornerstone of most tumor infiltration and plays an important role in disease progression or distant metastases.[5–7]

In breast cancer (BC) patients, TAM infiltration is common. A recent study showed abundant macrophage infiltration in malignant tissues, which positively correlated to increased angiogenesis and poor prognosis.[8] Other investigators published evidence that TAM promoted primary site angiogenesis, tumor cell invasion, distant seeding, and suppression of antitumor immune responses.[9] Given that TAM initiate an inflammatory microenvironment, some acute and chronic inflammatory cytokines and serum markers including tumor necrosis factor (TNF)-α, interleukin (IL)-6/8, IL-1α/β, C-reactive protein (CRP), or serum amyloid A have been used to detect tumor-promoting, disease progressive activity, and as indicators of prognosis or treatment response.[10,11]

Tartrate-resistant acid phosphatase (TRACP) is a unique biomarker expressed in activated macrophages, dendritic cells, and osteoclasts.[12] Serum TRACP5b is specifically released by bone-resorbing osteoclasts. There is a large literature documenting the clinical relevance of serum TRACP5b in diseases of bone metabolism as it reflects the systemic number of osteoclasts.[13] Our group has shown that serum TRACP5b activity has diagnostic and prognostic significance in BC with bone metastasis.[14] The TRACP5a isoform is selectively secreted from macrophages and dendritic cells while the 5b isoform remains intracellularly. As such serum TRACP5a[15–17] protein is a potential marker for disease severity in cardiovascular disease,[18] rheumatoid arthritis,[19] and perhaps chronic kidney disease.[20] From these and other studies,[21,22] it is postulated that serum TRACP5a protein levels reflect the systemic burden of inflammatory macrophages.

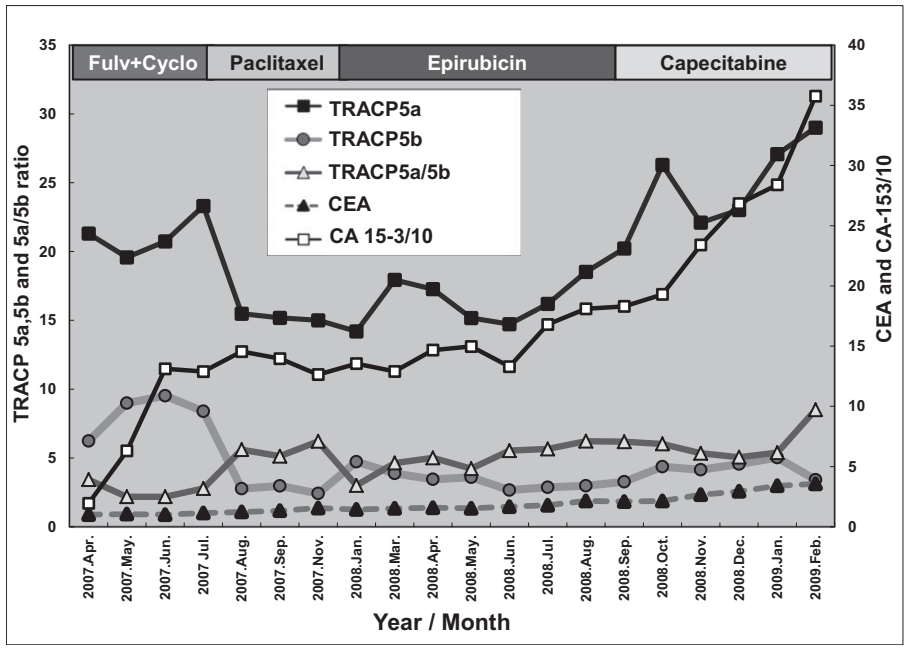

FIGURE 1. Representative patient with metastatic breast cancer. Schematic representation of the illustrated metastatic breast cancer patient demonstrated the sequential lines palliative chemotherapy regimen received, change of serum tartrate-resistant acid phosphatase (TRACP) isoforms, carcinoembryonic antigen (CEA), and cancer antigen 15-3 (CA 15-3) throughout the treatment period.

