

Expression of Bone Morphogenetic Proteins in Human Metastatic Prostate and Breast Cancer

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- Aim** To analyze the expression of bone morphogenetic proteins (BMPs) in prostate and breast cancers with established metastasis in bone, where prostate cancer causes osteoblastic metastases, and breast cancer osteolytic metastases.
- Methods** Primary tumor specimens from 20 patients with prostate cancer and 15 with breast cancer were studied for BMP-2/4, -3, -5, -6 and -7 immunohistochemistry. All patients had multiple bone metastases proven by bone scan. We also examined BMPs expression in normal prostate and breast tissues. BMPs expression was compared with clinicopathological and biochemical parameters.
- Results** Cytoplasmic BMPs immunostaining was observed in both prostate cancer and normal prostate tissue. Expression of BMP-2/4, -5, -6, and -7 proteins was detected in all normal prostate samples, with the predominance of BMP-2/4 ($87.8 \pm 11.4\%$ positive cells) and BMP-7 ($94.6 \pm 0.9\%$ positive cells). In prostate cancer tissues, we found variable expression of all BMPs. BMP-2/4 ($83 \pm 11.6\%$ positive cells) was predominantly expressed in prostate carcinoma, whereas the expression of BMP-7 ($24.3 \pm 19.2\%$ positive cells) was significantly lower than in the normal prostate. In all breast cancers tissues, we found nuclear staining only for BMP-7. In normal breast tissue, the BMP expression was not detectable. The percent of BMP-7 positive cells in breast cancer ($86.4 \pm 7.3\%$) was higher than in prostatic cancer. Comparing BMP expression levels and clinicopathological parameters, we did not find statistical difference, except for serum alkaline phosphatase, which was significantly higher in patients with prostate cancer.
- Conclusion** The expression of BMPs differs between prostate and breast cancer cells. Identifying the BMP proteins in cancers may be useful for monitoring the tumor status with reference to metastases.

Prostate and breast cancers frequently metastasize to bone (1,2). When an excessive amount of new bone formation takes place, the lesion is described as osteoblastic or osteosclerotic. Human prostatic adenocarcinoma produces osteoblastic metastases in bone in approximately 90% of cases (3). Conversely, the majority of bone secondaries from breast cancers are osteolytic lesions with increased bone resorption and osteoclastic activity (4,5). The mechanisms of the metastatic

process to bone are poorly understood. Paget's theory of metastasis suggests that the migration of cancer cells to bone may result from the adhesion and growth properties of cancer cells (6). Exploring the origin of the prostate and breast carcinoma bone metastasis, Jacob et al (7) suggested that specific homing factors therein facilitated cancer growth in bone. Previous studies have demonstrated that several growth factors are known to stimulate bone formation: transforming growth

factor (TGF)- β , fibroblast growth factor (FGF), insulin growth factor (IGF), epidermal growth factor (EGF), and bone morphogenetic proteins (BMPs), are expressed in benign and malignant prostate samples, as well as in malignant breast cells (8,9).

An important group of bone-inducing factors are the bone morphogenetic proteins (BMPs), which have the capacity to induce new bone formation *in vivo* (10-12). Fifteen BMPs are currently recognized, and with the exception of BMP-1, all are members of the TGF- β superfamily (13). BMPs stimulate the replication and differentiation of normal cells of the osteoblast lineage. Nevertheless, they are not only restricted to the bone tissue. BMPs play a critical role during embryogenesis in the process of mesoderm induction, neural tissue differentiation, and morphogenesis of various tissues (14,15). Finally, several studies demonstrated that solid tumors also express BMPs (16-20).

