Breast Cancer Res Treat (2009) 117:61–68
DOI 10.1007/s10549-008-0135-x

CLINICAL TRIAL

Cyclooxygenase-2 expression in primary breast cancers predicts dissemination of cancer cells to the bone marrow

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Received: 27 May 2008 / Accepted: 14 July 2008 / Published online: 29 July 2008

Abstract *Purpose* Cyclooxygenase-2 (COX2) plays a role in breast cancer progression at various stages starting from pre-malignant phenotype to clinical metastasis. Breast cancer metastasizes commonly to the bone and preclinical studies suggest an involvement of COX2 in this process. Detection of disseminated tumor cells in the bone marrow of patients at the time of surgery correlates with the subsequent development of clinical bone metastasis. Therefore, to investigate whether COX2 is important for breast cancer metastasis in humans, we analyzed COX2 protein expression by immunostaining of primary tumors from 112 operable stages I, II, or III patients and determined its correlation with bone marrow micrometastasis (BMM). *Methods* We detected COX2 protein in primary tumors by immunostaining with a monoclonal antibody, and tumor cells present in the bone marrow by immunostaining for epithelial cytokeratins and by morphological criteria. *Results* COX2 expression in primary breast cancer correlated with BMM in a highly statistically significant manner ($P = 0.006$). Our statistical analyses of correlations of the COX2 positivity in primary tumor with other clinically relevant indicators revealed that COX2 positivity correlates with high nuclear grade ($P = 0.0004$). Furthermore, we were able to detect COX2 protein in BMM by immunostaining. *Conclusions* These studies indicate that COX2 produced in primary breast cancer cells may be vital to the initial development of BMM that may subsequently lead to osteolytic bone metastases in patients with breast cancer, and that COX2 inhibitors may be useful in halting this process.

Keywords Breast cancer · COX2 · Bone marrow micrometastasis · Disseminated tumor cells · Minimal residual disease

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Introduction

There is strong evidence from several clinical studies that breast cancer patients with bone marrow micrometastasis (BMM; defined by the presence of single cancer cells or microscopic cancer cell-clusters in the bone marrow) have a shorter time to recurrence and decreased overall survival. In an analysis of combined individual patient data from nine studies involving 4,703 patients with stages I, II, or III

breast cancer, Braun et al. [1] evaluated patient outcomes over a 10-year follow-up period (median, 5.2 years), and found that the presence of BMM was a significant prognostic factor with respect to poor overall survival and breast-cancer-specific survival. Similarly, published studies have shown that systemic chemotherapy does not consistently eradicate BMM in breast cancer patients known to have these micrometastases prior to the start of systemic therapy [2]. Currently, little is known about the molecular mechanisms that govern (1) dissemination of cancer cells to the bone marrow, (2) their survival and dormancy in this important niche, and (3) progression from BMM to clinical metastasis. Since many advanced breast cancer patients develop bone metastasis, it is crucial to understand the basis of BMM.

Another compartment in the body where circulating cancer cells (CTC) can be analyzed is blood. Although the disseminated tumor cells (DTC) that colonize the bone marrow pass through the blood, emerging evidence suggests that cancer cells detected in the bone marrow are different from those in the blood [3–5]. This is not surprising if we consider that the microenvironment plays an important role in supporting or inhibiting the cancer cells of a given molecular phenotype in the context of metastasis [6]. From the perspective of clinical practice, it is easier to obtain blood than the bone marrow, and detection of CTCs provides significant prognostic and diagnostic information regarding the metastasis in advanced breast cancer patients [7]. However, further studies are needed to understand the significance of CTCs in operable breast cancer patients.