Since inflammatory markers and cytokines are useful for early detection of tumor progression, and TRACP5a may be prognostically useful in many chronic diseases, our hypothesis is that TRACP5a protein can also be a useful serum marker to predict advanced or distant metastases in cancers, which are associated with systemic inflammation. Therefore, we aim to examine the relationship between serum TRACP5a protein and cancer-related inflammation to address this hypothesis. We also intend to investigate whether serum TRACP5a protein value can be a prognostic/predictive marker for cancer progression, distant metastasis, and treatment resistance.

PATIENTS AND METHODS

Illustrative Case Presentation

A 61-year-old woman had stage IIIA infiltrating ductal carcinoma of right breast (pT3N1M0, positive for Estrogen receptor/Progesterone receptor and negative for HER-2/neu) and had undergone modified radical mastectomy in 1993. After surgery, she received sequential adjuvant chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil and radiotherapy. The maintenance hormonal therapy with tamoxifen was administrated for 5 years. Six years later, whole body Tc-99m MDP imaging showed multiple ribs, scapular and thoracic-lumbar metastases. Her disease remained stable status after receiving therapy with anastrozole and pamidronic acid followed by exemestane and zoledronate. During follow-up, repeat Tc-99m MDP imaging showed stable metastatic disease without evident clinical progression. However, due to subsequent clinical disease progression and increased bony metastases salvage therapy including fulvestrant, cyclophosphamide, and Zoledronate was given since 2007. At this time laboratory tests for serum TRACP5a protein, TRACP5b activity, IL6, CRP, carcinoembryonic antigen (CEA), and cancer antigen 15-3 (CA 15-3) were performed regularly. In April 2007, chemotherapeutic regimen was shifted to paclitaxel because of pulmonary metastases with multiple soft-tissue nodules over the pleura and bilateral malignant pleural effusions. In August 2008, she experienced further disease progression with increased size and numbers of hepatic and bony metastasis. Supportive treatment was provided because of poor performance status, and the patient succumbed to disease progression several months later. A schematic representation summarizing the therapeutic regimen, clinical response, and related inflammatory markers throughout the treatment period are depicted in Figure 1. These longitudinal data demonstrated that serum TRACP5a protein fluctuations paralleled the disease course and the tumor marker CA15-3/10. Several issues were raised from this case: Rising serum TRACP5a protein, with CA 15-3, reflected the tumor aggressiveness either bone or visceral organ metastasis; TRACP5a protein declined when a newline of chemotherapy was initiated with clinical response; TRACP5b activity remained stable from April 2007 to February 2009 after initially falling upon bisphosphonate use then fell again as it was continued further. From this clinical observation, it prompted us to conduct a pilot study to examine retrospectively the association between serum TRACP5a and the disease course of metastatic BC.

Study Design

This was a retrospective study of 34 patients with pathologically proven BC with bony and/or visceral metastases. Patients were diagnosed to have metastatic diseases according to either of the following modalities: histologically proven by aspiration biopsy, cytological evaluation, or bone biopsy, or in those without histologic diagnosis, the presence of both relevant clinical symptoms of bone or visceral organs metastasis and radiographic evidence including computerized tomography, magnetic resonance image, ^{99m}Tc-hydroxymethylenediphosphaonate (^{99m}Tc-MDP) whole-body bone scintigraphy, or positron emission tomography (PET) scan. Additional

supporting laboratory tests included the serum tumor markers CEA and CA 15-3 as clinical practice.

All patients had received standard therapies including neoadjuvant, adjuvant chemotherapy, radiotherapy, hormone therapy, and targeted therapy accordingly. In addition, relevant image studies and tumor markers were followed regularly. The disease progression in distant visceral or bone metastases was recognized by tumor marker elevation, image studies, and clinical judgment of physicians. Serum for biomarker assays were collected at the time upon disease progression. Patients with active or unresolved infection and chronic inflammatory conditions such as cardiovascular disease or rheumatoid arthritis were excluded from study. Patients were stratified into 3 groups based on progression-free interval to the metastasis-related therapies including chemotherapy, hormone therapy, or targeted therapy. Group A was defined as stable/indolent clinical symptoms and bone/visceral lesions without active radiographic progression under current treatment and progression-free interval of >10 months. Group B was defined as patients with progressive clinical symptoms, bone/visceral metastasis, rising tumor markers, and a response to treatment with progression-free interval of 6 to 10 months. Group C was defined as patients with aggressive clinical symptoms, bone/visceral lesions refractory to treatment with progression-free interval of <6 months.[23] Overall survival was measured from the date of observed clinical metastases until death. Surviving patients and those who were lost to follow-up were censored from overall survival analysis when they were last known to be alive.