Recent research showed different BMP expression in human benign and malignant prostatic tissue. Bentley et al (21) demonstrated that BMPs-1 to -6 were expressed in human prostatic adenocarcinoma and they were the first to suggest that BMPs may have a role in the formation of skeletal metastasis in prostate cancer. BMP-6 was strongly expressed in the majority of skeletal metastases from prostate carcinoma and was introduced as a potential mediator of osteoblastic metastases in prostatic cancer (3,21-24). Other studies have investigated BMP expression in normal human and rat prostate and in prostate cancer cell lines demonstrating various patterns of gene and protein expression (25,26). BMPs are also expressed in the fetal and postnatal mammary gland, as well as in breast cancer (27). BMP-6 expression was found in normal breast tissue, breast cancer, and cancer cells lines (28). Arnold et al (29) detected expression of BMP-2, -3, -5, and -6 in breast carcinoma cells, whereas Schwalbe et al (30) reported BMP-7 expression in these cells and suggested that BMP-7 was associated with the differentiation of carcinoma cells.

Because of the unique roles of BMPs in the formation of new bone and their expression in prostate and breast carcinoma tissues, we think that BMPs may play role in the formation of different metastatic bone lesions. The aim of the study was to investigate the expression of BMP-2, -3, -4,

-5, -6, and -7 in cancers with established bone metastases of different types.

Materials and Methods

Clinicopathological Data

Clinicopathological data were obtained from patients' medical records at the Surgical Department, Rijeka University Hospital Center, and from pathologic reports. The study included patients with prostate and breast cancer with established skeletal metastases. Archival tissue samples of prostatic and breast cancer were obtained from the Department of Pathology, Rijeka University School of Medicine. Normal tissue samples were obtained from autopsy cases or non-malignant lesions and were used as controls. Tissue specimens were fixed in 4% buffered formalin, embedded in paraffin, and routinely stained with hematoxylin and eosin.

Twenty-five male patients, aged 54 to 81 years (mean \pm SD, 67.2 ± 6.2) were studied. Five presented normal prostate glandular tissue and 20 had clinically confirmed prostate cancers. Clinical staging of prostate cancer patients was determined on the basis of digital rectal examination, serum prostate specific antigen (PSA) estimation, transrectal needle core biopsy, and isotope bone scanning, which verified the presence of multiple skeletal metastases. All studied cancer samples showed extracapsular tumor extension, regional lymph node metastases, and distant bone metastasis. The nature of each prostate tissue biopsy sample was confirmed by standard histological examination and graded using Gleason scoring system according to the World Health Organization (WHO) classification (31).

Twenty female patients with breast carcinoma, aged from 48 to 77 years (62.2 ± 10.8) were included in the study. Out of them, 15 cases were an invasive ductal carcinoma with bone metastasis; and 5 non-tumor cases were used as control. All women were postmenopausal. Tissue sections were examined by two independent pathologists (E. M. and G. D.) to confirm the presence of malignant tissue and to be validated histopathologically, using the classification of breast tumors according to the WHO (32). The size of the tumors, lymph node status, and histological and nuclear grade were recorded. For histological grading, we used the three-tier systems for describing tumor structure in terms of tubule formation, nu-

clear grade, and mitotic count. Each element was scored on a scale from 1 to 3 and the final grade was determined by the sum of scores. Nuclear grade was based on assessment of nuclear polymorphism and was scored from 1 to 3 (33).

Concomitant serum prostate specific antigen (PSA) and alkaline phosphatase (ALP) values were obtained from patients' charts. Serum PSA was measured using IMx assay (Abbott Laboratories, Abbot Park, IL, USA); the reference range for this assay was 0 to 4.0 ng/mL. Serum total ALP activity was determined using a kinetic color test on Olympus analyzer (Olympus, Tokyo, Japan) in the laboratory of the University Hospital Rijeka, using p-nitrophenylphosphate substrate at pH 10.4 (normal range for adults 30-120 U/L) (34).

Bone metastases were established by bone scintigraphy; bone lesions, which showed as hot spots on scintigraphy, were confirmed by plain radiography.

Immunohistochemistry

For immunohistochemistry, tissue slices were collected on glass slides coated with 3-aminopropyltriethoxy silane (APES, Sigma, St. Louis, MO, USA), air-dried, and stored at 4 °C until processing for indirect immunoperoxidase staining. Briefly, tissue slices were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase and nonspecific binding were blocked by incubation in 0.3% H₂O₂ in methanol and 5% non-immune serum. The sections were incubated with primary antibody for 60 minutes at room temperature. Anti-BMP-2, -3, -5, -6, and -7 were goat polyclonal antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After the incubation with a primary antibody, secondary biotinylated antibody was applied according to the manufacturer's protocol (DAKO, LSAB[®]+ Kit Peroxidase, Carpinteria, CA, USA). Peroxidase conjugated streptavidin was added and the site of antigen binding was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen. Sections were counterstained with hematoxylin. Slides used as a control were processed either with normal serum replacing the specific primary antibodies or with the secondary antibody alone.