Cyclooxygenase-2 (COX2), which mediates the production of prostaglandins and thromboxanes from arachidonic acid, is induced in inflammation and cancer [8–10]. The important mediator of tumorigenic effects of COX2 is prostaglandin E_2 (PGE_2). Because of its nature as a small diffusible molecule, PGE_2 has a potential of modifying the surrounding microenvironment, which may be important in tumorigenesis/metastasis. We have shown that in a xenograft mouse model of breast cancer, cells producing a high level of PGE_2 are selected to colonize and grow in the bone marrow, highlighting the importance of COX2 in bone metastasis [11]. Overexpression of COX2 in breast cancer cell lines increases cell migration and invasion [12], and results in a significantly greater production of interleukin-8 (IL-8) [13] and IL-11 [14] as compared to their parental lines. Several recent studies have implicated increased production of IL-8 and IL-11 as important factors in the development of bone metastases in patients with breast cancer [15–18]. Presence of IL-8 in the serum of these patients is associated with worse overall survival [19]. We have shown that COX2 inhibition results in the inhibition of bone metastasis from breast cancer in a mouse model [11]. Significantly, COX2 inhibition reduces both spontaneous metastasis and experimental metastasis in the mouse models [11, 20]. Bone is a common site of metastasis in human breast cancer and bone metastases are a cause of significant morbidity. Our ability to target and prevent metastases to bone would represent a significant improvement in the treatment of breast cancer patients. Based on our preliminary data, we hypothesized that COX2 protein expression in primary breast cancer would facilitate dissemination of cancer cells into the bone marrow and would correlate with the occurrence of BMM. Identification of COX2 as a facilitator of BMM could result in targeted therapy to prevent or eradicate BMM before they develop into clinical metastases. The purpose of this study was to determine if COX2 protein expression in primary breast cancer is associated with other disease characteristics, specifically BMM.

Materials and methods

Immunostaining of primary tumor

Sections of the primary tumor in the breast were fixed in formalin, routinely processed, embedded in paraffin, cut at 5 μm thickness and subsequently used for immunostaining. Immunostaining was performed by the avidin biotin peroxidase technique using appropriate positive and negative controls in each run. The chromogen used was diaminobenzidine. After antigen retrieval in a citrate buffer, COX2 was stained with a monoclonal antibody (Cayman Chemical, Ann Arbor, MI) at 1:50 dilution. Positive staining for COX2 was recognized as brown cytoplasmic and/or membranous staining. The proportion of cells demonstrating COX2 staining was semiquantitatively scored on a scale of 1–100% and intensity of staining for COX2 was recorded visually on a scale of 1+ to 3+. Immunostaining of more than 5% of tumor cells of any intensity was regarded as positive for COX2 protein expression. A single dedicated pathologist (S.K.) reviewed all of the immunostains of the primary tumor specimens and provided grading for COX2 staining.

Information about the status of clinically useful biomarkers (e.g., ER, PgR, HER2, and Ki-67) and other clinical parameters (e.g., lymph node metastasis, sentinel lymph node metastasis, lymphovascular invasion, and nuclear grade) was obtained from the pathology reports in clinical records. The status of ER and PgR was determined by immunostaining using 10% or more cells staining as the cut-off value. HER2 status was determined by both immunostaining for protein overexpression as first line test, followed by fluorescence in situ hybridization (FISH) to detect gene amplification in all 2+ and 3+ cases.

Detection of cancer cells in the bone marrow

Under an institutional review board approved protocol, and after obtaining informed consent from each patient, participants went to the operating room for bone marrow aspiration at the time of either the definitive operation for their primary tumor and lymph nodes, or at the time-of port-a-catheter placement in patients who would subsequently be undergoing neoadjuvant chemotherapy. After the induction of general anesthesia, and after sterile prepping and draping of the appropriate areas, approximately 10 ml bone marrow was aspirated from bilateral anterior superior iliac crests using a separate Jamshidi needle. Aspirates were placed in labeled EDTA-primed tubes and sent to the laboratory immediately. The bone marrow specimen was layered with 4 ml of Lymphoprep (Axis-Shield PoC AS, Norway), centrifuged at 1,500 rpm for 10 min. The buffy coat was separated and washed in PBS, subsequently centrifuged. From the cell pellet 10 cytospin smears were made and used for immunostaining.

Immunostaining was performed by the avidin biotin peroxidase method using diaminobenzidine as the chromogen. Monoclonal antibodies were used against cytokeratin cocktail comprised of AE1/AE3 (DAKO), CAM5.2 (BD Biosciences), MNF116 (DAKO), and cytokeratin 8 and 18 (Zymed) at a dilution 1:50 for the first three antibodies and 1:25 for the last two). Cytoplasmic and/or membranous staining in cells was regarded as positive for cytokeratin. We used strict cytomorphologic criteria to detect cytokeratin positive tumor cells in the cytospin smears so as to avoid misdiagnosing spurious positivity in hematopoietic cells as tumor cells positive for CK. One dedicated pathologist (S.K.) prepared and reviewed all of the bone marrow aspirates for this study. Only those patients with both COX2 and BMM results available were included in the final analysis.

Detection of CTCs

Blood was collected prior to surgery and CTCs were detected by immunomagnetics (Veridex, LLC) as described previously [7]. From every patient, three tubes containing 7.5 ml blood in each tube were analyzed. Presence of one or more CTCs in either tube of blood was considered positive.