Serum and Tissue Samples

Venous blood was drawn after overnight fast, allowed to clot at room temperature for 30 min, then sera separated and stored at -80°C until used for biomarker assays. Biopsy samples from metastatic sites were collected when clinically required. After serving their clinical purposes the left-over specimens were reserved, frozen at -70°C or fixed in 4% neutral buffered formalin. Patients were treated in the Division of Hematology/Oncology of the Tri-Service General Hospital, a 1200-bed Medical center in northern Taiwan. Informed consent, approved by Institutional Review Board, was obtained from the patients. The Institutional Review Board of the Tri-Service General Hospital approved the use and analysis of sera and biopsy samples.

Biochemical Markers

Previously collected frozen sera were thawed at room temperature immediately before subsequent biochemical analyzing. Serum CEA and CA 15-3 were tested as routine clinical practice. Serum CRP was analyzed by peroxidase-labeled and unlabeled commercial rabbit polyclonal antiserum to human CRP (DAKO Denmark, Copenhagen, Denmark) in a sandwich enzyme-linked immunosorbent assay. IL-6 level was determined by the commercial human IL-6 enzyme-linked immunosorbent assay kit (RayBiotech, Atlanta, GA). Serum TRACP5a protein and TRACP5b activity immunoassays were measured according to previously reported methods.[19]

Immunohistochemical and Immunofluorescence Staining

Four-micron sections from formalin-fixed paraffin embedded tissues were collected onto glass slides and processed for immunohistochemical staining for CD68 (1:200, Bioss Inc, China) using an amplified polymer technology (Thermo, Bio SB, USA, TL-060-QPH). Briefly, after blocking nonspecific binding sites with 5% nonfat milk and 0.1% Triton X-100 in PBS, specimens were incubated for 1 h at 37°C with rabbit polyclonal anti-CD68, washed with PBS (3×5 min), then incubated for 1 h at 37°C with HRP polymer. Peroxidase activity was disclosed with DAB and H_2O_2 according to kit directions. For double-labeled immunofluorescence staining, fresh frozen specimens were sectioned, mounted onto glass slides, washed with PBS (3×5 min), and reacted for 1 h at 37°C with a combination of primary antibodies: mouse monoclonal anti-TRACP (1:100 dilution, Bio SB) and rabbit polyclonal anti-CD68 (1:200 dilution, Bioss Inc). Subsequently, slides were washed with PBS (3×5 min) and reacted for 1 h at 37°C with combined secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG and phycoerythrin (PE)-conjugated donkey anti-rabbit IgG (both from Santa Cruz Biotechnology, Inc, USA). Stained slides were mounted using 3% n-propyl gallate and 50% glycerol in PBS, and examined using a Zeiss epifluorescence microscope (Carl Zeiss, Oberkocheu, Germany), images being captured and digitized using a Nikon, Japan DIX digital camera.

Statistical Analysis

All statistical analyses were performed using SPSS version 18.0 statistical software (Chicago, IL). All descriptive data are expressed as median (range) and for the comparison of more than 2 groups, 1-way analysis of variance (ANOVA) was done. A P value < 0.05 was considered statistically significant. Data are expressed as mean $\pm$ standard deviation. The Kaplan–Meier method was used to calculate time-to-event end points (overall survival), which were compared using the log-rank test.

RESULTS

Patient Characteristics

The demographic, baseline biochemical parameters, hormone receptor status, received anti-osteoclast treatments, and HER2-directed therapies are displayed in Table 1 and Table 2. All patients received multiple ''lines'' of variable therapies as determined by standards of care and clinical judgment for each patient based on individual need. Because of the small cohort size and variable treatments, patients could not be grouped based on treatments. Instead, ''lines of therapy" and duration of progression-free interval for each line in the 3 groups are available in supplemental material (Fig. S1).

