Human Cancer Biology

αB-Crystallin: A Novel Regulator of Breast Cancer Metastasis to the Brain


North Carolina; Breast Cancer Unit, Medical Oncology Department, Vall d’Hebron Institute of Oncology, Barcelona, Spain; Center for Developmental Therapeutics, Northwestern University, Evanston, Illinois; Department of Pathology and Lab Medicine, Carolinas Medical Center, Charlotte, North Carolina; Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota; and Department of Medicine and Department of Pathology, Duke University Medical Center, Durham, North Carolina

Abstract

Purpose: Basal-like breast tumors are typically (ER/PR/HER2) triple-negative and are associated with a high incidence of brain metastases and poor clinical outcomes. The molecular chaperone αB-crystallin is predominantly expressed in triple-negative breast cancer (TNBC) and contributes to an aggressive tumor phenotype in preclinical models. We investigated the potential role of αB-crystallin in brain metastasis in TNBCs.

Experimental Design: αB-crystallin expression in primary breast carcinomas and brain metastases was analyzed by immunohistochemistry among patients with breast cancer with brain metastases. αB-crystallin was overexpressed or silenced in two different TNBC cell lines. The effects on cell adhesion to human brain microvascular endothelial cells (HBMEC) or extracellular matrix proteins, transendothelial migration, and transmigration across a HBMEC/astrocyte coculture blood–brain barrier (BBB) model were examined. In addition, the effects of overexpressing or silencing αB-crystallin on brain metastasis in vivo were investigated using orthotopic TNBC models.

Results: In a cohort of women with breast cancer brain metastasis, αB-crystallin expression in primary breast carcinomas was associated with poor overall survival and poor survival after brain metastasis, even among patients with TNBC. Stable overexpression of αB-crystallin in TNBC cells enhanced adhesion to HBMECs, transendothelial migration, and BBB transmigration in vitro, whereas silencing αB-crystallin inhibited these events. αB-crystallin promoted adhesion of TNBC cells to HBMECs, at least in part, through an α3β1 integrin–dependent mechanism. αB-crystallin overexpression promoted brain metastasis, whereas silencing αB-crystallin inhibited brain metastasis in orthotopic TNBC models.

Conclusion: αB-crystallin is a novel regulator of brain metastasis in TNBC and represents a potential biomarker and drug target for this aggressive disease. Clin Cancer Res; 1–12. ©2013 AACR.

Introduction

Brain metastases are a devastating complication in 10% to 15% of women with advanced breast cancer that result in neurologic deficits, including headaches, cognitive problems, seizures, and/or motor deficits (1). Brain metastases are often a late occurrence in women with preexisting metastatic disease in other organs and typically follow a rapidly progressive course with a 1-year survival rate less than 20%. Unfortunately, the incidence of brain metastases appears to be increasing with the advent of improved systemic therapies, which are frequently ineffective against brain metastases because of their limited ability to cross the blood–brain barrier (BBB), a distinctive permeability barrier composed of human brain microvascular endothelial cells (HBMEC) interconnected by tight junctions and reinforced by astrocytes, pericytes, and a basement membrane (2, 3). The BBB is also a formidable obstacle for circulating tumor cells to extravasate into the brain, a critical step in brain metastasis.

Recent studies in animal models have provided new insights into the mechanisms by which tumor cells cross the BBB and colonize the brain. Circulating tumor cells arrest in the brain microvasculature, often at capillary branches, where they adhere to HBMECs and/or the subendothelial basement membrane, distending the capillary and extravasating through gaps in the microvessel wall (2–5). Tumor
cells proliferate in the brain in close proximity to the exterior surface of capillaries from which they extravasate, a process termed “vascular cooption,” although some metastatic tumor cells rely on angiogenesis for perivascular growth (2–6). The metastatic tumor cells initiate a localized inflammatory response through reciprocal interactions with reactive astrocytes and microglia that promote tumor growth (2, 3, 5). Hence, dynamic interactions between tumor cells and diverse cell types in the brain contribute to brain metastasis.