Statistical analysis

Patient characteristics were tabulated and compared between COX2 groups with the χ^2 test or Fisher's exact test, as appropriate. Fisher's exact test was used when any one of the observed values in the 2×2 contingency table was <5. Odds ratios were calculated for some variables for additional descriptive purposes. Five percent (5%) was used as the threshold to dichotomize COX2 as negative/positive. Statistical analyses were performed with STATA and power calculations were performed with NQuery Advisor 6.01 (Statistical Solutions, Saugus, MA). *P*-values <0.05 were considered statistically significant.

Results

COX2 expression and BMM

To determine whether COX2 expression in primary tumor would correlate with BMM, we immunostained primary tumors with a monoclonal antibody against COX2 and scored COX2 positivity based on 5% or more tumor cells staining positive. An experienced pathologist (SK) performed all the COX2 scoring. The cancer cells present in the bone marrow were detected by cytokeratin (CK) immunostaining using a cocktail of antibodies; CK-positive cells also met strict cytomorphologic criteria for malignancy. An important aspect of our study design was the optimal detection of CK-positive cancer cells with the use of a unique cocktail of antibodies that would detect a broad-spectrum of cytokeratins. The expression of cytokeratins differs greatly in breast cancer cells, owing to the differences in the cell of origin and the degree of differentiation. Sample images of COX2 staining of primary tumor and cytokeratin staining of bone marrow are shown (Fig. 1).

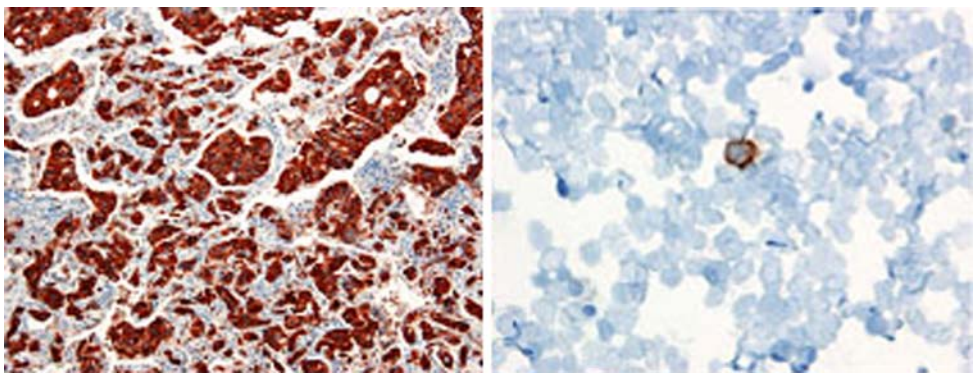

Fig. 1 Detection and characterization of breast cancer cells. We immunostained a primary tumor and a bone marrow sample as described in Materials and Methods. Left: A primary tumor stained with a COX2 antibody shows strong COX2 expression. Right: Cytokeratin immunostain detects a single cancer cell in the bone marrow

Table 1 Patients characteristics

Variable	Status	N	Percent
Tumor size	T1	48	32
	T2	63	42
	T3	13	9
	T4	25	17
Lymphovascular invasion	(+)	46	32
	(−)	96	68
Estrogen receptor status	(+)	93	62
	(−)	56	38
Progesterone receptor status	(+)	73	49
	(−)	76	51
HER2 status	(+)	21	14
	(−)	128	86
Lymph node status	(+)	68	49
	(−)	71	51
COX2 staining in primary tumor	≥5%	39	35
	<5%	73	65
CTC detection	(+)	44	34
	(−)	87	66
BMM detection	(+)	28	24
	(−)	87	76

To improve the chances of translation, we performed this study on operable breast cancer patients (n = 149). Patient characteristics are summarized in Table 1. Mean age was 54 years (range 25–92) and 48 (32%) had a primary tumor less than or equal to 2 cm in size. Ninety-three patients (62%) were estrogen receptor (ER) positive, 73 (49%) were progesterone receptor (PgR) positive and 21 (14%) were positive for HER2 gene amplification. Based on the threshold value of 5% for COX2 positivity, 39 (35%) patients were positive for COX2 and 73 (65%) were negative for COX2 in the primary tumor. In addition, 44 patients (34%) had detectable CTCs and 28 (24%) were positive for BMM. All of our BMM-positive cases showed isolated cytokeratin-positive cells in the bone marrow; we did not observe clusters of tumor cells in any of our cases with BMM.

