

COX-2 Drives Metastatic Breast Cells from Brain Lesions into the Cerebrospinal Fluid and Systemic Circulation

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Abstract

Breast cancer is among the most common malignancies that metastasize to the brain, with 15% to 20% of patients with metastatic breast cancer eventually developing brain metastases. We previously reported a method to enumerate tumor cells in the cerebrospinal fluid (CSF) of patients with breast cancer with central nervous system (CNS) metastases, a setting that lacks sufficiently informative biomarkers. Here, we show that breast cancer cells can spontaneously disseminate into the CSF from brain lesions in mice in a COX-2–dependent manner and can escape from the CNS to systemic circulation. Enumeration of tumor cells in the peripheral blood (circulating tumor cells, CTC) and CSF (cerebrospinal fluid tumor cells, CSFTC) of nine breast cancer patients with brain metastases revealed dynamic changes in tumor cell burden in both the peripheral blood and CSF compartments that correlated with clinical disease progression. Interestingly, four of the enrolled patients exhibited rapid intercompartmental transitioning of the disease reflected in the CTC and CSFTC counts that preceded corresponding evidence by clinical imaging or neurologic symptoms. Two of these patients had systemic disease recurrence involving the primary malignant site. Intercompartmental cycling of tumor cells may represent an important mechanism for disease persistence and recurrence that may involve tumor self-seeding. Our findings demonstrate the involvement of COX-2 in the genesis of CSFTCs and suggest that COX-2 inhibitors should be investigated in patients with breast cancer with brain metastases for their ability to reduce CSFTC counts and prevent systemic recurrence. *Cancer Res*; 74(9); 1–6. ©2014 AACR.

Introduction

In addition to brain lesions, metastatic breast cancer can cause leptomeningeal metastatic disease, which has been reported to occur in approximately 5% of all patients with cancer (1). The diagnosis of leptomeningeal disease is often elusive, involving clinical presentation of neurologic symptoms, gadolinium-enhanced MRI, and cerebrospinal fluid (CSF) cytology. Unfortunately, these diagnostic techniques have low sensitivity and specificity in this setting and therefore are not optimal for early detection, which contributes to the dismal survival following diagnosis of leptomeningeal disease that can be as short as a few weeks without treatment. Tumor

cells remaining in the CSF represent minimal residual disease, are nearly undetectable with standard means, and may potentiate relapse.

We previously reported a novel adaptation of the CellSearch system for use in patients with metastatic breast cancer with central nervous system (CNS) metastases to accurately and sensitively enumerate CSFTCs, a concept that has now been recapitulated by other investigators (2, 3). In our prior studies, CSFTC counts correlated with the clinical course of the patient and response to treatment, and demonstrated the superiority of CSFTC counts over CSF cytology. Here, we examined the relationship between tumor cells found in the peripheral blood and CSF of metastatic breast cancer models and patients with CNS involvement.

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Materials and Methods

Cell culture studies

MDA-MB-231 and MDA-MB-468 human breast cancer cells were obtained from American Type Culture Collection and cultured under manufacturer-recommended conditions. RNA was harvested with RNeasy Mini Kit (Qiagen) and reverse transcriptase PCR (RT-PCR) was performed with SuperScript II reverse transcriptase (Invitrogen) and FastStart Taq polymerase (Roche) according to the manufacturer's instructions. Primers were COX-2 forward (GACAGTCCACCAACTT-ACAAT), COX-2 reverse (CATCTCTCATCAATTATCTGAT), BHEGF forward (ATGAAGCTGCTGCCGTCGGT) BHEGF

reverse (CAGTGCTTGTGGCTTGGAGG), ST6GALNAC5 forward (GTCTGGCAGTGTGTTTAGCG), and ST6GALNAC5 reverse (CCCCAGAAGATGAACACGGT). Transwell migration assays were performed with BD Matrigel Transwell migration 24-well assays using serum-free media in the chamber and complete media (10% serum) in the well. At endpoint, cells were stained with Hoechst 33342 (Invitrogen) at 100 ng/mL, Matrigel was removed with a cotton swab, and the membrane was removed with a scalpel and mounted on a slide for fluorescent microscopy imaging. Scratch assays were performed by plating 5×10^6 cells in 12-well plates and making multiple scratches with a 200- μ L tip the following day to allow for adherence.