Serum Biomarker Association With Disease Progression

We aim to determine whether TRACP5a protein can be a predictive marker for disease progression or longer duration of responsiveness compared with other inflammatory and tumor markers. Table 2 summarizes the results of 1-way ANOVA assessing differences of biomarkers among 3 groups. TRACP5a protein was significantly higher in group C with treatment-refractory metastatic disease, compared with groups A and B with more stable/indolent metastasis (Fig. 2A and B), and longer progression-free interval. Furthermore, TRACP5a levels were significantly higher in the patients with visceral metastasis compared to nonvisceral metastasis (Fig. S2). TRACP5b, a marker for osteoclastic bone resorption, was significantly higher in group A, compared with groups B and C (Table 2).

TABLE 1. Characteristics of the 34 Breast Cancer Patients With Visceral/Bone Metastases

Variables	Numbers or Values (Range)
Female/male	34/0
Median age, y	47.68 (29–74)
Median time from initial diagnosis to enrollment, y	4.69 (0.00–15.46)
Median overall survival, y	0.87 (0.00–3.96)
Median initial TRACP5a protein, nmol/L	12.47 (6.5–19.5)
Median initial TRACP5b protein, μkat/L	1.92 (0.79–5.54)
Median serum IL-6 level, pmol/L	19.06 (0.39–155.00)
Median CEA, nmol/L	17.51 (0.3–175.0)
Median CA 15-3, μkat/L	39.70 (4.18–132.00)
ER	
Positive	25
Negative	7
Unknown	2
PR	
Positive	24
Negative	8
Unknown	2
Her-2/neu	
Positive	6
Negative	26
Unknown	2
Triple negative	1
Visceral metastasis	21
Bone metastasis	25
Both metastases	18

CA 15-3 = cancer antigen 15-3, CEA = carcinoembryonic antigen, ER = estrogen receptor, Her-2/neu = human epidermal growth factor receptor 2, IL-6 = interleukin 6, PR = progesterone receptor; TRACP = tartrate-resistant acid phosphatase.

There were no significant differences in serum CRP or IL-6 among the groups ($P = 0.053$ and 0.758), and no significant correlation between TRACP5a and CRP or IL-6.

Immunohistochemistry and Fluorescence Immunostaining

Two patients were compared, 1 from group A and 1 from group C, who had low serum TRACP5a (6.73 ng/mL) and high serum TRACP5a (22.51 ng/mL), respectively, and whose PET scans showed similarly extensive metastatic diseases (Fig. 3A and F). Both patients also had similarly large numbers of tumor-infiltrating macrophages (TIMs) in their liver metastatic tumors as judged by CD68 IHC (Fig. 3B and G). However, co-localization immunofluorescence stains showed that the macrophages within group A patient's tumor had fewer TIMs that were TRACP+ compared with the group C patient's tumor (Fig. 3D–F, H–J). These results suggest that higher serum TRACP5a protein probably reflected more TRACP+ TIMs modulating tumor microenvironment, which in turn could have contributed to the observed more rapid progression than the patient with low serum TRACP5a and fewer TRACP+ TIMs.

Overall Survival as a Function of Serum TRACP5a

At the time of our analysis, 25 of the 34 patients had died. Nine patients were still alive whether their disease status was stable or worsening. There is no statically different overall survival among the 3 groups; however, there is a trend toward shorter survival in group C patients with the most disease aggressiveness as determined by clinical criteria (Fig. 2C). We re-stratified all patients into 2 equal groups based on the median value of initial serum TRACP5a protein. There was a trend toward longer median survival in patients with serum TRACP5a <12.4 ng/mL compared to those with initial serum TRACP5a >12.4 ng/mL although statistical significance was not achieved (Fig. 2D).