BMM was detected much more frequently in patients who were positive for COX2 (43%) versus patients negative for COX2 (15%). The odds ratio for BMM associated with COX2 status was 4.22 ($P = 0.006$, Fig. 2). In this analysis, we had 90% power to detect an odds ratio 4.22. We also analyzed correlation between COX2 expression and BMM by a method that considers both proportion (scores: 0, 0%; 1, 1–5%; 2, 6–10%; 3, 11–30%; 4, 31–60%; 5, 61–100%) and intensity (scores: 0–3) of COX2-positive cells rather than a fixed cut-off value (5% cells being COX2-positive). This analysis also demonstrated a statistically significant correlation between COX2 and BMM

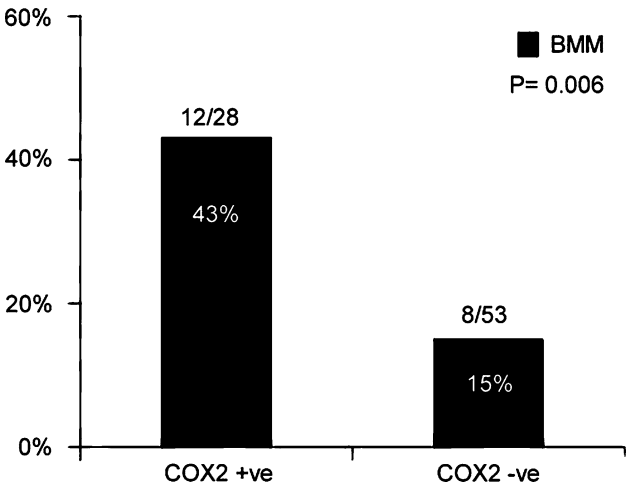

Fig. 2 Association between the COX2 expression in primary tumor and the incidence of BMM. The COX2 positivity in primary tumors and the incidence of BMM were determined as described in Materials and methods. Percent COX2 positivity in BMM-positive and BMM-negative subgroups of patients, along with the number of patients in each group is displayed. The statistical significance for this association was determined by the χ^2 test ($P = 0.006$)

($P = 0.006$ based on a cut-off score 1). Thus both methods used to determine level of COX2 expression provided the same result.

A noteworthy observation in this analysis was that the presence of COX2-positive tumor cells in primary tumors was always associated with the presence of COX2-positive inflammatory cells, both lymphocytes and histiocytes, around the tumor. The phenomenon involving infiltration of tumor by inflammatory cells is well established [21, 22]. The adjacent breast tissue also showed COX2 positivity in cases that showed COX2 expression in the primary tumor cells; this may be explained if one considers that COX2 positive tumor cells would produce PGE_2 which would then act on surrounding tissues to induce COX2 expression [23].

Table 2 also shows the tabulation of other primary tumor characteristics by COX2 staining on primary breast cancer tissue. Significantly, patients positive for COX2 also had a higher nuclear grade of the primary tumor (69.2% vs. 34.2% in COX2-positive versus negative patients respectively, $P = 0.0004$). We also observed this correlation upon analyzing the data wherein COX2-positivity was based on both proportion and intensity of staining as described above. COX2-positivity did not correlate significantly with Ki-67 expression, ER, or PgR status of the primary tumor (Table 2). The information about the ER, PgR and Ki-67 status was obtained from the clinical records of pathological evaluation. The cut-off values for ER and PgR positivity were 10% of cells immunostaining positively. We used common clinical criteria for high nuclear grade (including nuclear

Table 2 Correlations of COX2 staining of primary breast cancer with BMM and high nuclear grade

Variable	COX-2 (+)	COX-2 (−)	Odds ratio (OR)	95% CI for OR	*P*-value
ER (+)	56.4% (22/39)	68.5% (50/73)	0.59	0.27–1.33	0.204
PgR (+)	41.0% (16/39)	57.5% (42/73)	0.51	0.23–1.13	0.096
HER2 (+)	5.1% (2/39)	12.3% (9/73)	0.38	0.08–1.88	0.19*
Ki-67 (+)	61.9% (13/21)	44.4% (12/27)	2.03	0.64–6.50	0.230
High nuclear grade	69.2% (27/39)	34.2% (25/73)	4.32	1.87–9.95	0.0004
CTCs (+)	37.1% (13/35)	34.3% (22/64)	1.13	0.47–2.66	0.783
LN (+)	56.4% (22/39)	46.3% (32/69)	1.50	0.68–3.30	0.317
SLN (+)	44.4% (12/27)	33.3% (18/54)	1.60	0.62–4.12	0.329
LVI (+)	35.9% (14/39)	33.8% (24/71)	1.09	0.48–2.49	0.825
BMM (+)	42.8% (12/28)	15% (8/53)	4.22	1.46–12.18	0.006