Molecular profiling of breast tumors has provided additional insights into brain metastasis. Brain metastases are most prevalent in HER2/ErbB2-positive and basal-like breast tumors; the latter express basal epithelial genes and are often ‘triple (ER/PR/HER2)-negative’ (7–9). Brain metastases in triple-negative breast cancer (TNBC) are particularly challenging given the lack of targeted agents and poor clinical outcomes. In one cohort, brain metastases were present in 46% of patients with metastatic TNBCs and were associated with median survival of 4.9 months after diagnosis (10). Moreover, patients with early-stage TNBC have a high incidence (4.7%) of brain metastasis as a first site of relapse (11). Although a brain metastasis gene signature was recently reported (12), the specific genes that regulate brain metastasis in TNBC are poorly understood. Identification of these genes is a critical first step in the development of biomarkers and targeted therapies for brain metastases.

The molecular chaperone αB-crystallin is predominantly expressed in basal-like breast cancer/TNBC and is associated with poor outcomes (13–15). αB-crystallin has been linked to many biologic characteristics of these aggressive tumors. αB-crystallin promotes apoptosis resistance, at least in part, by inhibiting caspase-3 activation, thereby enhancing cell survival in the setting of oncogenic stress, growth factor depletion, chemotherapy, and other cellular stressors (16–19). αB-crystallin promotes cell migration and invasion and localizes to the infiltrative edge of malignant glioblastomas (13, 20–22). These effects are likely mediated by direct interactions of αB-crystallin with actin and intermediate filaments, which regulate cytoskeletal stability and dynamics (23, 24). In addition, αB-crystallin expression is dramatically increased in two highly metastatic TNBC cell lines identified by in vivo selection (including GILM2 cells used in our experiments) compared with the less metastatic parental cells (25). However, the functional role of αB-crystallin in metastasis has not been studied. We postulated that αB-crystallin might contribute to the observed proclivity of TNBCs to metastasize to the brain.

Here, we report that αB-crystallin is commonly expressed in breast cancer brain metastases and show that its expression in primary breast carcinomas predicts poor survival in patients with brain metastasis. Stable overexpression of αB-crystallin in TNBC cells enhanced adhesion to HBMECs, transendothelial migration, and transmigration through a BBB model in vitro, whereas silencing αB-crystallin inhibited these events. αB-crystallin promoted adhesion of TNBC cells to HBMECs, at least in part, by an α3β1 integrin-dependent mechanism. Moreover, αB-crystallin promoted brain metastasis in vivo in orthotopic TNBC models. Our findings indicate that αB-crystallin is a novel regulator of brain metastasis in TNBC and point to αB-crystallin and α3β1 integrin as potential drug targets for this devastating disease.

Materials and Methods

Breast cancer brain metastasis cohort and immunohistochemical analyses

Patients with a diagnosis of breast cancer and brain metastases who were treated at the University of North Carolina at Chapel Hill (1989–2006) and Duke University Medical Center (1985–2005) with available tumor tissue (breast, brain, or both) and survival data were included. Additional data included age, gender, race, tumor estrogen receptor (ER)/progesterone receptor (PR)/HER2 status, and therapies. For cases with sufficient tissue, ER, PR, and HER2 status was determined by immunohistochemistry (IHC). Eighty-seven formalin-fixed, paraffin-embedded (FFPE) tissues (49 brain metastases and 38 breast tumors, including 11 paired tumors) were available from 76 patients. The study was approved by the respective Institutional Review Boards.

FFPE tissue sections were incubated in 3% hydrogen peroxide/methanol for 10 minutes, followed by antigen retrieval in steaming citrate buffer for 30 minutes. Sections were preincubated in horse serum (Vector Laboratories) and then incubated for 60 minutes with antibodies against ER (1D5, 1:50, Dako), PR (16, 1:70, Vision Biosystems), HER2 (CB11, 1:100, BioGenex), or αB-crystallin (1B6.1-3G4, 1:200, Enzo Life Sciences/Stressgen) using a DakoCytomation autostainer. An avidin/biotin complex
(VectorStar Elite) was applied for 30 minutes followed by
diaminobenzidine (Innovex) and hematoxylin (DakoCy-tomation). ER and PR staining were scored using the Allred system (26). HER2 was scored using ASCO/CAP guidelines (27). Breast cancer subtype was assigned by primary tumor
IHC as ER/PR or hormone receptor (HR)-positive/HER2-
negative (HR+/HER2−), triple-negative (HR−/HER2−), or
HR-positive/negative and HER2-positive (HER2+). β-crystallin was scored as negative (0%) or positive (>0%) based
on tumor cell expression.

