Lectin Affinity Electrophoresis of Serum Alkaline Phosphatase in Metastasized Breast Cancer

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The use of serum alkaline phosphatase (ALP) isoenzymes as markers of breast cancer metastases and treatment efficacy has received little attention. Twenty-six breast cancer women (56 ± 13 years, all post-menopausal) were prospectively evaluated during their first and third course of chemotherapy (4-week interval). Serum samples were analyzed for ALP isoenzymes (bone, liver, and intestine) using a lectin affinity electrophoresis kit (Hydragel 15 ISO-PAL[®], Sebia) adapted on a semi-automated Hydrasys[®] system (Sebia). Results were compared with imaging techniques for the presence of metastases; bone ALP isoenzyme (B-ALP) results were

compared with C-Terminal degradation products of type I collagen (S-CTX) (Cross-Laps[®], IDS Nordic). Serum B-ALP, but not S-CTX, confirmed the presence of bone metastases (BM) ($n = 15$) with 67/100% sensitivity/specificity (using a 69 UI/L ROC cut-off); ROC AUC was 0.806 ($P = 0.0004$) (NS for S-CTX). Chemotherapy reduced serum B-ALP by 24% over 4 weeks ($P = 0.0012$); there was no change for S-CTX. There was no specific clinical pattern for other ALP isoenzymes (liver and intestine). In conclusion, serum B-ALP, but not S-CTX, could help confirm the presence of BM in breast cancer patients. *J. Clin. Lab. Anal.* 24:20–24, 2010. © 2010 Wiley-Liss, Inc.

Key words: tumor markers; isoenzymes; breast cancer; metastases; chemotherapy

INTRODUCTION

Lymph node, bone, liver, and lung metastases are frequent in advanced breast cancer. Bone metastases (BM) represent up to 70% of cases, responsible for fractures, neuronal compression, and overall reduced quality of life. Biochemical markers of bone turnover have been evaluated for BM detection (currently done with ^{99m}Tc-labelled biphosphonate scintigraphy) with conflicting results (reviewed in (1)). BM often being of osteolytic, the most studied markers are the N- and C- (CTX) Terminal degradation products of type I collagen (1,2). Based on the essential role of osteoblasts in the metastatic process of bone destruction, bone formation markers might also be of interest (3,4). Alkaline phosphatase (ALP) (5) possesses limited bone specificity when total serum activity is measured. A large variety of physiological (such as normal growth, pregnancy), malignant (liver, bone, lymphoma) and non-malignant diseases (such as cirrhosis, hepatitis, Paget disease, diabetes, etc.) increase ALP serum levels. Tissue-specific isoforms are present in bone (B-ALP),

liver (L-ALP), kidney, intestine, and placenta. They have been proposed as potential tumor makers (6), but their electrophoretic separation remains challenging due to limited glycosylation and post-translational modifications. A sample pre-treatment (such as differential inactivation by heat or immuno-precipitation) is often required to isolate B-ALP. Launched in 2001, the Hydragel ISO-PAL kit (Sebia) (7) is based on wheat germ lectin (WGL) affinity for sialic acid residues of ALP isoforms, a concept first developed more than 30 years ago (8). This technique allows, in a single run, the separation and quantification of main ALP isoenzymes: bone (the highest sialated one) and two liver isoenzymes (L1 and L2) (7). Intestinal isoenzymes are

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found in only 40% of normal subjects and placental isoforms are present during pregnancy.

In this prospective study, total ALP and its isoenzymes were measured by WGL affinity electrophoresis in a group of women with metastasized breast cancer. Marker efficacy to detect BM and/or other metastases was compared with serum CTX (S-CTX), a marker previously validated by us for BM detection in breast cancer (9,10). All patients being treated by conventional chemotherapy (no biphosphonates or hormonal therapy), the effect of treatment on bone turnover was also investigated.

METHODS

Subjects and Samples

A group of 26 consecutive post-menopausal women with advanced breast cancer and hospitalized 24 hr for conventional chemotherapy prospectively entered this study (Table 1; exclusion criteria: renal failure, recent bone fracture). Post-menopausal status (physiological or secondary to treatment) was established by serum follicle stimulating hormone (FSH > 30 UI/L), luteinizing hormone (LH > 30 UI/L), and estradiol (E2 < 100 pmol/L) (9,10). Patients were separated into two groups according to the absence ($n = 11$) or the presence of BM ($n = 15$) identified by imaging techniques (X-rays or magnetic resonance imaging) and ^{99m}Tc scintigraphy; other metastases are listed in Table 1. Conventional chemotherapy was administered in a 6 courses/2-week interval protocol (Table 1).