Mouse studies

Animal studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Penn State Hershey Medical Center (Hershey, PA). Intracranial injection was carried out with 1×10^5 cells per injection as previously described in athymic nude mice (4). Brains were fixed in 4% paraformaldehyde, paraffin embedded, and stained with hematoxylin and eosin by the Histology Core Facility at Penn State Hershey Medical Center. Bioluminescence imaging of intracranial tumors was carried out as previously described (4). Circulating tumor cell (CTC) detection of human cancer cells in mouse blood is described in Supplementary Fig. S1.

Human studies

All subjects provided informed consent for testing of their CSF as approved by the Institutional Review Board at the Penn State Hershey Medical Center. Inclusion criteria were newly discovered breast cancer with metastatic disease involving the CNS confirmed with radiologic or cytologic findings and the commencement of intrathecal chemotherapy. All subjects were diagnosed with primary breast cancer before enrollment into this study and had undergone neurosurgical intervention in terms of placement of a ventricular access device, which was accessed to obtain CSF for testing. CSF was obtained every 2 to 3 weeks for each patient and often coincided with intrathecal chemotherapy deposition. For CTC analysis, 7.5 mL of venous peripheral blood was drawn into CellSave tubes (Veridex, LLC) and processed using the standard operating procedures for the CellSearch system by a certified assay operator (Veridex, LLC). CSFTCs and CTCs were evaluated by a blinded trained investigator. The neuro-oncologist providing treatment and evaluating the patients was blinded to the CSFTC analysis. CSFTC analysis was performed as previously described, as was standard CTC analysis using the CellSearch system (2).

Statistical analysis

For pairwise comparisons, we analyzed data with the Student two-tailed *t* test in Excel (Microsoft). Log-rank statistical analysis was performed with a web-based script that interfaces with the statistical package R (<http://bioinf.wehi.edu.au/software/russell/logrank/>). Western blot band intensities were quantified using NIH ImageJ software.

Results and Discussion

Given the apparent clinical importance of CSFTCs, we sought to create a mouse model to study the behavior of breast cancer cells in the CNS. COX-2 expression is elevated in approximately 40% of breast cancers (5) and has been previously shown to mediate the migration of breast cancer cells to the brain *in vitro* and *in vivo* in MDA-MB-231 cells (6) as well as to bone (7). Among available human breast cancer cell lines, MDA-MB-231 cells have a well-documented strikingly high expression of COX-2 compared with MDA-MB-468 cells that we confirmed by Western blot analysis (Supplementary Fig. S1; ref. 8). We injected luciferase-expressing MDA-MB-231 and MDA-MB-468 human triple-negative breast cancer cells into the brains of athymic nude mice. We observed a high tumor take rate of 80% in both cell lines at the primary site of injection that was confirmed by histology and bioluminescence imaging (Fig. 1A and B). Interestingly, the MDA-MB-231 cohort of mice exhibited a rapid and heterogeneous spread of tumor cells into the neuroaxis of several mice, unlike the MDA-MB-468 cohort of mice. This difference in tumor cell dissemination among cohorts of mice suggests that this is likely driven by intrinsic properties of the cell lines, such as COX-2 expression, rather than a dissemination of tumor cells resulting from surgical manipulation. Furthermore, the inoculate site was selected to prevent ventricular injection, which might promote dissemination into the CSF. The MDA-MB-231 cohort also had a decreased overall survival compared with the MDA-MB-468 cohort of mice, which is in line with our previously reported relationship between disease burden and CSFTCs (Fig. 1C). Analysis of extracted CSF isolated from inoculated mice revealed that the MDA-MB-231 cohort of mice possessed an abundance of CSFTCs by imaging and enumeration, unlike the MDA-MB-468 cohort of mice (Fig. 1D).