DISCUSSION

Chronic inflammation is an important contributor to oncogenesis and cancer progression.[24] Macrophage infiltration, associated with inflammation surrounding tumor, has been identified as 1 of the poor prognostic factors in several types of cancer.[25] Therefore, activated macrophage-related products have been studied as surrogate markers for progression of cancer. TRACP is abundantly expressed in differentiated and activated monocyte-derived cells.[17] In vitro extracellular TRACP5a is selectively secreted by macrophages while TRACP5b is retained intracellularly. In vivo, activated macrophages are the likely source of serum TRACP5a, and bone-resorbing osteoclasts are responsible for serum TRACP5b.[26] In BC, the interval change of TRACP5b activity after treatment represented a prognostic role in skeletal metastasis.[14] However, in this study, TRACP5b activity was significantly reduced in groups B and C probably attributed to the common use of anti-osteoclastic agents (zoledronate or denosumab) in these patients. Assuming that expanding tumor tissue would contain proportionally more TRACP+ TIM, then serum TRACP5a should reflect cancer progression and poor prognosis.[27] Zenger et al[28] had found that higher concentration of TRACP5a protein in BC-related bone metastasis and postulated TRACP5a protein resulted from local inflammatory responses caused by TIMs. In addition, Honig et al[29] showed marked elevation in serum TRACP to be correlated to TRACP-positive macrophage infiltration to tumor. Our data also demonstrated that the TRACP5a protein could be an effective marker to monitor the disease activity, being associated with TIM infiltration and systemic inflammation.

Cancer leads to progressive lymphocyte recruitment and activation, within the tumor microenvironment. Other common biomarkers used to assess inflammation are IL-6 and CRP. Previous studies showed higher serum CRP and IL-6 in patients with colorectal cancer and BC.[30,31] However, a recent study showed no obvious correlation between the TAM and IL-6 concentration in animal models.[32] Our data showed a trend toward higher serum CRP in group C compared with other groups but not in IL-6. There was also a significantly higher TRACP5a level in group C patients, but it did not correlate to either IL-6 or CRP. This finding is not surprising. We had previously shown in a cardiovascular disease setting that TRACP5a correlated weakly or not at all with CRP and IL-6, respectively.[18] We postulated that these inflammation markers may not correlate due to cell source differences and

TABLE 2. Characteristics of Molecular Subtypes, Received Therapeutic Modalities, and Relevant Inflammatory Markers Among 3 Groups

Variables	Group A (N = 9)	Group B (N = 13)	Group C (N = 12)	*P* Value
Hormone receptor (ER or PR) positive	6 (66.67%)	11 (84.61%)	11 (91.66%)	
Triple negative	–	–	1 (8.33%)	
Lines of received treatment (mean)	2.67	2.23	2.75	
Received denosumab	2 (22.22%)	10 (76.92%)	7 (58.33%)	
Received bisphosphonate	6 (66.67%)	1 (7.69%)	–	
Received chemotherapy	7 (77.78%)	13 (100%)	12 (100%)	
Anti-hormone therapy	6 (66.67%)	9 (69.23%)	11 (91.66%)	
Received HER2-directed therapy	2 (22.22%)	4 (30.77%)	3 (25%)	
IL-6, pmol/L	22.40 ± 39.96	13.57 ± 16.44	22.88 ± 42.44	0.758
CRP, mmol/L	2.04 ± 1.97	3.79 ± 2.14	4.64 ± 2.47	0.053
TRACP5a, nmol/L	11.30 ± 3.50	11.17 ± 2.24	14.76 ± 3.97	0.019*
TRACP5b, μkat/L	2.56 ± 1.33	1.81 ± 0.27	1.56 ± 0.59	0.021*
TRACP 5a/5b ratio	4.83 ± 1.24	6.21 ± 1.18	10.05 ± 2.39	0.001**

Significant difference among 3 groups by 1-way ANOVA test.
CRP = C-reactive protein, ER = Estrogen receptor, IL-6 = interleukin 6, PR = progesterone receptor, TRACP = tartrate-resistant acid phosphatase.
* $P < 0.05$.
** $P < 0.01$.

temporal differences in their expression during the inflammatory response.

Examination of the clinical course in group C patients with the highest serum TRACP5a, showed them to be more refractory to treatment and have shorter periods of response duration and trend toward shorter survival. However, the small cohorts indicate caution to be used in interpretation.

