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Determination and biological relevance of serum cross-linked type I collagen *N*-telopeptide and bone-specific alkaline phosphatase in breast metastatic cancer

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Abstract

Bone metastasis is a frequent complication of cancer disease. The metastatic spread of cancer to bone is common to many different malignancies, particularly breast (ca. 73%), prostate (ca. 68%) and lung (ca. 36%) cancers. Metastases to bone cause increased bone resorption both from direct effects of the tumor itself and thought osteoclastic activation. The diagnosis and follow-up of bone metastatic cancer patients usually relies on skeletal X-ray and bone scintigraphy. However, the development of biochemical markers, used as indicators of bone metabolism, provides data useful in the clinical practice. The most important markers for bone remodeling process, bone formation and resorption, are bone-specific alkaline phosphatase (BAP) and *N*-telopeptide of type I collagen (NTx), respectively. In this report, we applied two solid-phase immunoassays used for the determination of BAP and NTx in serum of breast cancer (BC) post-menopausal women with bone metastasis and healthy individuals. BAP level in patients was found to be 45.72 ± 12.92 U/l, while the normal range for healthy individuals was 14.2–42.7 U/l. The respective level of serum NTx was 19.20 ± 8.87 nM bone collagen equivalents (BCE) for patients and 15.9 ± 3.8 nM BCE for healthy women. Correlation of the obtained data showed elevated levels for both markers indicating high rate of bone degradation in breast metastatic cancer.

Keywords: Breast cancer; Bone metastasis; Tumor biochemical markers; Bone-specific alkaline phosphatase; Collagen I *N*-telopeptide

1. Introduction

Breast cancer (BC) is the most common malignant disorder that affects women. The frequency of this type of cancer is one in 12 women and approximately 44,000 women die each year in US from this disease [1]. The majority of these patients die not because of the tumor in the primary site, but rather because it has spread to other sites. Metastasis is the process by which tumor cells travel from the primary tumor to a distant site via the circulatory system and establish a secondary tumor. The process of metastasis is a complex cascade of organised, sequential and interrelated steps, including angiogenesis, local invasion, intravasation and extravasation [2]. Bone is one of the most

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preferential target sites for cancer metastasis. Breast cancer has a predilection for spreading to bone, and bone metastasis is one of the major causes of increased morbidity and eventual mortality in breast cancer patients. Patients with advanced breast cancer almost always develop bone metastases, and the chances are high that, in patients who are originally diagnosed with breast cancer, the bulk of the tumor burden at the time of death will be in bone [3].

Bone metabolism is a dynamic and continuous process, called remodeling cycle, which is normally maintained in a tightly coupled balance between resorption of old or injured bone and formation of new bone. Bone mass depends on this crucial balance. Bone resorption is mediated by the action of osteoclasts, derived from fusion of cells of monocyte lineage, while bone formation is mediated by the action of osteoblasts, derived from precursor blood cells [4]. In pathological states, such as bone metabolic diseases including bone metastases, there is a tuning off this balance and the remodeling process becomes uncoupled. The imbalance to the side of bone resorption, when resorption exceeds formation, can result in substantial changes in integrity of bone, such as bone loss and, therefore, can lead to osteoporosis or to the disordered bone tissue of Pagetic lesions.

There are two basic types of bone in the human skeleton, cortical and cancellous bone. Cortical bone, accounting for more than 80% of the skeleton, is the major component of long bones because it is dense due to its extended calcification. Cancellous or trabecular bone, comprising the remaining 20% of the skeleton, shows high metabolic activity due to its cancellate surface area. Both types of bone mainly consist of type I collagen, which comprises over 90% of the organic bone matrix. Type I collagen is synthesized by osteoblasts and is derived from a larger protein termed procollagen I. During the synthesis and fibril formation process, large extension peptides, known as propeptides, on both carboxyterminal and aminoterminal ends of procollagen I, are cleaved by specific proteinases. Structurally, type I collagen has a triple helix form and one of the three α-chains has a high proportion of the amino acids proline, lysine and their hydroxylated residues, hydroxyproline and hydroxylysine. The major difference bone and other tissues collagen is that the first type contains hydroxylysylpyridinoline and lysylpyridinoline crosslinks. Crosslinking occurs between lysine and hydroxylysine residues on the nonhelical carboxyterminal and aminoterminal ends of mature type I collagen, termed telopeptides, and the helical portions of an adjacent collagen. This process results in forming the pyridoline and deoxypyridoline crosslinks, which provide rigidity and strength in the collagen molecule [5].