To gain a molecular understanding of the difference in CSFTC generation between these cell lines, we investigated differences in transcriptional levels of genes previously reported to be involved in breast cancer cell migration to the brain (6). Among the examined genes, we found that MDA-MB-231 cells have strikingly higher expression of BHEGF and COX-2 messenger RNA (Fig. 1E). COX-2 has been heavily implicated in cancer cell motility and metastasis in several cancers, including colon and breast cancer. Celecoxib is a small-molecule selective COX-2 inhibitor that has been shown to decrease breast cancer metastasis to the lungs in murine models (9). To study the effect of COX-2 inhibition on invasive properties of breast cancer cells, we selected a subcytotoxic dose of celecoxib that inhibited COX-2 (Supplementary Fig. S2A and S2B), as indicated by elevated COX-2 expression driven by a previously reported negative feedback loop (10, 11). At this dose, celecoxib inhibited the pronounced migration and invasion capacity of MDA-MB-231 over MDA-MB-468 cells in Transwell migration and scratch assays (Supplementary Fig. S2C–S2E).

Given these observations, we directly assessed the ability of celecoxib to affect CSFTC generation *in vivo*. The celecoxib-treated cohort of mice inoculated with MDA-MB-231 intracranial tumors possessed a strikingly lower tumor burden at the primary site of injection compared with vehicle cohort of mice, several of which developed hydrocephalus and lost