Immunohistochemistry was also performed on paraffin embedded sections from breast cancer patients for the demonstration of estrogen receptor (ER) and progesterone receptor (PR). For

these receptor proteins, 1D5 and PgR636 monoclonal antibodies (DAKO, Glostrup, Denmark) were used at dilutions of 1:50. A semiquantitative estimation based on staining intensity and percentage of positive cells was performed. Staining for ER and PR was evaluated using the H-score system (35), based on a summation of the proportion of tumor cells showing different degrees of reactivity: no reactivity – 0, weak – 1, moderate – 2, strong – 3. This gives a maximum total score of 300 if 100% of cells show strong reactivity. Tumors with an H-score ≥ 100 for both antigens were considered positive. On the basis of H-score cut-off, receptor status was determined as follows: ER+/PR+ tumors – hormonal dependent, ER+/PR- or ER-/PR+ tumors – probably hormonal dependent, and ER-/PR- tumors – not hormonally dependent.

To determine BMP-expression of the cancer cells, 1000 cancer cells for each specimen were counted on 15 high power field ($\times 400$ magnification), chosen by two independent pathologists (E. M. and G. Đ). Cells counting was performed using image analyzer system equipped with software package (Issa, VAMS, Zagreb, Croatia) by two independent observers (I. M. and S. Z.). The results were expressed as a percentage of immunopositively stained cells. Staining score of counted cells revealed a significant interobserver concordance (Pearson's $r=0.683$, $P<0.05$).

Statistical Analysis

Data were described and analyzed using Statistica 6.1 software package (StatSoft.Inc., Tulsa, OK, USA). The data were described by means \pm standard deviations, or with medians with 10th to 90th percentile range, where suitable. Groups were compared using nonparametric tests (Kruskal-Wallis ANOVA for comparison of multiple groups or Mann-Whitney *U* test for comparison of two groups), since in most cases the distribution of data was not normal (a check for normality performed with Shapiro-Wilks test), but the distributions had similar shapes. A value of $P<0.05$ was considered statistically significant.

Results

Cancer tissue obtained from patients with prostatic and breast carcinomas with established bone metastases were assigned according to pathohistological staging. The prostatic carcinoma samples were categorized into three classes

based on the Gleason score: well-differentiated cancers (1-4; n=10), moderately differentiated cancers (5-6; n=7), and poorly differentiated cancers (7-10; n=3). For breast cancer, 6 cancers (40%) had low (I) histological grade, 6 cancers (40%) intermediate (II) grade, and 3 cancers (20%) high (III) histological grade. With regard to nuclear grade, 12 (80%) breast cancers had grade II and 3 cancers (20%) had grade III. In addition, a total number of 15 breast cancers were analyzed for ER/PR receptors: 6 cancers (40%) were classified as hormonal dependent, 7 cancers (46.67%) as possible dependent, and 2 cancers (13.33%) as hormonal independent. On the basis of estrogen and progesterone receptor status, the majority of breast cancers were hormonal dependent tumors. The results indicated that the most of the prostate and breast carcinomas were well or moderately differentiated cancers.