The follow-up of bone metabolism is very important, because it provides information for the rate of both formation and resorption and the grade of bone metastasis in cancer patients. There are three available diagnostic methods to estimate the metabolic state of bone: bone density, bone biopsy, and measurement of specific biochemical markers. Bone density measurements are essential for the diagnosis of osteoporosis and biopsies are useful for assessing bone metastases, but both techniques are painful for the patients and cannot be used in clinical practice. During the recent years, there is a prominent effort for the development of new biochemical markers that can be used as indicators of bone metabolism. There are markers for both formation and resorption, which are extensively accepted as research tools in clinical studies. The measurement of specific biochemical markers provides analytical data regarding of bone metabolism. Substantially, these biochemical bone markers are products, which are released during the metabolic cycle and can be detected in serum or urine [6]. Markers of bone formation assess either osteoblastic synthetic activity or post-release metabolism of procollagen. These are total and bone-specific alkaline phosphatase (BAP), osteocalcin, a relatively small protein produced by osteoblasts during the mineralization of bone matrix and type I procollagen *C*- and *N*-propeptides (PICP and PINP, respectively). Resorption markers reflect osteoclast activity or collagen degradation. These are urinary calcium, acid phosphatase, hydroxyproline, pyridoline and deoxypyridoline crosslinks and type I collagen *C*- and *N*-telopeptides (CTx and NTx, respectively).

Among these markers, BAP and NTx in serum play an important role for the determination of bone metabolic state. Alkaline phosphatase is an enzyme associated with the plasma membrane of cells. Although it appears to be involved in the transport of substances from intracellular to extracellular matrix,

its exact function is still unknown. There are four specific isoforms of alkaline phosphatase in circulation for the following tissues: liver, placental, intestine and bone. The bone-specific alkaline phosphatase isoenzyme, a tetrameric glycoprotein, predominates during childhood and adolescence and represents less than 40% of the total serum concentration [7]. BAP is produced by osteoblasts during the bone formation phase by clipping off the membrane and released into circulation. It is produced in extremely high amounts and is, therefore, an excellent indicator of bone formation activity. Type I collagen *N*-telopeptide fragments (NTx) are released into circulation during the bone resorption phase by the action of osteoclasts as a stable end-product of bone degradation, as described before [8,9]. NTx in serum is a specific biochemical marker of bone tissue breakdown due to the unique amino acid sequences and orientation of the cross-linked α-2 (I) *N*-telopeptide. Other tissues comprised of type I collagen, e.g. skin, produce different fragments during degradation since are not metabolized by osteoclasts. Measurement of NTx in serum reflects the rate of bone resorption and is used as an important indicator [10].

The aim of this experimental study was to determine, with specific solid phase immunoassays, the levels of BAL and NTx in serum of breast cancer patients with bone metastasis and in healthy individuals in order to evaluate whether these specific biochemical markers may be at importance in the detection of breast cancer bone metastasis.

2. Material and methods

2.1. Biologic material

The study was carried out in 24 post-menopausal women. Twelve were breast cancer patients with substantiate bone metastasis (age range 47–63). The inclusion criteria were histologically confirmed breast cancer with evidence of bone metastasis. The remaining 12 (age range 49–56), used as control group, were healthy individuals. Serum samples were obtained from Metaxas memorial cancer hospital. Sera were collected the cubical vein using a syringe without additives. Serum aliquots were and stored at −80 °C until to be assayed.