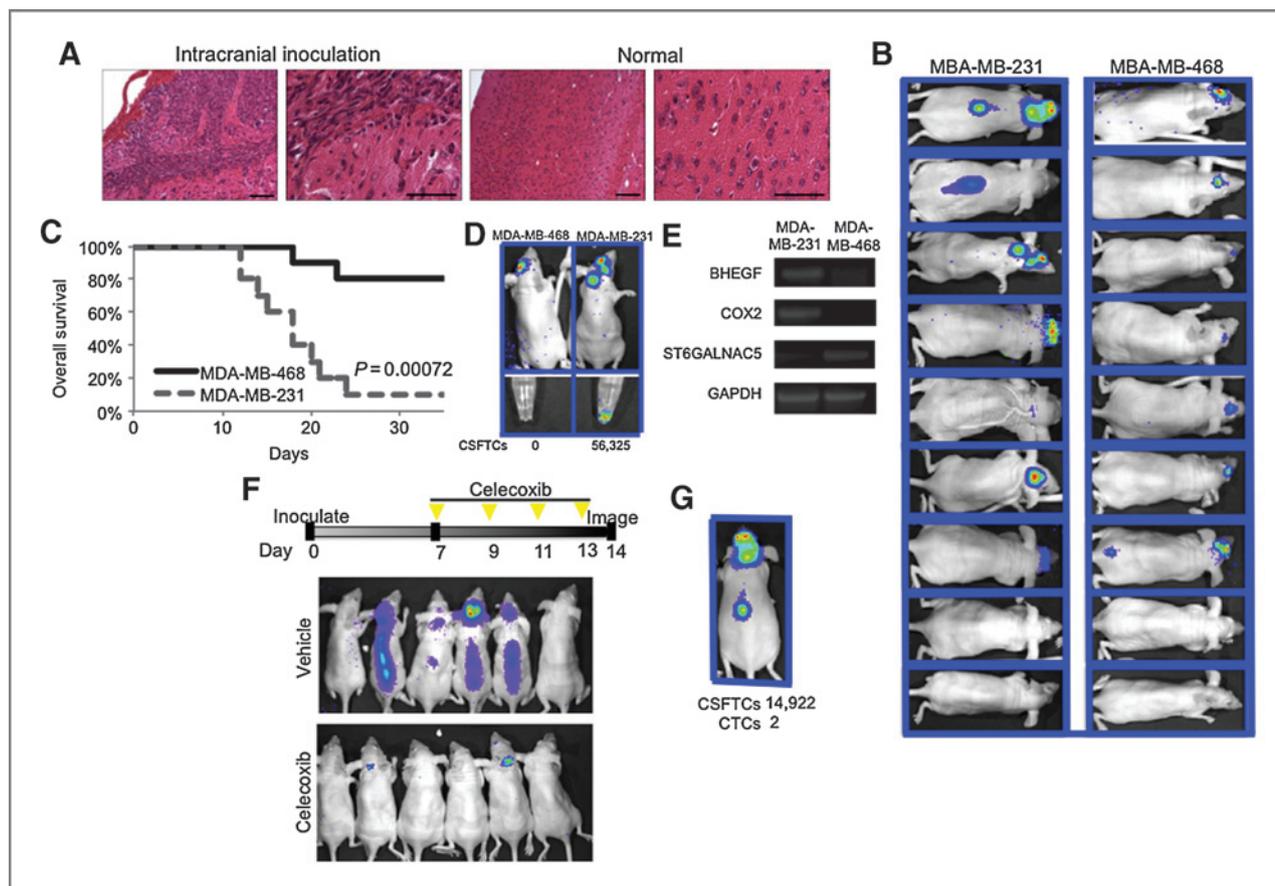


Figure 1. Inoculation of human breast cancer cells into the brains of mice results in primary brain lesions and heterogeneous dissemination into the CSF and blood of mice. **A**, hematoxylin and eosin staining of brains harvested from a normal mouse or a mouse harboring an intracranial MDA-MB-231 xenograft tumor. Scale bars, 100 μ m. **B**, bioluminescence imaging of athymic nude mice 10 days following intracranial injection of MDA-MB-231 or MDA-MB-468 cells. **C**, survival of inoculated mice described in **B**. **D**, exemplary mice from MDA-MB-231 or MDA-MB-468 cohorts harboring intracranial tumors and extracted CSF (bottom). **E**, RT-PCR analysis of genes involved in breast cancer metastasis to the brain in MDA-MB-231 and MDA-MB-468 cells harvested from cell culture. **F**, bioluminescence imaging of mice that received intracranial MDA-MB-231 inoculation and either vehicle or celecoxib every other day 1 week following inoculation (25 mg/kg, i.p.). Timeline of events is shown at the top. **G**, concomitant enumeration of CTCs and CSFTCs in a mouse harboring an intracranial MDA-MB-231 tumor exhibiting tumor cell dissemination into the CNS.

weight (Fig. 1F and Supplementary Fig. S3A). Furthermore, tumor cell dissemination throughout the neuroaxis was inhibited in the celecoxib cohort of mice (Supplementary Fig. S3B). In accordance with these observations, extraction of CSF corroborated live imaging observations, revealing a lower CSFTC burden in the celecoxib cohort of mice compared with the vehicle cohort of mice (Supplementary Fig. S3C). Although apparent, the effect of celecoxib on tumor cells burden only approached significance ($P = 0.12$), which is likely due to the heterogeneity of the model.

Because of the high burden of CSFTCs observed in MDA-MB-231 cohort of mice and the aggressiveness of the model, we sought to simultaneously evaluate the burden of tumor cells in the CSF as well as the peripheral blood of mice using the CellSearch system (Supplementary Fig. S4). Concomitant analysis of CSFTCs and CTCs in a mouse with intracranial MDA-MB-231 tumor cells corroborated a strong burden of 14,992 CSFTCs and also identified two CTCs (Fig. 1G). This observation suggests that some CSFTCs in the CNS compartment may

be capable of egress into systemic circulation, which warranted clinical investigation.

