Short Communication

Alpha CTX as a Biomarker of Skeletal Invasion of Breast Cancer: Immunolocalization and the Load Dependency of Urinary Excretion

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Abstract

We recently showed that increased urinary excretion of the cross-linked, nonisomerized form of the C-telopeptide of collagen type I (α1CTX) could be a sensitive indicator of the presence of bone metastases in breast cancer patients. The present study was sought to investigate (a) the localization of α1CTX epitopes in the proximity of a bone metastasis and (b) the relationship between number of metastases and the urinary excretion of α1CTX. Adjacent bone sections from breast cancer patients were stained for the presence of tumor cells (anti-cytokeratin antibody), osteoclasts (TRAcP activity), and α1CTX (anti-α1CTX antibody). The association between the extent of metastatic bone disease and urinary excretion of α1CTX measured with ELISA was assessed in 90 breast cancer patients (45 with bone metastasis and 45 without bone metastasis). Immunohistochemistry revealed accumulation of TRAcP-positive osteoclasts and intense staining for α1CTX epitopes in the proximity of cytokeratin-positive bone metastasis. Areas of α1CTX staining showed unstructured bone tissue under polarized light. In addition, there was a significant linear association between the number of bone metastases and the urinary levels of α1CTX in breast cancer patients with metastatic bone disease, independent of age and body mass index (r = 0.56, P < 0.001). The estimated relative increases in α1CTX associated with the presence of one, two, or three metastases are 38%, 57%, and 81%, respectively. Taken into account the 17% intrapatient variation of the assay, α1CTX could be a sensitive biochemical marker for the close monitoring of cancer patients aiming the facilitation of early metastasis detection. (Cancer Epidemiol Biomarkers Prev 2006;15(7):1392–5)

Introduction

Early diagnosis of skeletal metastases in breast cancer patients has important consequences for the prognosis of the primary disease. The diagnosis of bone metastases routinely relies on skeletal X-rays and bone scintigraphy. However, limited sensitivity and potential harm of serial exposure to X-ray makes these techniques inadequate for the close monitoring of cancer patients.

Metastatic bone disease is characterized by acceleration of bone remodeling at sites of the metastases. Collagen type I constitutes the main component of the extracellular matrix. The epitope 1207EKAHGDDR1214 is located in the C-telopeptide α1 chain of collagen type I and found as an α-form in the GD motif of newly synthesized collagen (1). It undergoes spontaneous enzymatic β-isomerization with aging. In a previous study, Houze’ et al. showed a significant correlation between scintigraphic scores of metastatic bone invasion and urinary βCTX levels in breast cancer patients (2). However, in pathologic situations of high bone remodeling, quantification of degradation products from the newly synthesized collagen (α1CTX) can be expected to provide an even more relevant biomarker. Indeed, three recent studies (1, 3, 4) undertaken in breast and prostate cancer patients illustrate the higher sensitivity of α1CTX for indicating the presence of bone metastases compared with β1CTX.

The aim of this study was to investigate (a) whether α1CTX epitopes are detectable in histologic sections of tumor infiltrated bone in breast cancer patients, (b) how number of bone metastases influence the urinary excretion of α1CTX in breast cancer patients compared with those without bone metastases, (c) the potentials of this marker for the early detection of skeletal invasion (one to three metastases).

Materials and Methods

Subjects. The histologic analyses were done on seven iliac crest or transpedicular bone metastasis bone biopsies of the spine of metastatic breast cancer patients from archive material in Hamburg (G.D.). Specimens were fixed in buffered formalin (4%), decalcified by ultrasonic sound in combination with constant temperature at 24°C, and embedded in paraffin. This method is in use as routine process for immunohistochemical characterization of primary or secondary bone tumors.

The use of measuring urinary α1CTX for the detection of bone metastases was assessed in 90 breast cancer patients (45 without bone metastases and 45 with bone metastases). All patients were routinely scanned by radiography at the Cancer Institute Hospital in Tokyo, Japan (M.K.). Positives for bone metastases were verified by Tc99 bone scintigraphy together with computer tomography and/or magnetic resonance imaging to verify the presence and determine the number of
Figure 1. Histology and immunohistochemistry done on bone tumor secondary from primary breast cancer: (A) Pan-cytokeratin (CK-MNF, DAKO), (B) TRACP-positive staining of osteoclasts, (C) immunolocalization of αCTX epitopes, (D) control (adjacent sections), (E and F) area of woven and lamellar bone in the proximity of bone metastasis and αCTX staining, (G and H) area of healthy bone structure distant from the metastatic invasion with corresponding αCTX staining. Magnification, ×10 (A-D and G-H) and ×60 (E-F). E and G, bone microstructure (woven versus lamellar bone) under polarized light.
bone metastases as described previously by Soloway et al. (5). All patients with skeletal complications were newly diagnosed, and none of them had received therapies known to influence bone turnover for the past 2 years before entry to the study. All participants signed an approved written consent, and the studies were done in accordance with the Helsinki Declaration II and Standards of Good Clinical Practice. Local ethical committees have approved study protocols.