The expression of BMPs in prostatic and breast carcinoma with established bone metastases was analyzed using immunohistochemistry. For comparison, normal samples of prostatic and breast tissues were analyzed. Fig. 1A shows representative immunohistochemical staining in malignant prostatic epithelial cells, which was typically cytoplasmic, whereas in breast carcinoma the staining was exclusively nuclear (Fig. 1B). As shown in Table 1, normal and cancer prostatic tissues expressed almost all BMPs, but the percentage of positive cells differed for each BMP. All malignant prostate tissues were positive for the expression BMP-2 to -7 proteins and statistically significant differences among BMPs expression in prostate cancer cells was found ($P \leq 0.001$, ANOVA, Kruskal-Wallis test). BMP-2/4 and -5 were expressed in all samples of carcinoma tissue and the highest expression was detected for BMP-2/4 ($83 \pm 11.6\%$ positive cells). In normal prostate tissue, the highest BMP expression was found for BMP-2/4 ($87.8 \pm 11.4\%$ positive cells)

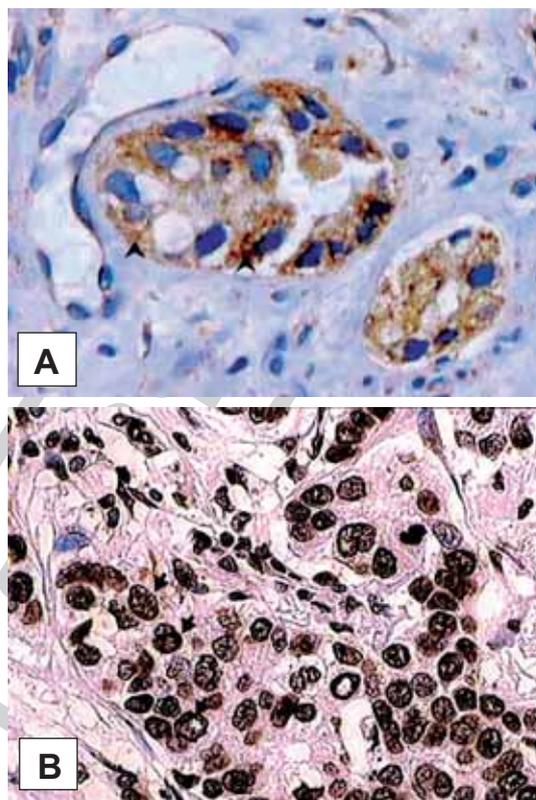


Figure 1. Microphotographs of bone morphogenetic proteins immunohistochemical stainings on prostate and breast cancer. **(A)** BMP-2/4 immunostaining showing strong cytoplasmic signals in prostate cancer cells (arrow heads). **(B)** BMP-7 immunostaining of breast cancer cells exhibited strong nuclear staining (arrows) ($\times 400$ magnification).

and BMP-7 ($94.6 \pm 0.9\%$ positive cells). The former was significantly greater than in prostate cancer. We also found that normal prostate tissue expressed significantly lower level of BMP-5 than prostate cancer. No demonstrable immunoreactivity for BMP-3 was found in normal prostate tissue. Breast cancer cells showed high expression only for BMP-7, whereas other BMPs were not ex-

Table 1. Bone morphogenetic proteins positive cells (% , mean \pm SD) in human prostate and breast carcinoma, and in normal human prostate and breast

BMP	Prostate				Breast			
	normal (n=5)		cancer (n=20)		normal (n=5)		cancer (n=15)	
	No. of samples	cells (%)	No. of samples	cells (%)	No. of samples	cells (%)	No. of samples	cells (%)
BMP-2/4	5/5	87.8 \pm 11.4	20/20	83.5 \pm 11.6	5/5	not detected	0	not detected
BMP-3	5/5	not detected	3/20	22.5 \pm 20.3*	5/5	not detected	0	not detected
BMP-5	5/5	42.6 \pm 12.4	20/20	57.8 \pm 17.2*	5/5	not detected	0	not detected
BMP-6	5/5	34.5 \pm 12.9	11/20	34.0 \pm 18.8	5/5	not detected	0	not detected
BMP-7	5/5	94.6 \pm 0.9	8/20	24.3 \pm 19.2*	5/5	not detected	15/15	86.4 \pm 7.3†

* $P \leq 0.001$, Kruskal-Wallis test.

† $P \leq 0.001$, prostate cancer vs breast cancer, Mann-Whitney U-test.

pressed in cancer cells (Table 1). All breast cancers showed positive nuclear staining for BMP-7 (Fig. 1B). Normal breast tissue revealed complete absence of BMPs expression. The comparison of BMP-7 expression in prostate and breast cancers showed significant difference in the percentage of positive cells ($P \leq 0.001$, Mann-Whitney U test). The percentage of BMP positive cells in prostate and breast carcinomas are presented in Table 1.