We monitored the number of CSFTCs and CTCs in 9 patients with breast cancer with metastatic spread to the CNS undergoing intrathecal chemotherapy with or without systemic therapy (Supplementary Table S1). Positive CSF cytology generally correlated with higher CSFTC and CTC burden, though three samples contained high CSFTC burden that were negative by CSF cytology (Fig. 2A). This observation supports our previous findings that CSFTC enumeration is more accurate and sensitive than CSF cytology (2). Examining immediate changes in disseminated tumor cell burden in relation to changes in clinical symptoms indicated that changes in neurologic symptoms may be positively correlated with changes in CSFTC burden (Supplementary Fig. S5A). Similarly immediate changes in systemic clinical symptoms correlated positively with changes in CTC counts (Fig. 2C). The majority of the clinical samples contained <100 CSFTCs and approximately half of the samples contained <5 CTCs,

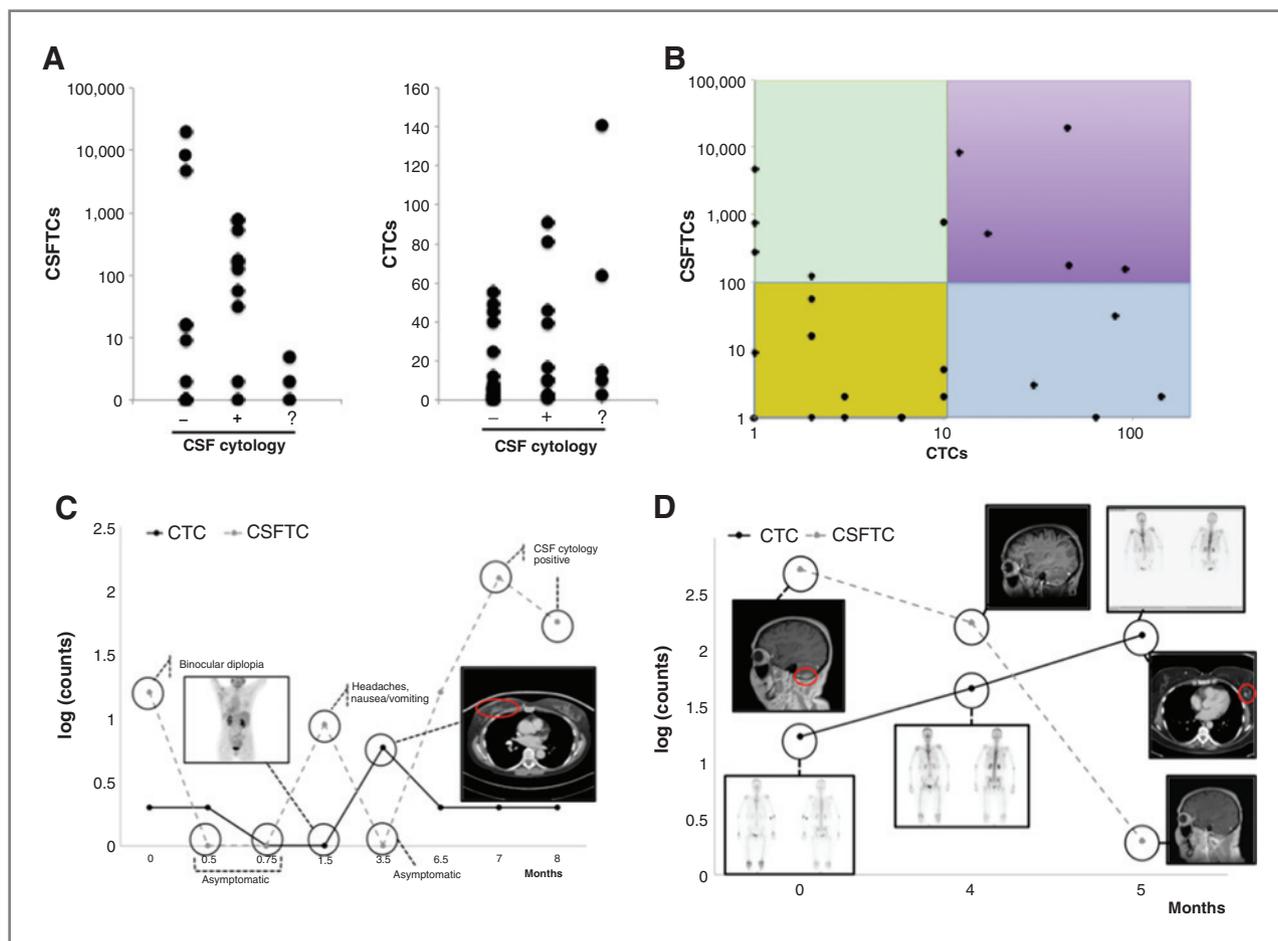


Figure 2. Dynamic changes in CTC and CSFTC counts in patients with breast cancer undergoing treatment. **A**, CSFTC and CTC counts in 9 patients with metastatic breast cancer with CNS involvement as a function of CSF cytology conducted in parallel. CSF cytology results shown as negative (–), positive (+), or ambiguous (?). **B**, plot of CSFTC versus CTC concomitant enumeration in 9 patients with metastatic breast cancer over the course of treatment. Data points in the upper right purple quadrant represent samples with high CSFTC and CTC burden. These samples correspond to patients A1, A2, A3, and A4. Examples of patients with metastatic breast cancer demonstrating intracompartmental disease transitioning with systemic recurrence involving the primary site (A4; **C**) as well as bone metastasis (A3; **D**). Tumor cell burden was assessed by CSFTC and CTC counts during treatment. MRI and PET imaging are shown in the bottom panels for this patient. Red circles indicate evidence of malignant disease.

which is the U.S. Food and Drug Administration-approved prognostic cutoff value for metastatic breast cancer (Supplementary Fig. S5B).