2.2. Determination of bone-specific alkaline phosphatase in blood serum

The levels of BAP in serum samples were determined by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Metra™ BAP kit, Metra Biosystems, San Diego, CA, USA). All required reagents were supplied with the kit and the procedure was carried out according to the manufacturer's instructions. ELISA was performed on sterile 96-well round bottomed microplates. Each well contained immobilized monoclonal anti-BAP IgG murine antibody. The antibody captures serum BAL and the product can be quantified spectrophotometrically. In brief the assay steps are the following. After the addition of 125 μl of assay buffer, containing magnesium chloride, zinc sulfate and sodium azide (0.05%) as a preservative, and applying 20 μl of the samples to each well (all runs in triplicate), the mixtures were incubated for 3 h at room temperature. Microplates were washed four times with wash buffer (250 μl per well). The next step was to add 150 μl of substrate comprised of paranitrophenol phosphate (pNPP) in 2-amino-2-methyl-1-propanol and to incubate for 30 min in order to color development. The reaction was determined with 1N NaOH and optical density (OD) was measured at 405 nm in Molecular Devices E-max photometer. Calibration and validation of results was performed using the SOFT max PRO software (Version 2.4.1). All quantitative results for BAP were expressed as units per liter (U/l) where 1 unit represents 1 μmol of pNPP in the substrate buffer. The used standard BAP levels were 0, 2.0, 20.0, 50.0, 80.0 and 140.0 U/l.

2.3. Determination of NTx in blood serum

Quantitative determination of NTx in human serum was also performed by ELISA using the Osteomark® NTx Serum test (Ostex International Inc., Seattle, USA). This assay is a competitive inhibition ELISA format in which the NTx antigen, which has been absorbed to the surface of microtiter plate wells, specifically the a-2 chain of *N*-telopeptide fragment, is recognized by a horseradish peroxidase labeled monoclonal antibody. After adding 100 μl of each diluted (1:5) calibrator, control and samples into the wells (all runs in rtiplicate) and 100 μl of antibody

conjucate diluent (1:101), the plate was incubated for 90 min at room temperature. The next steps included five washes (350 μl per well) with diluted (1:101) wash solution, addition of the chromogen reagent (200 μl per well) and incubation for 30 min. Finally, the developed blue color in the wells turned to yellow when added 100 μl 1 M H_2SO_4 as stopping reagent. Optical density was measured at 450 nm with a 630 nm reference filter.

The results were analyzed with the same version of SOFT max PRO software as referred above. The levels of serum NTx were expressed in nanomoles of bone collagen equivalents (BCE) per liter (nM BCE). BCE are units of immunoreactive NTx standarized in moles of type I collagen in human bone that on digestion in vitro yield the same assay response as immunoreactve NTx [11]. The used calibrator concentrations of purified NTx were 0, 5, 10, 20 and 40 nM BCE.

2.4. Statistical analysis

All results are presented as mean values ± S.D. A quadratic calibration curve fit was used for accurate results in the measurement of BAP levels. Serum NTx values were analyzed using a four-parameter logistic curve fitting equation. Statistical analysis was performed by *t*-test. A value of $P = 0.001$ was considered to indicate a statistically significant difference.

3. Results

3.1. Method's quality parameters for BAP and NTx determination in blood sera

Each well of the microplate, in the Metra BAP assay, contains bone-specific alkaline phosphatase antibody. It is selective and has high affinity for the BAP isoform and presents low or negligible grade of cross-reactivity with the other isoforms (liver, intestinal and placental). The detection limit of this assay, determined as a signal-to-noise ratio equal to three, was 0.7 U/l. The intra-variation from well to well in the in the same ELISA microplate, as calculated by analysis of three standards with known concentrations, was very low (R.S.D. = 2.6%). Inter-variations, examined by performing the same immunoassay on different days or by different analysts, showed excellent reproducibility (R.S.D. = 3.4%). The low intra- and inter-assay variations demonstrated that the ELISA for BAP determination has a very high degree of repeatability, reproducibility and robustness. Calibration graph used for determination of BAP is given in Fig. 1A.

For the Osteomark NTx serum assay, the CVs of the intra- and inter-assay variations, determined by examining five serum samples with NTx levels (nM BCE) which were distributed throughout the assay's calibration curve, were 4.6 and 6.9%, respectively. The total assay precision was estimated by testing two serum samples, provided with the kit and used as control NTx levels (9.4 nM BCE and 30.0 nM BCE, respectively). CVs were 1.2 and 0.9% for the low and high serum NTx level, respectively. Antigen recovery, representing the resulted assay values and calculated as a percent of the expected serum levels, was 94–105% across the assay range. Calibration graph used for the determination of NTx is shown in Fig. 1B.