Comparison of BMPs expression with clinicopathological parameters of breast carcinoma such as tumor size, histological and nuclear grade, hormonal receptor status, and lymph nodes status showed no statistically significant difference. For prostate cancer, there was also no significant difference between BMPs expression and grade of prostate malignancy according to the Gleason score.

Analysis of serum ALP level as a bone formation marker illustrated that mean ALP level of patients with prostate cancer was 295 ± 283.6 U/L. On the other hand, patients with breast carcinoma (104 ± 53.5 U/L) displayed significantly lower levels of serum ALP. The level of serum PSA, a prognostic factor for the progression of prostate cancer, was 164.42 ± 190.37 ng/mL. We did not find any significant difference between values of both biochemical markers (ALP, PSA) and BMPs expression in prostate and breast carcinomas.

Discussion

Our study showed different patterns and extent of BMP expression in prostate and breast carcinoma tissue. Positive immunohistochemical staining for BMPs was detected in cytoplasm of prostate malignant cells, whereas in breast carcinoma cells nuclear staining was found. Malignant and normal prostate tissues expressed all BMPs; prostate cancer tissue predominantly expressed BMP-2/4, whereas normal prostate tissue BMP-2/4 and -7. On the other hand, the breast cancer cells expressed only BMP-7, whereas in normal breast tissue, we did not detect BMP expression. In prostate cancer tissue, the expression of BMP-7 was significantly lower, whereas in breast carcinoma tissue the expression was significantly higher than in normal tissue.

Prostate and breast cancer typically metastasize to bone, characteristically forming osteoblastic and osteolytic lesions, respectively. Recently, due to osteogenic properties of the BMPs, a

link between BMPs activity and tumor progression into the bone has been suggested (36). It was hypothesized that BMPs and other growth factors, such as TGF- β and PTHrP, which are produced by cancer cells turn the local bone homeostasis into the blastic metastatic phenotype (23,36). BMPs, as well as TGF- β , stimulate the proliferation of fibroblasts and inhibit osteoclasts, thus causing increased bone formation, as can be seen in osteoblastic metastases (37).

In the present study, we found that prostate cancer cells expressed BMP-2/4, -3, -5, -6, and -7. The expression of BMP-2/4 and -5 was detected in all of the prostate cancer samples, whereas BMP-6 expression was observed in 55% of the cancer samples. These results correlated well with the results of Bentley et al (21) and Masuda et al (23) who reported similar data for prostatic adenocarcinoma with skeletal metastases.

We found the highest immunoreactivity for BMP-2/4 in normal and prostate carcinoma tissues. This corresponds to data of Harris et al (25) who found that normal human prostate and carcinoma cell lines PC3 produce a high level of BMP-4 mRNA. Ide et al (38) analyzed the influence of BMP-2 on prostate cancer cell lines and found that BMP-2 decreased the growth rate of androgen-sensitive prostate cancer LNCaP cells, whereas androgen receptor-negative prostate cancer cell lines (TSU-PR1, PC3, and DU145 cells) were insensitive to its growth-inhibitory effect. Masuda et al (23) found the BMP-7 expression in almost all normal prostate tissue samples and in 50% of prostate carcinoma samples. Our results are in concordance with this study, as BMP-7 was expressed in all normal prostate and in 40% of prostate carcinoma samples. In addition, Masuda et al (39) found significantly higher levels of expression of BMP-7 mRNA in normal prostate tissue in comparison with prostate carcinoma. Our results showed a high percentage of BMP-7 positive cells in normal prostate tissue samples, whereas the percentage of BMP-7 positive cells in prostate carcinoma was significantly lower.

