Serum Bone Sialoprotein in Patients with Primary Breast Cancer Is a Prognostic Marker for Subsequent Bone Metastasis

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Abstract

Bone sialoprotein (BSP) is a noncollagenous bone matrix protein that is important for both mineralization and cell-cell interactions. Tissue studies in primary breast cancers have shown that immunohistochemical expression of BSP is associated with a high incidence of bone metastases in the course of the disease. We used a RIA to investigate the importance of serum BSP as a marker for subsequent bone metastases. Between 1994 and 1996, preoperative blood samples were collected from 388 consecutive patients with nonmetastatic breast cancer and from 30 control patients with benign breast disease. Serum BSP concentrations were measured in a blinded fashion by RIA. The cutoff for elevated serum BSP values was 24 ng/ml, i.e., two SDs above the normal mean value. Serum BSP was correlated with the risk of metastasis and analyzed with regard to its prognostic value. After a median follow-up period of only 20 months, 28 patients had developed metastases. Fourteen patients had bone metastases only, 9 visceral metastases only, and 5 a combination of osseous and visceral metastases. Of the 19 women with skeletal metastases, 17 had preoperative serum BSP values in excess of 24 ng/ml (median BSP values: 48.3 ng/ml for isolated metastatic bone disease, 30.6 ng/ml for combined metastases), whereas none of the women with visceral metastases only had elevated serum BSP concentrations (median BSP value: 12.3 ng/ml). The median serum BSP value in the control group (benign breast disease) was 8.8 ng/ml serum BSP; levels correlated with the size of the primary tumor and other prognostic factors. Using a multivariate regression analysis, serum BSP was found to be the most important independent prognostic factor for the development of skeletal metastasis (P < 0.001; relative risk, 94); its specificity was 96.7%, and its sensitivity was 89.5%. Our study shows that patients with preoperatively elevated serum BSP levels are at high risk of subsequent bone metastases in the first years after primary surgery. The mechanism of BSP in the pathogenesis of skeletal metastases is unclear. Because BSP contains an integrin recognition sequence, its expression in tumor cells may facilitate their adhesion to the bone surface. However, it is possible that a proportion of circulation BSP is derived from normal or tumor-induced bone turnover. Breast cancer patients with elevated serum BSP levels may benefit from osteoprotective adjuvant therapy with bisphosphonates.

Introduction

Bone metastases commonly occur in the course of malignant tumor disease. Although any tumor may metastasize to bone, only five solid tumors (carcinomas of the breast, prostate, lung, kidney, and thyroid) are responsible for >80% of all skeletal metastases. Approximately 75% of women who die of breast cancer display bone metastases at autopsy (1, 2).

For many years, attempts have been made to identify factors for metastatic organ selection in breast cancer to understand the pathogenetic processes of skeletal metastasis. It is known, for example, that well-differentiated and/or steroid receptor-positive breast tumors are more likely to produce skeletal metastases (3–5). Furthermore, there is a correlation between immunohistochemical detection of PTHrP2 in the primary tumor and osteotropic metastasis (6, 7).

BSP is a glycosylated and phosphorylated protein with a high content of sialic acid (15% of carbohydrates). Like osteocalcin and osteopontin, BSP is one of the noncollagenous proteins in the extracellular bone matrix. It plays an important role in the mineralization and in the adhesion of osteoclasts to the bone surface (8–13). In the last few years, there has been increasing evidence that breast cancers that express BSP immunohistochemically metastasize to bone more frequently than BSP-negative tumors (14, 15).

Recently, a specific RIA has been developed for the measurement of human BSP in serum (16, 17). The aim of our study was to determine serum BSP preoperatively in patients with primary breast cancer and to assess its value as a prognostic factor for subsequent metastatic disease.

Patients and Methods

Patients. The study was carried out in 388 consecutive patients with primary breast cancer who underwent surgery at the Women’s Hospital of the University of Heidelberg between October 1994 and October 1996. The inclusion criteria were histologically confirmed breast cancer (T1–T4, N0–N2, M0) and the consent of the patient to preoperative blood collection. The exclusion criteria were diagnosis of distant metastases in the perioperative period, previous or simultaneous secondary malignant disease, neoadjuvant chemotherapy or hormone therapy,

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2 The abbreviations used are: PTHrP, parathyroid hormone-related protein; BSP, bone sialoprotein; RR, relative risk; UIICC, Union International Contre Cancer.
known metabolic bone disease (including clinically apparent osteoporosis with fractures), therapy with substances with a pronounced effect on bone metabolism (exception: calcium supplements and hormone replacement therapy with estrogens/progestins), severe disturbances of liver and kidney function, and pregnancy. To exclude apparent distant metastases, liver ultrasounds, chest X-rays, and bone scans were performed in all patients perioperatively. Computed tomography was carried out in the event of suspicious skeletal findings. Patients with suspected or evident bone disease were excluded from the analysis.