Examining the relationship between CSFTC and CTC counts obtained from 9 patients with metastatic breast cancer revealed heterogeneous results with a slight inverse correlation (Fig. 2B). This observed correlation is paradoxical to the trend expected with the linear unidirectional metastatic disease progression model that would predict a positive linear slope in this plot. Our data suggest that at any given time, such patients are more commonly burdened with disease in their systemic circulation or their CSF, but rarely both. Interestingly, patients with samples that contained high simultaneous tumor burden in the peripheral blood and CSF (>10 CTCs and >100 CSFTCs, respectively) were the same subset of patients (A1, A2, A3, A4) that exhibited transitioning of tumor cells burden from one compartment to another unlike the other patients (Fig. 2B). Interestingly, these same patients had a longer median

survival time, from the onset of enrollment, than the rest of the patients in our cohort (392 days vs. 190 days; log-rank $P = 0.0325$). This suggests that intercompartmental cycling is not a late event in the course of this disease. Together, these results suggest that high disease burden in both systemic and CNS compartments may represent a transient phenomenon during the course of disease progression.

Intercompartmental cycling of malignant disease during therapy was apparent in 4 of the 9 enrolled patients with metastatic breast cancer (patients A1, A2, A3, A4). One patient (A4) possessed approximately 1,000 CSFTCs before intrathecal therapy and had malignant disease involving the cerebellum but lacked systemic disease involvement as determined by MRI and positron emission tomography (PET) imaging (Fig. 2C). Over the course of intrathecal therapy, the patient's CSFTC count dropped more than two orders of magnitude and the malignant disease in the CNS resolved by MRI. Interestingly, the patient's CTC count rose significantly during

the same timespan, and clinical imaging revealed evidence of systemic disease involving the spinal column as well as the breast. Another patient (A3) also had recurrence of disease at the primary site, i.e., breast (Fig. 2D). These observations are in line with compelling preclinical evidence that suggests that tumors exhibit tumor self-seeding, whereby CTCs return to the primary site to colonize, and that these self-seeded colonies may represent a considerable amount of the primary tumor (12).