The COX2 positivity was defined as ≥5% cells staining positive in a primary tumor. If not indicated otherwise, we determined the statistical significance by a χ^2 test, which is indicated by *P* values; *, the *P*-value was determined by the Fisher's exact test

pleomorphism, enlarged nuclear size, and presence of mitotic figures) and for high Ki-67 (more than 40% cancer cells staining positive for Ki-67). While COX2 expression was highly associated with BMM, it did not correlate with CTCs. We observed that 37.1% of patients with COX2-positive primary tumors had CTCs versus 34.3% with COX2-negative primary tumors.

Several signaling pathways, including the NF kappa B pathway, can influence COX2 expression. HER2 can also induce COX2 expression [24, 25] but the association between HER2 gene amplification and COX2 expression in primary breast cancers is not strong [26]. In our study, only 2/39 primary tumors positive for COX2 were also positive for HER2 (5.1%), versus 9/73 (12.3%) of primary tumors negative for COX2 ($P = 0.19$; Table 2).

In current clinical practice, operable breast cancer patients are routinely evaluated for lymph node metastases. There is also a significant interest in determining the prognostic significance of CTCs in operable breast cancer patients. Therefore, we determined whether the presence of cancer cells in the bone marrow correlated with the presence of cancer cells in the blood and/or lymph nodes. Forty-four of 131 (33.6%) patients were found to have one or more CTCs (Table 1). There was no significant correlation between BMM and CTCs ($P = 0.724$). Similar to another published study [27], there was no significant correlation between BMM and lymph node metastases ($P = 0.696$; Table 2), suggesting independent biology and routes of dissemination of these cancer cells.

COX2 expression in BMM

To provide direct evidence for our hypothesis that COX2 is important for BMM, we determined whether the COX2 protein is present in BMM. For this purpose, we first enriched BMM by immunomagnetic separation with EpCAM (CD326) affinity columns (Miltenyi Biotec). After confirming the presence of BMM in the enriched material by immunostaining for CK as above, we stained additional slides with COX2 antibody (Fig. 3). In this manner, we detected COX2 protein in DTCs in concordance with COX2 expression in primary tumors; we detected COX2 on DTCs in both (2/2) of the patients tested whose primary tumors were COX2 positive, but did not detect COX2 on DTCs from both (2/2) patients tested whose primary tumors were COX2-negative.

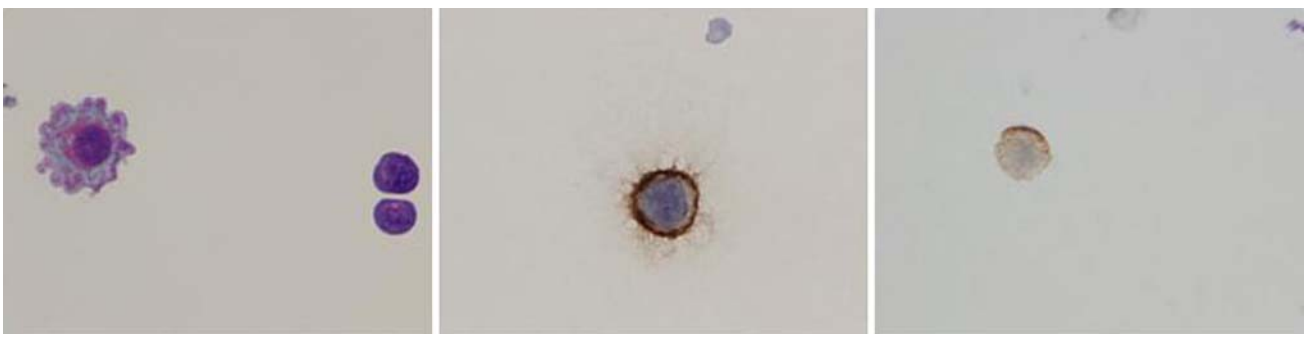

Fig. 3 Detection and phenotyping of BMM. About 1.5 ml aliquots of bone marrow samples were enriched for DTCs with EpCAM affinity columns (Miltenyi Biotec) according to manufacturer's protocol. The purified cell populations were pelleted onto two slides for immunostaining. Left panel, DTC detected with Papanicalaou stain (two cells seen on the right are not DTCs but immature hematogenic progenitor cells); middle panel, DTC confirmation by staining for cytokeratin; right panel, DTC showing a COX-2 positive stain