The control group consisted of 30 consecutive patients with histologically confirmed benign breast lesions (21 fibroma/fibroadenoma, 6 cystic mastopathy, and 3 papilloma).

**Sample Collection**

Immediately before anesthesia and surgery (between 8 and 12 a.m.), 10 ml of blood were collected from all 418 patients via the cubital vein using a syringe without additive. The blood was allowed to clot for 30–45 min at room temperature and then centrifuged immediately (1000 g) for 10 min. Aliquots of serum were frozen at −20°C within 2 h of collection.

**Determination of BSP in Serum**

The serum concentrations of human BSP were determined by an RIA (Immundiagnostik, Bensheim, Germany) using a polyclonal chicken antibody against purified human BSP (16–19). In brief, 100 μl of serum were mixed with 100 μl of chicken antihuman BSP antibody (dilution, 1:200) and 100 μl of 125I-labeled BSP. After incubation for 24 h at 4°C, 100 μl of second-antibody solution (raised in donkeys against chicken IgY; 1:15) were added. After incubating for an additional 2 h at 4°C, the reaction mixture (containing antibody-bound radioactivity) was centrifuged for 10 min at 2000 × g. The supernatant was discarded, and the radiactive pellet was washed with 250 μl of an aqueous buffer (60 g/l polyethylene glycol and 9 g/l sodium chloride) before being centrifuged again (10 min at 2000 × g). After aspiration of the supernatant, the radioactivity in the pellet was measured in a Berthold gamma counter for 1 min.

The results were calculated by interpolation of the unknown samples with a calibration curve (constructed by use of a four-parameter curve-fitting algorithm). The intraassay coefficient of variation was 5.3% (mean concentration, 9.8 ng/ml; n = 20). The lower limit of detection in the present RIA (determined by 20 replicate analyses of the zero calibrator and calculation of the concentration corresponding to the 95th percentile of the counts obtained) was 0.7 ng/ml. The assay showed a recovery rate of 99.4% (range, 92–108%). All analyses were performed in duplicate within a single run. The normal serum BSP value is 9.0 ± 3.8 ng/ml in premenopausal women and 13.3 ± 4.8 ng/ml in postmenopausal women (n = 90; age, 20–80 years; Ref. 16). A cutoff for normal BSP values of 24 ng/ml was selected for the statistical analysis of the 418 serum samples from our patients, which corresponds to 2 SDs above the normal value for postmenopausal women.

**Therapy of the Patients**

The primary surgical therapy consisted of either breast-conserving measures (lumpectomy or segmental resection plus 50 Gy of radiotherapy to the breast) or modified radical mastectomy for large tumors and multicentric/multifocal lesions. Axillary lymph node dissection (levels I and II) was performed in all patients. Tumor size was collected from the primary tumor for determination of steroid hormone receptors and S-phase fraction and for tumor grading.

**Table 1 Clinical and pathological characteristics of 388 breast cancer patients**

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>No. of patients</th>
<th>BSP ≥ 24</th>
<th>BSP &lt; 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>195</td>
<td>8</td>
<td>187</td>
</tr>
<tr>
<td>T2</td>
<td>156</td>
<td>15 (10)</td>
<td>141</td>
</tr>
<tr>
<td>T3</td>
<td>25</td>
<td>2 (8)</td>
<td>23</td>
</tr>
<tr>
<td>T4</td>
<td>12</td>
<td>4 (33)</td>
<td>8</td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>252</td>
<td>16 (6)</td>
<td>236</td>
</tr>
<tr>
<td>N+</td>
<td>136</td>
<td>13 (9)</td>
<td>123</td>
</tr>
<tr>
<td>Estrogen receptor (n = 357)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>226</td>
<td>18 (8)</td>
<td>208</td>
</tr>
<tr>
<td>Negative</td>
<td>146</td>
<td>11 (7)</td>
<td>120</td>
</tr>
<tr>
<td>Progesterone receptor (n = 358)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>228</td>
<td>19 (8)</td>
<td>209</td>
</tr>
<tr>
<td>Negative</td>
<td>130</td>
<td>10 (8)</td>
<td>120</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>127</td>
<td>8 (6)</td>
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<tr>
<td>Post</td>
<td>261</td>
<td>21 (8)</td>
<td>240</td>
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<tr>
<td>Grading (n = 377)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>247</td>
<td>19 (8)</td>
<td>228</td>
</tr>
<tr>
<td>III</td>
<td>130</td>
<td>10 (8)</td>
<td>120</td>
</tr>
<tr>
<td>S-phase fraction (n = 358)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5%</td>
<td>106</td>
<td>11 (10)</td>
<td>95</td>
</tr>
<tr>
<td>≥ 5%</td>
<td>252</td>
<td>17 (7)</td>
<td>235</td>
</tr>
<tr>
<td>Osteocalcin (n = 264)</td>
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<td></td>
</tr>
<tr>
<td>&lt; 15 ng/ml</td>
<td>111</td>
<td>7 (6)</td>
<td>104</td>
</tr>
<tr>
<td>≥ 15 ng/ml</td>
<td>153</td>
<td>9 (1)</td>
<td>144</td>
</tr>
</tbody>
</table>

* Tumor staging according to UICC criteria.