BMP-6 appears to be of particular importance as a useful marker for metastatic prostate cancer. These findings were confirmed by Bentley et al (21) who detected BMP-6 cDNA in over half of the patients with metastatic prostate cancer, but not in bone scan negative prostate cancers. BMP-6 mRNA and protein were detected in both rat nor-

mal prostate and rat malignant cell lines, but no difference in BMP-6 expression was found between metastatic and nonmetastatic cell lines (26). Some other authors (21,22,25) compared the expression of BMP-6 in prostate cancer with normal prostate and found higher BMP-6 expression in prostate cancer. Autzen et al (3) reported positive signals for BMP-6 in 85% of bone metastases from prostate carcinoma, compared with 29% of positive bone metastases from non-prostatic malignancies. According to Masuda et al (23), BMP-6 was positive in 66% of prostate carcinoma with skeletal metastases and in all prostatic skeletal metastases. Our finding about BMP-6 expression in malignant prostate tissue with bone metastases was in agreement with previous reports, but its expression was not significantly different than expression in normal tissue.

Further, BMP immunohistochemistry in breast tissue showed in all analyzed breast cancer samples positive and highly expressive of BMP-7 protein only, whereas in normal breast tissue, BMPs immunoreactivity was not detectable. These results are partially in concordance with the study of Weber et al (40) who found BMP-7 expression in 30% of breast cancer cells and in 6% of normal breast tissue. BMP-7 expression in breast carcinoma cell lines was also described by Schwalbe et al (30). They described BMP-7 expression in both normal breast tissue and breast carcinoma, but indicated specific expression of BMP-7 in different areas of the mammary gland. BMP-7 was expressed in end buds in normal breast tissue, but was not expressed in lactiferous ducts, whereas immunohistochemistry of invasive-ductal breast carcinoma showed marked BMP-7 expression in the solid area of the tumor. In contrast to these findings, Arnold et al (29) were not able to detect BMP-7 in breast cancer cell lines. Using RT-PCR, they found the presence of mRNA for BMP-2 and -3 but not for BMP-4 and -7. BMP-2/4 was also detected in fetal and postnatal mammary gland (27), as well as in breast tumor where this growth factor inhibits proliferation of breast cancer cells (29). In contrast, our results indicate complete absence of BMP-2/4 in both breast metastatic cancer and normal breast tissue.

The comparison of cancer BMP expression with clinical prognostic factors of these tumors, such as pathohistological grade, hormonal receptor status, and lymph nodes status revealed

no statistical significance. In respect to pathohistological grade, BMP expression was mostly found in well- or moderately- differentiated cancers, as reported in other studies (26,27). A recently published study revealed that biochemical markers of bone turnover showed significant differences between prostate carcinoma patients with and without bone metastases (41). In our study, the level of ALP and PSA in serum of prostate cancer patients with bone metastases was elevated, suggesting that these biochemical markers have a direct relationship with bone progression. Serum ALP level was also analyzed in patients with breast cancer. Significantly higher serum ALP values were observed in patients with prostate cancer than in patients with breast cancer, suggesting a higher osteoblastic activity in bone metastases.

Normal and malignant prostate tissues express many growth factors that have a possible role to act in osteoblastic activity seen in skeletal lesions secondary to prostate cancer (42). Indications in favor of a possible role of BMPs in carcinoma tissue come from the biological effects in developmental processes. BMPs are involved in morphogenesis of tissue and in epithelial-mesenchymal cell interactions (14). Our study demonstrated that prostate and breast carcinoma cells showed different patterns of BMPs expression, including both number of cases and percent of positive cells. Prostate cancer tissue expressed all BMPs but predominantly BMP-2/4, whereas breast cancer showed a high expression of BMP-7 only. Also, in metastatic prostate cancer tissue, the expression of BMP-5 and -2/4 was found in all tissue samples. Although we found low BMP-7 expression in prostate cancer, we suppose that the presence of osteoblastic metastases can be associated with high expression of all BMPs in prostate tissue. The expression of BMPs is more common for prostate than for breast cancer. These results confirmed the affinity of both tumors to produce bone metastases, but different patterns of BMP expression in prostate and breast metastatic cancers could be linked to the presence of different types of bone metastases in these cancers. In our study, the reason for limited understanding of this process may be because of our statistically low sample sizes. Further characterization of the effect of the BMPs on the progression and metastasis of cancers with bone metastasis is needed.

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