To limit diurnal variations, blood samples were obtained after the first course of chemotherapy (just after drug infusion) between 11:00 and 13:00 (CTX nadir in serum) before lunch (11). Blood was drawn by

venous puncture in a 7 mL glass tube without anti-coagulant (Becton Dickinson, France). Following centrifugation at 3,000g for 10 min at +20°C, serum was separated in two aliquots and stored at -20°C until assay. A second blood sample was obtained in a subgroup of 18 patients after the third course of chemotherapy (1 month later).

This study was in accordance with the ethical standards of the Helsinki Declaration of 1975, revised in 1983. Informed consent was obtained from all participants and the trial was approved by the ethical committee of Saint Louis Hospital.

ALP Electrophoresis

ALP isoenzymes were separated by WGL affinity on alkaline buffered (pH 9.1) agarose (1 g/dL) gel (Hydragel 15 ISO-PAL[®] kit) using Hydrasys[®] semi-automated electrophoresis system (Sebia, France), as before (7). Each serum sample was run in duplicate with 10 μL applied in two adjacent wells. Migration conditions were as follows: 20 W constant power at 35° followed by 30° until 80 V hr have accumulated. From anode to cathode, the migration pattern (in the absence of WGL) is as follows: liver 1 (L1)+bone (B) (unseparated), liver 2 (L2), intestine 1 (I1), 2 (I2), and 3 (I3) (Fig. 1). In the presence of 10 μL of WGL deposited anodally from the sample's point of application, B-ALP is specifically precipitated during sample migration. Separated ALP fractions are visualized using a specific chromogenic substrate: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (in aminomethyl propanol buffer at pH

Fig. 1. Separation of serum alkaline phosphatase (ALP) isoenzymes by WGL affinity electrophoresis. ALP isoenzymes were separated by wheat germ lectin (WGL) affinity on alkaline buffered (pH 9.1) agarose (10 g/L) gel (Hydragel 15 ISO-PAL[®] kit, Hydrasys[®] electrophoresis system, Sebia, France) (7). Each serum sample was run in duplicate in two adjacent wells. From anode to cathode, the migration pattern (in the absence of WGL) is as follows: liver 1 (L1)+bone (B) (unseparated), liver 2 (L2), intestine 1 (I1), 2 (I2), and 3 (I3). Track 3 (without WGL) and 3' (with WGL): patient S. (59 years) with BM, total ALP = 424 IU/L, B-ALP = 225 IU/L.

TABLE 1. Patient Characteristics and Treatments Stratified by the Absence/Presence of Bone Metastases

	Without bone metastases (BM-)	With bone metastases (BM+)
Number	$n = 11$	$n = 15$
Age (years)	53 ± 14	58 ± 12 (NS)
Other metastases	None: $n = 2$	Lung: $n = 2$
	Lymph nodes: $n = 7$	Lymph nodes: $n = 1$
	Liver: $n = 1$	Liver: $n = 1$
	Lung: $n = 1$	Brain: $n = 1$
		Liver and lung: $n = 1$
Chemotherapy	Cyclophosphamide+ Epirubicine: $n = 8$	Cyclophosphamide+ Epirubicine: $n = 9$
	Epirubicine+Docetaxel: $n = 1$	Docetaxel+5-Fluorouracil: $n = 3$
	Ifosfamide+Gemcitabine: $n = 1$	Cyclophosphamide+ Docetaxel: $n = 2$
	Docetaxel: $n = 1$	Ifosfamide+Vinorelbine: $n = 1$

NS, not significant vs. BM-patients.

10.0) (incubation at 37° for 45 min and at 50° for 15 min). Quantification of electrophoretic ALP fractions (in %) was obtained by densitometric integration at 570 nm of the dried gel. In this approach, the value of the B-ALP is calculated by subtracting the densitometric value of L1 (lectin track) from the L1+B (track without lectin). All other ALP fractions are determined by densitometric integration on the track without lectin (Fig. 1). Results (in %) are then multiplied by total ALP activity (in IU/L) measured using a DGKC method on a Modular PP® system (Roche Diagnostics, France) (normal upper cut-off: 160 UI/L). According to the manufacturer, the detection limit for individual isoenzymes ranges from 2 to 3 IU/L with a linearity up to 600 IU/L (B-ALP) and 1,000 IU/L (L1). Interassay precision ($n = 10$ runs, CV) and normal values ($n = 50$ women, age: 20–50 years) were as follows: L1 (6.6%; <90 IU/L), B (4.1%; <88 IU/L), L2 (6.2%; <16 IU/L), and when present I1 (7.1%), I2 (3.5%), and I3 (13.9%; <16 IU/L).