3.2. Application to the analysis of BAP and NTx in patients' serum

BAP levels were measured for both groups of post-menopausal women, patients and healthy controls. Reportedly to the literature, the normal range for healthy individuals BAP is 14.2–42.7 U/l of serum with mean value of 25 U/l. A statistically significant increase was observed in the measurement of BAP levels of patients group. For breast cancer patients with bone metastasis ($n = 12$) the level of BAP was 45.72 ± 12.92 U/l (Fig. 2A), expressed as mean ± S.D., indicating a significant increase of bone formation rate due to the high rate of bone resorption.

The determination of serum NTx also showed elevated levels in breast cancer patients. Mean baseline value in healthy individuals is 15.9 ± 3.8 nM BCE. For breast cancer patients, NTx level was 19.20 ± 8.87 nM BCE, suggesting high osteoclastic activity (Fig. 2B).

4. Discussion

Bone remodeling is a process underlying normal and abnormal skeletal metabolism. The building and degradation processes, bone formation and resorption respectively, are closely related in healthy conditions and in many bone diseases, including bone metas-

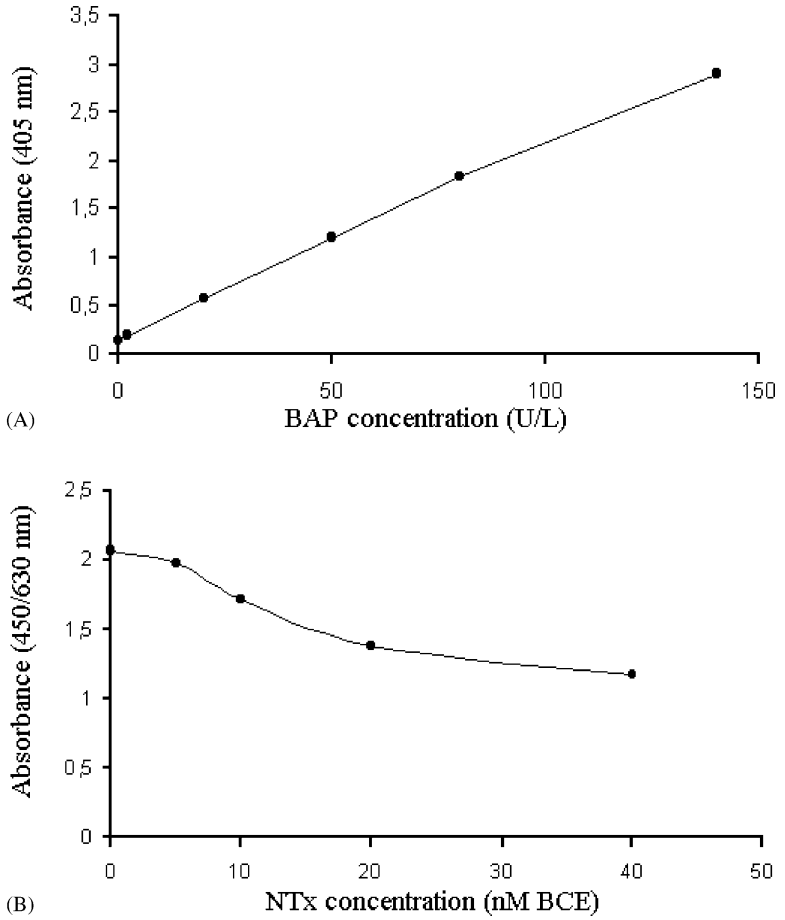

Fig. 1. Calibration graphs for BAP (A) and NTx (B). Graph for BAP resulted using a quadratic calibration curve fitting equation [$y = A + Bx + Cx^2$, $A = 0.14$, $B = 0.023$, $C = -2.02 \times 10^{-5}$, $R^2 = 1$]. Graph for NTx resulted via a four-parameter logistic curve fitting equation [$y = (A - D)/(1 + (x/C)^B) + D$, $A = 2.078$, $B = 2.133$, $C = 13.253$, $D = 1.084$, $R^2 = 1$].