It has been reported that infiltrating $CD68^+$ or $CD163^+$ TAMs or TIMs correlate with poor outcome in solid tumors.[33,34] It is believed that recruited monocytes within

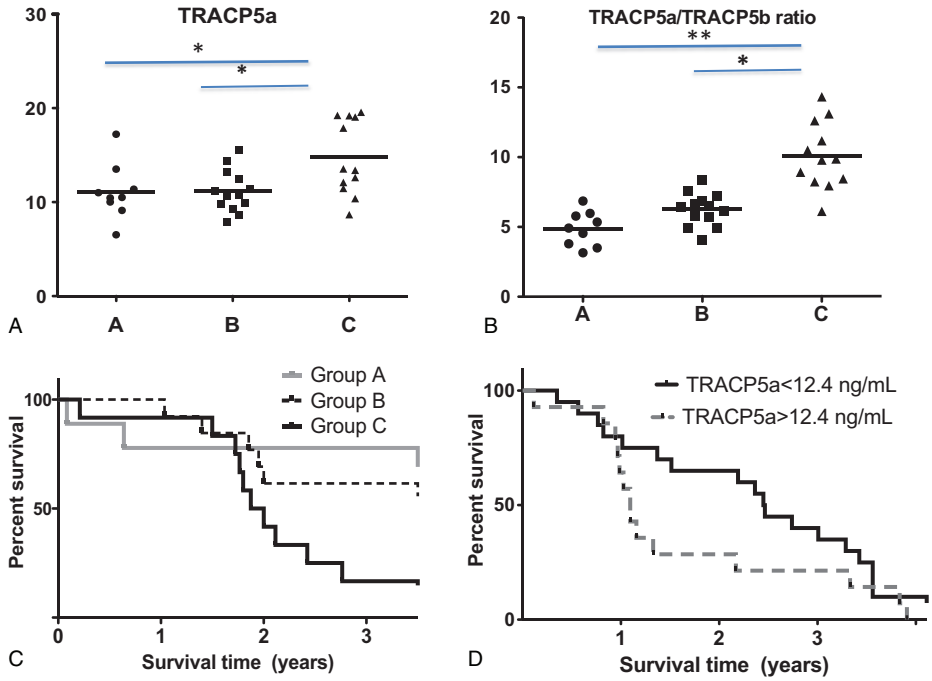

FIGURE 2. Serum TRACP5 level in metastatic breast cancer patients and overall survival. (A) The mean and standard deviation of serum TRACP5a protein in groups A, B, and C are shown (* $P < 0.05$). (B) The mean and standard deviation of serum TRACP5a/TRACP5b ratio in groups A, B, and C are shown (* $P < 0.05$, ** $P < 0.01$). Kaplan–Meier plot of survival from all 34 patients based on (C) 3 different clinical subgroups and (D) the serum level of TRACP5a.