**Follow-Up**

Follow-up investigations were carried out at the Women’s Hospital of the University of Heidelberg. The interval between the investigations was 2–4 months in the first 2 years. Every visit consisted of history and physical examination, whereas chest X-ray, bone scan, liver ultrasound, and mammography were performed yearly. Laboratory tests (blood count, serum tumor antigens, and tumor markers) were carried out at various intervals. If there was evidence of bone metastases, additional X-rays were taken of the areas affected. The pattern of metastasis was analyzed at the end of the study. Bone lesions seen on radiograms were assessed by two independent radiologists.
Statistical Analysis

The association of BSP values < and >24 ng/ml with established prognostic factors was analyzed by \( \chi^2 \) tests for contingency tables. Survival methods were applied to bone metastasis-free survival (defined as survival without the development of bony metastases). The survival curve was calculated by the Kaplan-Meier method, based on the log-rank test according to Mantel and Breslow. A stepwise multivariate Cox regression analysis was performed to assess the independent prognostic value of serum BSP in comparison with other prognostic factors. The impact of each variable in the Cox regression model was tested by the Wald \( \chi^2 \) test and described by the risk ratio (i.e., the hazard ratio). All reported \( P \)s are two-sided. The statistical analysis was done with the aid of SAS (SAS Institute, Inc., Cary, NC) and Systat (Evanston, IL) software.

Results

Characteristics of the Breast Cancer Patients. The median age of the patients was 55 years (range, 24–82). The clinical and pathological characteristics of all 388 patients with breast cancer are shown in Table 1. One hundred and ninety-five women (50%) had a primary tumor 2 cm in diameter (T1). In 156 patients (40%), the tumor had a diameter of between 2 and 5 cm, and 37 women (10%) had T3 or T4 tumors. There was no axillary involvement in 252 patients (65%), whereas 136 (35%) had histologically confirmed metastases in the axillary lymph nodes. Of 357 primary tumors examined, 226 (63%) were estrogen receptor positive; 228
of 358 tumors tested (64%) were progesterone receptor positive. A total of 127 patients (33%) were premenopausal; 261 (67%) were postmenopausal. With regard to grading, 247 of 377 tumors examined (66%) were assessed as grade I or II, and 130 (34%) were grade III. The S-phase fraction was $5% in 106 of 358 tumors (30%) and $5% in 252 cases (70%).

Results of Preoperative Serum BSP Determinations. The mean value for serum BSP for all patients with breast cancer was 14.1 ng/ml (range, 1.9–152.9). There were 29 patients (7.5%) with a value in excess of 24 ng/ml. The mean value for serum BSP in patients with benign breast disease was 8.8 ng/ml (range, 3.2–16.3).

Using the $^2$ test, a significant difference between patients with serum BSP values $\geq 24$ ng/ml and those $<24$ ng/ml with established prognostic factors was only seen for tumor size ($P = 0.001$; Table 1). Notably, the highest serum BSP values were seen in patients with T1 and T2 tumors (Table 2). There were no significant differences for nodal status ($P = 0.251$), estrogen-receptor content ($P = 0.885$), progesterone-receptor content ($P = 0.831$), tumor grading ($P = 1.0$), S-phase fraction ($P = 0.243$), or for menopausal status ($P = 0.539$).

Surgical and Adjuvant Systemic Treatment. Breast conserving therapy was possible in 261 of 388 patients (67%); mastectomy was performed in 127 patients (33%). Almost all patients ($n = 377$, 97%) received adjuvant systemic treatment (Table 3). Endocrine therapy was given to 72% (21 of 29) of the patients with a BSP value $\geq 24$ ng/ml and to 62% (223 of 359) of the patients with a BSP value $\leq 24$ ng/ml. Cytotoxic chemotherapy was used in 28% (8 of 29) of the patients with elevated BSP levels and in 30% (106 of 359) of those with normal BSP. The 19 patients (5%) who received a combination of chemotherapy and hormone therapy and the 11 women (3%) who received no systemic therapy all had normal BSP levels.