S-CTX Analysis

S-CTX analysis was performed using the Serum CrossLaps™ kit (IDS Nordic, Denmark), as previously (10). This ELISA assay uses a micro-titer plate coated with streptavidin and monoclonal antibodies recognizing C-Telopeptide fragments of collagen type I $\alpha 1$ chains containing the epitope Glu-Lys-Ala-His-Asp- β -Gly-Gly-Arg in an isomerized form. Analyses of standards, control, and samples were all realized in duplicates. According to the manufacturer, the detection limit of this assay is 100 pmol/L. Intra and interassay precision was assessed using the 200 pmol/L control ($n = 20$ repeats) with CV = 7.2 and 9.6%, respectively. A 2,300 pmol/L upper normal cut-off was previously established by us in a group of 30 post-menopausal women (range: 46–65 years) with no history of medical disease and no treatment affecting bone metabolism (10).

Statistical Analysis

According to the Kolmogorov–Smirnov test, all data followed a normal distribution; results are thus expressed as mean and standard deviation. The Student t -test for independent data was used to assess differences between the presence of BM and the absence of BM for each marker. The effect of chemotherapy on serologic markers was evaluated by the Student t -test for paired data. For all statistical tests, a P -value of less than 0.05 indicated a statistical significance.

RESULTS

After the first course of chemotherapy marker results were for total ALP: 192 ± 85 IU/L (% of patients above normal cut-off: 54%), B-ALP: 73 ± 51 IU/L (15%), L1-ALP: 95 ± 41 IU/L (46%), L2-ALP: 18 ± 17 IU/L (35%), and S-CTX: $2,561 \pm 1,579$ pmol/L (54%), intestinal fractions were observed in 7 out of 26 subjects (1–15 IU/L). Patients with BM had significantly higher serum B-ALP than those without BM (94 ± 58 vs. 45 ± 19 IU/L, $P = 0.0137$); there was no difference for total ALP, L1-ALP, L2-ALP (data not shown), and S-CTX (Fig. 2). Efficacy (sensitivity/specificity) to confirm the presence of BM was for total ALP: 75/64%, B-ALP: 27/100%, and S-CTX: 53/45% (manufacturer or established normal cut-off). At the ROC optimized cut-off of 69 IU/L, B-ALP efficacy to confirm BM was 67/100% vs. 60/82% for total ALP (cut-off: 197 IU/L) and 40/100% for S-CTX (cut-off: 985 pmol/L). Significant ROC AUC was obtained for B-ALP (0.806, 95th confidence interval: 0.605–0.933, $P = 0.0004$) and total ALP (0.721, 0.512–0.877, $P = 0.0273$), but not for S-CTX (0.585, 0.377–0.773, NS). Liver ALP isoforms were elevated in one out of three patients with liver metastases. During the first three courses of chemotherapy, no changes were noted for total ALP, L1 and L2-ALP (data not shown), and S-CTX (Fig. 3A), but a significant –24% reduction for B-ALP ($P = 0.0035$, $n = 18$) (Fig. 3B).

DISCUSSION

Current clinical guidelines do not recommend the routine use of serum tumor markers in breast cancer, including makers of bone turnover, for BM detection (1). Owing to their limited sensitivity, that they cannot replace ^{99m}Tc scintigraphy for BM screening and early

Fig. 2. Effect of bone metastases on total ALP, bone ALP and S-CTX levels. Metastases to bone (BM) were identified by imaging techniques: absent ($n = 11$) or present ($n = 15$). Serum samples were obtained at the first course of chemotherapy (just after drug infusion) between 11:00 and 13:00. NS, not significant vs. patients without bone metastasis (BM).

Fig. 3. Individual impact of chemotherapy on bone ALP and S-CTX levels. Follow-up during chemotherapy was available in a subgroup of 18 patients. Individual results were obtained after the first (baseline) and the third course of chemotherapy (1-month interval) between 11:00 and 13:00 just after drug infusion. (A): bone-ALP levels, $P = 0.0035$ between course #1 and #2. (B): S-CTX levels, NS, not significant; each symbol/line represents an individual patient.

diagnosis. However, imaging techniques are labor intensive, exposed to radiation and are thus limited in the number per year. Biochemical markers of bone turnover are non-invasive and can be determined at low cost on a regular basis. Some authors (1) suggest that they could be used together with imaging techniques to improve the detection and follow-up of BM in breast cancer patients.