**Histology and Immunohistochemical Localization of αCTX in Bone.** Human bone specimens were decalcified and embedded in paraffin for preparation of sections with 5 µm in thickness. The sections were blocked in TBS containing 0.5% casein and incubated overnight at 4°C in a moist atmosphere with primary antibody diluted in TBS containing 0.5% casein or control without antibody. The primary antibodies employed were monoclonal; F44 raised against the αCTX epitope (Nordic Bioscience, Herlev, Denmark) and another raised against cytokeratin against pan-cytokeratin (CK-MNF). Osteoclasts were visualized by their specific TRAcP activity. After incubation, the sections were thoroughly washed in TBS and incubated with secondary peroxidase-labeled antibody (Mouse EnVision; DakoCytomation, Glostrup, Denmark) for 30 minutes at room temperature. Finally, the peroxidase activity was visualized using 3,3'-Diaminobenzidine Plus (Sigma, St. Louis, MO), and the nuclei were counterstained using Ehrlich's hematoxylin. The bone slices were then dehydrated through a gradient of alcohol (70-99%) and toluene and mounted in DPX. Pictures were taken using an Olympus BX-60 light microscope equipped with an Olympus C-5050 Zoom digital camera.

**Quantification of αCTX in Urine.** Second morning void urine samples were collected from all patients and stored at −40°C until analysis. The concentration of αCTX fragments was measured by the ALPHA CTX ELISA (Nordic Bioscience, Herlev, Denmark) following the manufacturer's recommendations. This assay is a sandwich-format EIA that uses monoclonal antibodies raised against α-EKAHDDGR (αCTX) and measures cross-linked chains (ααCTX). The monoclonal antibody is highly specific for the α-form of CTX with <1% cross-reactivity with the β-form of CTX.

Urinary excretion of ααCTX was corrected for creatinine excretion, which was measured by standard routine method. The short-term intra-individual variation of the ALPHA CTX ELISA, determined in 17 healthy postmenopausal women providing five consecutive samples during a 14-day observation period, was 17%.

**Statistical Analysis.** Demographics of subjects with or without bone metastases was compared with Student's t test. The concentrations of ααCTX were calculated relative to the creatinine concentration and transformed logarithmically to obtain normality. The General Linear Models Procedure was used for the regression analysis assessing the relationship between increases in ααCTX level (relative to patients without bone metastases) and the number of metastases. Model check included test for normality and probability and residual plots. The Statistical Analysis System (SAS, Cary, NC) was used for all calculations.

### Table 1. Demographic and ααCTX data in breast cancer patients stratified by ± bone metastases

<table>
<thead>
<tr>
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<th>BC – BM</th>
<th>BC + BM</th>
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<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Mean age, y (1 SD range)</td>
<td>53.6 (43.1-64.1)</td>
<td>53.1 (41.6-64.6)</td>
</tr>
<tr>
<td>Mean BMI, kg/m² (1 SD range)</td>
<td>22.0 (18.9-25.1)</td>
<td>23.4 (19.8-27.0)</td>
</tr>
<tr>
<td>Mean ααCTX, µg/mmol (1 SD range)</td>
<td>0.39 (0.17-0.86)</td>
<td>1.23 (0.52-2.92)</td>
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Abbreviations: BC, breast cancer; BM, bone metastases; BMI, body mass index.

### Results

**Histology and Immunohistochemical Localization of ααCTX in Bone.** ααCTX staining was seen throughout all seven bone sections in the presence of tumor cells and consequently a large number of active osteoclasts. Figure 1A to D shows adjacent sections from a bone invaded by breast cancer. Areas with hyperchromatic nuclei and positive cytokeratin staining confirmed the presence of malignant tissue in these sections (Fig. 1A). In the proximity of the tumor, TRAcP-positive staining revealed numerous osteoclasts characteristic for osteolytic lesions (Fig. 1B). In addition, immunostaining also revealed diffuse presence of ααCTX epitopes with more intensive staining at the sites of high bone remodeling (Fig. 1C). Control using no antibody showed no staining (Fig. 1D).

Figure 1E shows a magnified area of bone tissue with both woven and lamellar bone structure as differentiated by polarized light. Figure 1F indicates that ααCTX staining is confined to areas of woven but not lamellar bone. This latter is also illustrated by sections of fully normal bone structure: large areas of lamellar bone do not reveal presence of ααCTX epitopes (Fig. 1G and H).