Discussion

Our data support the hypothesis generated from our preclinical studies that COX2 expression in primary tumor strongly correlates with BMM. This, combined with the strong evidence from the clinical studies for a correlation between BMM and clinical metastasis [1], indicates that COX2 is important for bone metastasis in human breast cancers. In the context of minimal residual disease, where disseminated cancer cells could survive for years often in dormant state, the survival of cancer cells would depend upon their ability to survive in a given microenvironment. In this regard, our data showing a correlation between COX2 expression and BMM but not lymph node metastasis or the presence of cancer cells in blood suggest that COX2 may be particularly important for bone marrow micrometastasis. Our results are consistent with the notion that cancer cells isolated from different sites in the body, i.e., bone marrow, blood, and lymph nodes, are most likely different from each other. Several published studies support this concept [1, 3–5, 27].

Several studies have examined the prognostic significance of COX2 expression in primary tumors [28–30]. However, our study is the first to specifically determine the correlation between COX2 expression in a primary breast cancer and the incidence of BMM in operable patients. Our results are significant as they showed a strong correlation between the COX2 expression and a high nuclear grade. One way to explain this correlation would be that high nuclear grade tumors contain a high percentage of tumor cells that are permissive for a high COX2 level, e.g., cells of basal subtype. Consistent with this interpretation, we have reported that COX2 is cytotoxic to ER-positive MCF7 breast cancer cells, but not to several breast cancer cell lines of basal subtype [12]. Furthermore, we have recently shown that COX2 expression induces genomic instability [31, 32], which would facilitate cancer progression.

The studies from the mouse models, including our own studies, provide support for the roles of COX2 in breast cancer metastasis to bone [11] and other organ sites like the lungs [33]. However, further studies are needed to understand the specific role(s) of COX2 in BMM. It is possible that COX2 contributes to tumor dormancy or the tumorigenic cancer cell phenotype that seems to increase in BMM as compared to primary tumor [34]. In support of the latter, it is noteworthy that the prominent COX2 metabolite PGE_2 inhibits apoptosis in mouse embryonic stem cells [35], and promotes their proliferation [36].

Our results showing a correlation between COX2 expression in primary tumor and BMM imply that therapies with COX2 inhibitors may help eradicate BMM before it has a chance to develop into clinical metastasis. For the BMM-positive patients, this may be a window of opportunity to reduce the risk of clinical metastasis. The concept that disseminated tumor cells and circulating tumor cells are suitable targets for therapy is gaining strength; several small studies have provided proof of concept [37]. Specifically in the context of a targeted therapy against COX2, although some COX2 inhibitors pose a significant risk of cardiac toxicity, other COX2 inhibitors are relatively safe [38, 39]. In addition to COX2, other targets on such cells could include HER2 and epidermal growth factor receptor against which effective therapies exist. We believe that the concept of individualized therapies to reduce the risk of recurrence should consider that DTCs and CTCs provide better information than does the primary tumor regarding the presence of therapeutic molecular targets for inhibiting the development of clinical metastasis.

In conclusion, COX2 staining in the primary breast cancers from operable patients was significantly associated with BMM. This is not an extremely large study, but the correlation between COX2 and BMM was highly significant, and it is likely that this correlation will remain significant even as we continue to accrue patients to the study. We also detected COX2 protein on the bone marrow micrometastases themselves from patients (2/2) who had COX2-positive primary tumors. A recent study involving rapid autopsies on ten consecutive metastatic breast cancer patients to compare primary tumor with the metastases indicated that progression towards metastasis involves loss of ER and PgR, and overexpression of COX2 (in 5/10 cases, all basal subtype cases in this study; reference [40]). Although that was a small study, it provides support to our model where COX2 plays an important role in BMM and development of subsequent metastases. The data from our study reveals important possibilities for novel targeted therapies against micrometastases, wherein they can be eradicated before developing into a clinical metastasis.

Acknowledgements These studies were supported in part by the grants DK067682 (AL) and CA16672 (Core) from the National Institutes of Health and DAMD17-03-1-0669 (AL) from the United States Army Medical Research and Material Command. We thank Gabriel Hortobagyi for helpful comments on the manuscript.

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