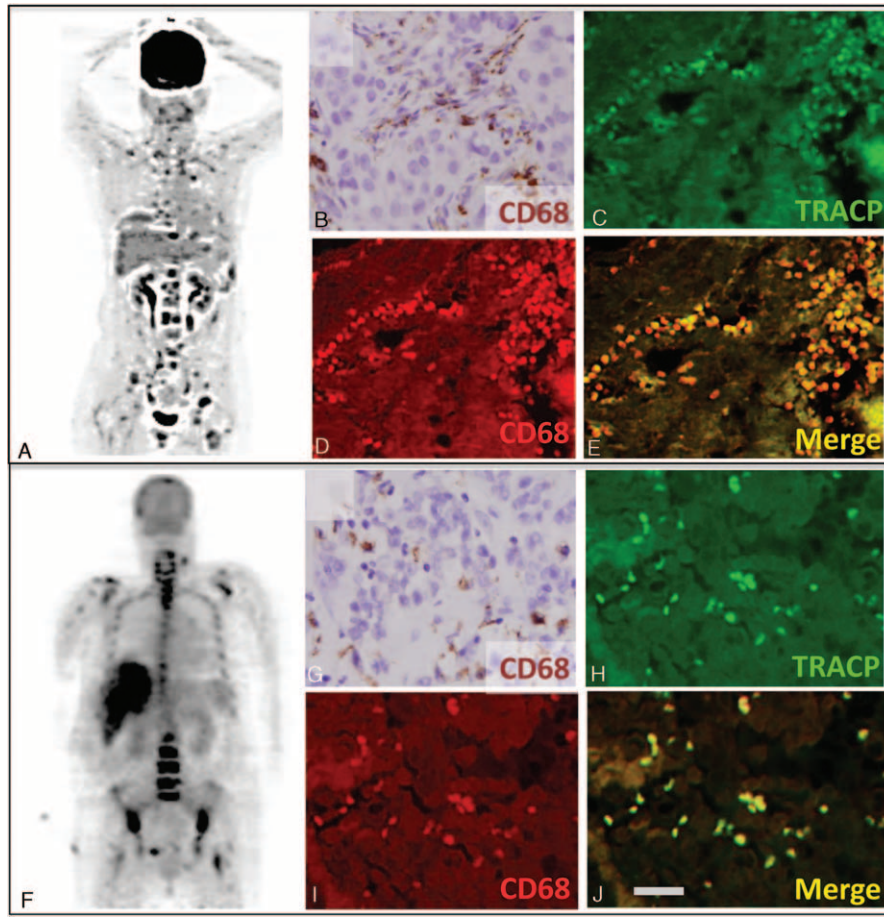

FIGURE 3. Immunohistochemical staining of tumor-infiltrating macrophages within breast cancer metastasis. PET scans in 2 representative specimens from 2 patients with extensive bone and visceral metastasis (A and F) but divergent serum TRACP5a levels (6.73 ng/mL in patient A and 22.51 ng/mL in patient F). CD68 were examined by immunohistochemical staining (B and G) and showed similar pattern of tumor-infiltrating macrophages. In fluorescence immunostaining, the mouse monoclonal antibodies against TRACP5a (green fluorescence, C and H) and rabbit polyclonal antibodies against CD68 (red fluorescence, D and I) were examined to elucidate the relationship between the TRACP expressions in the tumor-infiltrated macrophages. Colocalization of macrophage and TRAP (merge, E and J) is observed. More TRACP-expressed macrophages were found in patient F (J) compared with patient A (E). Scale bar, 50 μm. TRACP = tartrate-resistant acid phosphatase.

cancer become alternatively activated to a protumoral phenotype (M2), supporting angiogenesis, matrix remodeling, and cancer invasion. These cells can also suppress adaptive immunity and are distinct from classically activated, immunostimulating macrophages (M1). In this study, we sought to determine if serum TRACP5a is related to the number of TIMs or TAMs and their TRACP expression. Two representative patients from groups A and C, both having skeletal and visceral involvement with low and high serum TRACP5a, respectively, showed TRACP+ TIMs were lower in the group A than group C patient. From this observation, we postulate that serum TRACP5a is secreted by TIMs within the tumor microenvironment and may be associated with a more rapid clinical course and poor outcome. TRACP5a could be applied as a potential prognostic marker for cancer-related chronic inflammation.

It has recently been established in murine models that blocking TAM recruitment and/or survival in solids tumors improves efficacy of cytotoxic therapies.[35] Earlier studies have shown that macrophage-depletion strategies, combined with ''standard of care'' treatments such as chemotherapeutic drugs or immunotherapies, could be beneficial in a subpopulation of patients.[25,36] In this regard, serum TRACP5a level can be a stratification marker to select the patients who are eligible for adjuvant TAM-depletion therapy.

There are several limitations in this study: First, this was a small sample size retrospective study and patients did not receive uniform treatment. In addition, our patient population is heterogeneous in regard to ER, PR, HER2 status, and treatment regimens. Nevertheless, it does reflect the real context of clinical practice, and effects due to specific treatments were not our primary objective. Second, this was a proof-of-concept inquiry of small cohorts of patients. Although ours and previous studies by others show correlation between serum TRACP and TRACP+ TAMs/TIMs, further studies will be required to see whether co-existence of other inflammatory or infectious disease will affect the significance of serum TRACP5a in cancer. Finally, the small cohort size also limited our ability show a significant difference in survival based on serum TRACP5a stratification. However, the trend was clear and indicates that an expanded study is worthwhile.

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