Follow-Up and Pattern of Metastasis. During the median observation period of 20 months, distant metastases were detected in 28 patients. Metastases to bone only were seen in 14 patients, 9 women developed purely visceral metastases (liver, lungs, and...
Serum Bone Sialoprotein in Primary Breast Cancer

**Discussion**

In our investigation of the prognostic value of serum BSP in patients with primary breast cancer, we were able to show that patients with BSP values in excess of two SDs of the normal value for postmenopausal patients (>24 ng/ml) were at an enormous risk of developing skeletal metastases in the first 2–3 years after the diagnosis of disease. This is in keeping with previous immunohistochemical studies indicating an unfavorable prognosis for women with breast cancer in which the primary tumor demonstrated positive immunoreactivity for BSP (14, 15, 20, 21). The significance of the present observations, however, lies in the use of a specific and sensitive RIA that is simple to use in clinical routine and functions without the subjective assessment of immunohistochemical staining. The method also offers the possibility of carrying out follow-up determinations.

BSP, a phosphorylated glycoprotein with a molecular weight of about 70,000–80,000, accounts for ~10% of noncollagenous bone matrix proteins (8–10). BSP and its mRNA has been demonstrated in osteoclasts and osteoblasts but also in trophoblastic cells and in platelets. BSP contains an Arg-Gly-Asp (RGD) integrin recognition sequence. It improves the attachment of osteoclasts and osteoblasts, nucleates hydroxyapatite formation, and appears to enhance osteoclast-mediated bone resorption (22–33). The fact that microcalcifications in breast cancers consist of hydroxyapatite as well as calcium oxalate was the rationale for investigating BSP and other bone matrix proteins in primary tumors in breast cancer patients (34).

Ultimately, bone metastases are induced by the same mechanisms as all other metastases. There are, however, mutual interactions between tumor cells and the skeleton that determine organ selectivity. From *in vitro* studies by Mundy et al. (35) and Orr et al. (36), it is known that substances released from resorbing bone have a chemotactic effect on tumor cells. Furthermore, tumor cells that reach bone require adhesion molecules to become attached to the bone surface. BSP, with its integrin recognition RGD sequence, may play a part in the adhesion process. This has already been demonstrated for BSP-modulated adhesion of osteoclasts and osteoblasts (13, 29, 37, 38). It is possible that tumor cells are primed and coated with BSP in the primary tumor and are then able to adhere and bind to the bone surface. In cell attachment assays, Van der Pluijm et al. (39) have been able to show that the adhesion of breast cancer cell lines to bone surfaces could be successfully inhibited by application of BSP peptides.

To a large extent, the marked elevations in serum BSP seen in our study were probably attributable to paraneoplastic production by the primary tumor. This hypothesis is supported by the positive correlation between BSP and the size of the primary tumor (Table 1). However, circulating BSP may also be derived in part from normal or abnormal bone turnover. Seibel et al. (18) showed that serum BSP levels were elevated in patients with breast cancer and skeletal metastases, multiple myeloma, Paget’s disease, and primary hyperparathyroidism. In our study, an increase in bone turnover could be caused, for example, by paraneoplastic production of PTHrP, as has been reported in breast cancer in a number of studies (6, 7, 40, 41). Further studies including repeated measurement of BSP after primary surgery are presently under way to confirm this hypothesis.

Despite the fact that osteotropic metastasis in breast cancer has long been recognized, there is still no reliable marker to predict an elevated risk of subsequent bone metastasis. Although it is known that well-differentiated breast cancers (grade I and II) are associated with bone metastasis more frequently than poorly differentiated tumors (grade III), and although the same is true of primary tumors with a high steroid receptor content, these factors have no predictive impact for skeletal metastasis (3–5). It is also known that tumors that are immunoreactive to PTHrP also preferentially metastasize to bone. The presently available serum tests for PTHrP are not sufficiently sensitive to replace immunohistochemical detection. Elevated PTHrP values in serum can only be reliably detected in patients with humoral hypercalcemia of malignancy (42).

The determination of serum BSP might provide a reliable marker for subsequent bone metastasis. However, it should be pointed out that in our group of 388 patients, skeletal metastasis in the further course of the disease would be expected in 80–100 cases. In contrast, there were only 29 women with BSP values in excess of 24 ng/ml, which corresponds to only about a third of the number expected to develop bone metastases. Of these 29 patients, 17 (60%) already had osseous metastases after a median follow-up of only 2 years. We therefore suspect that BSP measurements at the time of surgery are primarily predictive of early osseous metastasis, and there may be different pathways for early and late bone metastases. Renewed follow-up in 1–2 years will provide a more exact answer to this question.

There is no doubt that breast cancer patients with elevated serum BSP constitute a high-risk group that might benefit from early treatment with osteoprotective drugs. A promising group of such substances are the bisphosphonates. In a recently published study, we showed that breast cancer patients who received adjuvant therapy with the bisphosphonate clodronate (1600 mg/day p.o. for 2 years) developed fewer bone metastases and fewer visceral metastases than patients in a control group (43). A further placebo-controlled study in more than 1000 patients confirmed our findings, at least with regard to the prevention of bone metastases (44).
References

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