Patients in this study were undergoing compartmentalized CNS-directed therapy alone or in combination with systemic chemotherapy. High-dose systemic chemotherapy has been proposed to combat neoplastic meningitis; however, poor perfusion into the CNS may explain its failure to show benefit. Our observations support a model whereby tumor cells may enter the CSF early in the course of the disease, evade otherwise successful systemic chemotherapy by virtue of the "privileged site" status of the CSF, and then re-emerge after the completion of systemic treatment and lead to extraneural relapse. Previous studies have found that lymphoma cells can migrate into the CSF as a site of refuge as an early event in leukemia and lymphoma patients (13–15). This paradigm is well established in acute lymphoblastic leukemia (ALL) and is the basis of the universal practice of up-front CSF prophylaxis in that disease (16). This practice is the primary reason that up to 90% of children with ALL are cured with current treatment regimens. A similar scenario is now widely believed to account for the behavior of aggressive diffuse large B-cell lymphomas (DLBCL) in the postrituximab era. This ontologic sequence has not been applied to solid tumors previously, but would recapitulate our experience with ALL and DLBCL.

There is mounting evidence in the literature that the natural history of solid tumor progression, including breast cancer, is related to microscopic disease behavior, and the increasing frequency of overt CNS metastases (20%–30% of patients with HER2-positive breast cancer and as many as 45% of triple-negative patients). Our data imply that the CSF is a relevant site of microscopic residual disease in this setting. If true, this hypothesis also suggests that incorporating CSF-directed therapy into the front-line treatment of newly diagnosed patients with high-risk breast cancer may be necessary to realize an increased cure rate similar to the increase seen three decades ago when this paradigm became standard care for leukemia.

Our molecular observations strongly suggest that COX-2 is responsible for the genesis of CSFTCs that may be capable of escaping into systemic circulation to potentiate recurrence. COX-2 is an enzyme involved in the initiation of prostaglandin synthesis that has been implicated in numerous aspects of cancer, including carcinogenesis, angiogenesis, and distant metastases. In breast cancer, a relationship between COX-2 overexpression in primary tumors and distant metastases has been demonstrated in a study of 29 patients (17). COX-2 has been linked to a worse prognosis in colorectal cancer (18), and inhibition of COX-2 by celecoxib or aspirin has proven effective in reducing colo-

rectal cancer risk (19). On the basis of our observations, the expression of COX-2 in metastatic brain lesions from breast cancer should be investigated for its prognostic potential, particularly with respect to relapse. In addition, future clinical studies should also evaluate the relationship between COX-2 expression in brain metastases from primary breast cancer and disease intercompartmental transitioning based on the clinical and murine studies presented herein. Although COX-2 seems to contribute to the genesis of CSFTCs in this study, "omic" analyses of CSFTCs and CTCs from patients with transitioning disease are warranted to identify other genetic determinants that may reveal additional therapeutic targets to preclude further disease dissemination.

A prior clinical study in 11 evaluable patients found that adding celecoxib to trastuzumab in trastuzumab-refractory metastatic breast cancer failed to demonstrate an effect on disease progression. This pilot study reported that the combination was well tolerated, though it is unclear whether any of these patients had brain metastases or high levels of COX-2 expression (20). Our findings with celecoxib and its relative safety indicate that COX-2 inhibitors should be evaluated for their ability to reduce metastatic tumor burden, CSFTC burden, and relapse specifically in patients with breast cancer with brain metastases that express COX-2.

Disclosure of Potential Conflicts of Interest

J.E. Allen has ownership interest (including patents) in an unlicensed patent. M. Glantz has received honoraria from the speakers' bureau and is a consultant/advisory board member of SigmaTau Pharmaceuticals. W.S. El-Deiry has ownership interest (including patents) in an unlicensed patent. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: J.E. Allen, A. Patel, D.T. Dicker, J.M. Sheehan, W.S. El-Deiry

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.E. Allen, A. Patel, V.V. Prabhu, D.T. Dicker, M. Glantz

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.E. Allen, A. Patel, V.V. Prabhu, D.T. Dicker, M. Glantz

Writing, review, and/or revision of the manuscript: J.E. Allen, A. Patel, D.T. Dicker, J.M. Sheehan, M. Glantz, W.S. El-Deiry

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