In this study, bone formation marker B-ALP was the only biochemical marker significantly elevated in patients with BM (+109% compared with patients free of BM); total ALP almost reached statistical significance (+41%, $P = 0.0562$). As expected, B-ALP was superior to total ALP for the detection of BM both in terms of ROC AUC and diagnostic performance. The manufacturer B-ALP cut-off was, however, clearly not adapted to the present post-menopausal population with only 27% sensitivity for BM. Using an ROC analysis approach, this cut-off was lowered to 69 IU/L with a

clinically more relevant (but still limited) 67/100% sensitivity/specificity. In a group of patients not receiving concomitant hormonal treatment (12), the area under the ROC curve was 0.822 for B-ALP vs. 0.724 for NTX; using a cut-off value of 50.0 U/L, B-ALP specificity and sensitivity were 92.3 and 70.6%, close to our results. It should be emphasized, however, that B-ALP (as other serologic markers) cannot be used for the localization of bone metastasis, a factor of considerable importance for prognosis. Recently published retrospective data (3) suggest, however, that B-ALP (and also NTX) could predict future skeletal events (such as fracture, medullar compression), disease progression and even death in breast cancer patients treated by biphosphonates. For example, a B-ALP serum level >146 IU/L was associated with a higher relative risk (RR) of breast cancer progression (RR = 1.77, $P = 0.0004$) and death (RR = 2.23, $P < 0.001$) (3). A strong correlation was observed between the extent of skeletal infiltration and six different markers of bone turnover (including B-ALP and S-CTX) in patients with breast ($n = 90$) or prostate cancer ($n = 42$) (2). In advanced breast cancer, specific treatments of BM are initiated to reduce the risk of pathological fractures (and other skeletal-related events) and to provide pain control. In this study, B-ALP (but not S-CTX and total ALP) responded to chemotherapy (24% decrease over 30 days). In a group of hormone refractory prostate cancer patients ($n = 40$), S-CTX and total ALP did not decrease with Docetaxel therapy in patients without BM (13). However, it decreased with Docetaxel/Zoledronate treatment in those with BM (13). In $n = 73$ breast cancer patients with BM under antitumor therapy and supportive pamidronate treatment, ALP was the only parameter which normalized more often in patients with clinical benefit as compared to progressive patients (14). Our results suggest that conventional chemotherapy might have a negative impact on bone tissue formation, without effect on bone resorption (no change for S-CTX) (see below).

Our results surprisingly suggest that S-CTX could not be reliably used to confirm the presence of BM regardless of the cut-off selected (ROC analysis), at least in our group of post-menopausal women. S-CTX was even not superior to total ALP, a non-specific marker of bone turnover. Extremely elevated S-CTX levels were found in patients without BM (6,258–6,698 pmol/L in a 40 year woman, over the first three courses of chemotherapy). These results contrast with some of our previously published data using serum (10) and urinary CTX (9). Post-menopausal patients with BM exhibited a +150% increase of S-CTX and urinary CTX compared with those without BM. In post-menopausal women, a difficulty is that the elevation of

bone resorption markers can also be secondary to osteoporosis (15). This might explain the lack of S-CTX efficacy to confirm the presence of BM in this study. The use of S-CTX is also limited by marked diurnal variations, high-interindividual biological variability (influence of age, hormonal status, kidney function) and requires a strict fasting state before sampling (9–11,16). Both diurnal and intraindividual variations have been reported to be more pronounced when CTX was measured in serum rather than urine (9–11). By contrast, bone formation markers, including ALP and osteocalcin, showed little or no circadian rhythm in breast cancer patients (16). Recently, urinary CTX in non-isomerized form $\alpha\alpha$ (an index of bone resorption of newly formed bone) showed the highest dynamic response of eight markers (increase $> 600\%$ increase in those with BM vs. absence of BM) (2). In our previous study (10), S-CTX was found to respond to chemotherapy, but mostly after the sixth course (2-month interval). Using urinary CTX (9), a slow decrease was noted during the course of chemotherapy infusions in women with bone involvement. At the end of the 6 cycles, urinary CTX values were similar to those recorded in women without BM. In nine pre-menopausal breast cancer patients with no distant metastases at baseline, S-CTX concentrations did not vary between baselines, the 4th and after the 9th chemotherapy cycle 998 ± 605 , 886 ± 562 , and $1,473 \pm 1,102$ pmol/L (NS) (17).