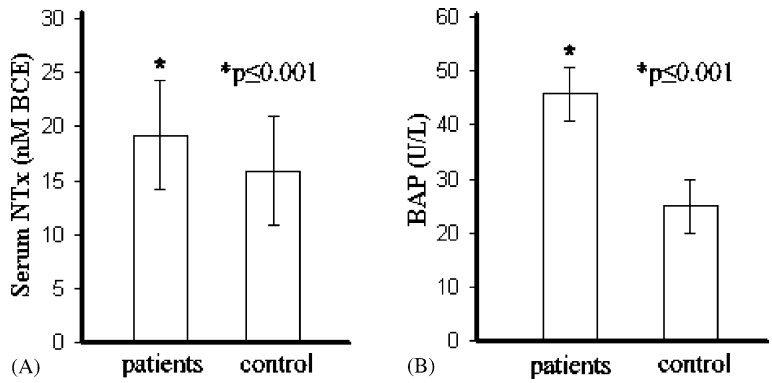

Fig. 2. Levels of NTx (A) and BAP (B) in serum samples from breast cancer post-menopausal women with bone matastasis ($n = 12$) and healthy post-menopausal individuals ($n = 12$). Bars indicate the mean ± S.D.

tasis from cancer. Since one-third of cancer patients will develop bone metastases of their primary tumor [12], the following up of bone metabolism is very crucial. Techniques such as radiology, bone scans and computer-assisted imaging are useful, but they cannot be performed to evaluate dynamic changes in skeleton metabolism [13]. Biochemical bone markers are considered as excellent indicators for bone formation and resorption. These secreted products allow detection of rapid changes in the synthesis and degradation of the components comprising bone tissue. There is a wide variety of methodologies for the detection of bone metabolism markers but immunoassays, specifically solid-phase assays, such as ELISA, using monoclonal antibodies seem to be the most sensitive and specific. This method also offers the capability of carrying out follow-up determinations.

Many investigators have been dealing with this issue, the determination of several bone markers in healthy and pathological states. Plebani et al. [13] examined 40 patients with different kinds of malignancies (breast, lung, prostate, Ewing's sarcoma) who received radiotherapy or chemotherapy and normal controls. The amount of BAP in patients was 118.4 ± 142.8 U/l while the respective level in controls was 23.4 ± 12.2 U/l measured by an immunoradiometric assay with efficiency of 0.80. Frenay et al. [14] underlined the usefulness of BAP for the prediction of bone metastasis from breast cancer in 40% of cases with lead time of 8.5 months. Berruti et al. [15] offered another use of markers, i.e. to assess both tumor burden and extent of bone pain. This investigation showed that the more skeletal involvement of the metastatic disease the higher markers' concentration (BAP, CTx, deoxypyridoline). For urine or serum NTx measurements several studies [11,16,17] concluded that the concentrations of this marker are elevated in metastatic cancer patients. These reports also designate that NTx is the most predictive indicator of bone metastases.

The majority of the previous studies focused on bone metastatic cancer patients treated with biphosphonates, adrenolate, estrogen/progestin and with other bone metabolic disorders, i.e. Paget's disease and osteoporosis. In the present report, we examined one marker for each process, BAP for bone formation and NTx for bone resorption, in blood sera from post-menopausal women with breast cancer and bone metastastic lesions without previous treatment and from healthy subjects. Increased levels were found for both analytes indicating higher rate of bone metabolism in patients than in controls. High levels of BAP reflects high rate of bone formation but, this finding leads to the opposite conclusion, that there is a high rate of bone resorption and, therefore, osteoblasts are activated to recover the bone loss ad the result is high secretion grade of BAP. On the other hand, high levels of NTx reflects high bone resorption rate, an expected result in patients with bone metastasis. The results of the present study suggest that both biochemical markers, BAP and NTx, are useful for detection of bone metastasis and might be useful for monitoring these patients during treatment.

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