**Urinary ααCTX in Breast Cancer Patients.** The demographic data of breast cancer patients with or without bone metastases has been published earlier (3). In brief, there were no statistically significant differences in terms of age or body mass index, but the urinary excretion of ααCTX was highly increased in patients with bone metastases (P < 0.001; Table 1). An aim of the study was to explore the utility of measuring ααCTX for the detection of metastatic invasion in breast cancer patients. Therefore, increases in urinary ααCTX in patients with bone metastases were expressed with reference to the mean value of patients without bone metastases. Figure 2 indicates the linear association between number of metastases and the relative increases in the urinary excretion of ααCTX in patients with distinctive metastases (n = 40; r = 0.56, P < 0.001): Superscan patients (75% of ribs, vertebrae an pelvic bone infected by bone metastases) were excluded. According to the model, the relative increases corresponding to the presence of one, two, and three metastases are estimated to be 38%, 57%, and 81%, respectively. We have previously reported that level and variance of urinary ααCTX in breast cancer patients without bone metastases and age-matched healthy controls are comparable (6).

### Discussion

The main result of the study were (a) the demonstration of the presence of ααCTX epitopes in bone tissue being in the close proximity of metastatic invasion and related osteolytic activity...
and (b) a significant association between urinary levels of αα-CTX and the extent of skeletal invasion (i.e., number of metastases) in breast cancer patients. The findings provide insights into why collagenous biomarkers, and in particular, αα-CTX is a valid marker for the detection of osteolytic metastases in breast cancer patients.

Breast carcinoma frequently metastasizes to specific organs, including lymph nodes, lung, and bone. The vast majority of bone metastases in breast cancer patients is osteolytic lesions (7), although mixed and osteoblastic metastases have also been observed (8, 9). The mechanisms of intercellular communication between bone and cancer cells have been recently proposed by Clines and Guise (10). Cytokines/hormones (interleukin-1, interleukin-6, and tumor necrosis factor-α) and parathyroid hormone-related protein secreted from breast cancer cells are known to stimulate receptor activator of nuclear factor-κB ligand and reduce osteoprotegrin expression in osteoblasts. This in turn leads to enhanced differentiation of osteoclast progenitor cells and formation/activation of osteoclasts. The result of these events is the disruption of normal bone remodeling with predominance of osteoclastic bone resorption-promoting osteolysis (11).

Secondary to the increased osteoclastic bone resorption, the activity of osteoblasts will also increase and facilitate continuous formation of new bone matrix at the margin of the metastasis. At the same time, growth factors (insulin-like growth factors, transforming growth factor-β, platelet-derived growth factor, and bone morphogenetic protein) and cytokines from osteoclasts enhance the growth and survival of tumor cells and further stimulate the expression of parathyroid hormone-related protein. These mechanisms will collectively result in the increase of a vicious cycle (12) driving the intensive remodeling. Presence of αα-CTX epitopes in bone tissue being in the proximity of a metastasis is a reflection of accelerated turnover of newly synthesized collagen type I. Another evidence of intensive remodeling is the lack of mature, lamellar structure in bone tissue revealing αα-CTX-staining. Importantly, in matured, lamellar bone tissue distant from the bone metastases, no αα-CTX staining can be detected. Thus, these findings indicate that αα-CTX is closely related to skeletal sites of intensive bone remodeling, such as bone being in the proximity of bone metastases.

Numerous efforts have been made to use biomarkers of bone formation, resorption, and osteoclastogenesis for the detection and quantification of bone metastasis from breast cancer. In our recent study (3), we provided further evidence that the collagenous bone resorption markers αα-CTX, ββ-CTX, NTX, and ICTP measured in serum or urine showed significant increases in breast cancer patients compared with those without bone metastasis. The largest relative increases were revealed by αα-CTX with increasing differences with the advancement of the metastatic disease. Our present findings indicated a significant association between the number of metastases and the increases in urinary αα-CTX relative to age- and body mass index–matched breast cancer patients without bone metastases. The estimated relative increases in αα-CTX corresponding to the presence of one, two, or three metastases were 2- to 5-fold higher than the intraindividual variation. Although the present study is of cross-sectional design, the aforementioned results also nurture the notion that the indicative value of an increase in αα-CTX levels for skeletal invasion can be improved when monitoring the same cancer patient with serial measurements.

In summary, our present analysis provide initial explanation to why αα-CTX is a promising marker for the first-line non-invasive diagnostic of skeletal invasion in patients with known breast cancer disease.

References
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