These preliminary data suggest that B-ALP could be used to confirm the presence of BM in breast cancer patients. Chemotherapy had a significant, but limited, negative impact on bone formation marker B-ALP. The resorption marker S-CTX (isomerized form) seems to be of limited value in this indication, at least in post-menopausal women.

REFERENCES

1. Tanko LB, Karsdal MA, Christiansen C, Leeming DJ. Biochemical approach to the detection and monitoring of metastatic bone disease: What do we know and what questions need answers? *Cancer Metastasis Rev* 2006;25:659.
2. Leeming DJ, Koizumi M, Byrjalsen I, Li B, Qvist P, Tanko LB. The relative use of eight collagenous and noncollagenous markers for diagnosis of skeletal metastases in breast, prostate, or lung cancer patients. *Cancer Epidemiol Biomarkers* 2006;15:32.
3. Coleman RE, Major P, Lipton A, et al. Predictive value of bone resorption and formation markers in cancer patients with bone metastases receiving the bisphosphonate zoledronic acid. *J Clin Oncol* 2005;23:4925.
4. Palma MA, Body JJ. Usefulness of bone formation markers in breast cancer. *Int J Biol Markers* 2005;20:146.
5. Moss DW. Alkaline phosphatase isoenzymes. *Clin Chem* 1982;28:2007.
6. Van Hoof VO, Van Oosterom AT, Lepoutre LG, De Broe ME. Alkaline phosphatase isoenzyme patterns in malignant disease. *Clin Chem* 1992;38:2546.
7. Gilles C, Moley M, Benlakehal M, Bousquet B, Le Bricon T. Séparation électrophorétique des isoenzymes de la phosphatase alcaline par le Kit Hydragel 15 Iso-PAL[®]. *Immunoanalyse et Biologie spécialisée* 2002;17:395.
8. Onica D, Sundblad L, Waldenlind L. Affinity electrophoresis of human serum alkaline phosphatase isoenzymes in agarose gel containing lectin. *Clin Chim Acta* 1986;155:285.
9. Houzé P, Bellik B, Extra JM, Bouro F, Bousquet B. Urinary carboxyterminal telopeptide of collagen I as a potential marker of bone metastases chemotherapy monitoring in breast cancer. *Clin Chim Acta* 1999;281:77.
10. Houzé P, Djerad A, Cottu PH, Bouro F, Bousquet B. Dosage sérique du C-télopeptide du collagène de type I: Intérêt dans le suivi des métastases osseuses du cancer du sein. *Immunoanalyse et Biologie Spécialisée* 2000;15:414.
11. Wichers M, Schmidt E, Bidlingmaier F, Klingmüller D. Diurnal rhythm of Crosslaps in human serum. *Clin Chem* 1999;45:1858.
12. Pectasides D, Farmakis D, Nikolaou M, et al. Diagnostic value of bone remodeling markers in the diagnosis of bone metastases in patients with breast cancer. *J Pharm Biomed Anal* 2005;37:171.
13. Leeming DJ, Hegele A, Byrjalsen I, et al. Biochemical markers for monitoring response to therapy: Evidence for higher bone specificity by a novel marker compared with routine markers. *Cancer Epidemiol Biomarkers Prev* 2008;17:1269.
14. Lüttner D, Richter A, Geppert R, Wernecke KD, Possinger K. Normalisation of biochemical markers of bone formation correlates with clinical benefit from therapy in metastatic breast cancer. *Anticancer Res* 2003;23:1017.
15. Koizumi M, Takahashi S, Ogata R. Bone metabolic marker in bone metastasis of breast cancer. *Int J Clin Oncol* 1999;4:241.
16. Generali DG, Tedoldi S, Tampellini M, et al. Circadian rhythm of bone turnover markers in breast cancer patients with bone metastases and in control subjects. *J Clin Oncol* 2005;23:737.
17. Kailajärvi ME, Salminen EK, Pajja OM, Virtanen AM, Leino AE, Irjala KA. Serum bone markers in breast cancer patients during 5-fluorouracil, epirubicin and cyclophosphamide (FEC) therapy. *Anticancer Res* 2004;24:1271.