Statistical analysis for associations between subtypes and
β-crystallin expression was conducted using the Fisher
exact test. Time-to-event analyses were done for overall
survival (time from breast tumor diagnosis to death or last
contact) and overall survival from brain metastasis (time
from the date of brain metastasis to the date of death or
last contact). The Kaplan–Meier method and log-rank sta-
tistics were used to estimate survival and to evaluate asso-
ciations with β-crystallin expression. Breast cancer subtype
was available for 71 of 76 patients. Date of brain metastasis
was available for 75 patients. Statistical analyses were con-
ducted with SAS 9.2 software.

Cell lines and culture

Human GILM2 and MDA-MB-231 TNBC cells expressing
mCherry fluorescent protein (231-mCherry) were described (28). 231-mCherry cells were grown in DMEM/F12 media
with 5% FBS, 100 units/ml penicillin/streptomycin, non-
essential amino acids, and 1 mmol/L sodium pyruvate
(Invitrogen). GILM2 cells were cultured in DMEM/F12
media with 10% FBS, 100 units/ml penicillin/streptomycin
and Insulin/Transferrin/Sodium Selenite mix (Invitrogen).
GILM2 cells were cultured in DMEM/F12
media with 5% FBS, 100 units/ml penicillin/streptomycin
and non-

Lentiviral and retroviral transduction

pL3.7Rsv-mCherry lentivirus was generated in 293T
cells using the ViraPower Lentiviral Expression system (Invi-
trogen) and used to stably transduce GILM2 cells. Retro-
viruses were produced in Phoenix cells and used to infect
cancer cells as described (13, 29). 231-mCherry cells
were infected with pLXSN or pLXSN-β-crystallin
retrovirus (13), whereas GILM2-mCherry cells were infected
with pSM2, pSM2-CRYABsh1, or pSM2-CRYABsh2 (Open
Biosystems, RHS1764-9393967 and RHS1764-9691062,
respectively). Cells were selected for growth in the appro-

Transendothelial migration and BBB transmigration

Transendothelial migration was assayed as described (30). Transwell inserts with 8-μm pores (BD Biosciences) were coated with fibronectin (20 μg/ml, Invitrogen), washed, and
blocked with 2% bovine serum albumin (BSA) for 1 hour at
37°C. Fifty thousand cells were added to each well, incu-
bated for 20 to 30 minutes at 37°C, washed, and the
attached cells were fixed, stained with crystal violet, and
scored per 10× field.

Adhesion to brain endothelium

HBMECs were grown on fibronectin-coated 24-well
plates until confluent. Fifty thousand cancer cells were
added to each well. After 2 hours (231-mCherry) or 4 hours
(GILM2-mCherry), cells were washed, and the attached cells
were fixed in 10% formalin and scored per 10× magnifi-
cation field (Leica MZ10F stereomicroscope). In some
experiments, cells were preincubated for 1 hour with integ-
rin blocking antibodies (Millipore).

Adhesion to extracellular matrix proteins

Twenty-four-well plates were coated with fibronectin,
collagen I, or laminin (20 μg/ml, Invitrogen), washed, and
blocked with 2% bovine serum albumin (BSA) for 1 hour at
37°C. Fifty thousand cells were added to each well, incu-
bated for 20 to 30 minutes at 37°C, washed, and the
attached cells were fixed, stained with crystal violet, and
scored per 10× field.

Immunocytochemistry of HBMECs

HBMECs (passage #4–5) were grown to confluence on
tissue-culture plastic, fixed with 4% paraformaldehyde (or
100% ice-cold methanol), and permeabilized with 0.1% Triton-X100. Cells were blocked with 10% goat serum and
then incubated with primary antibodies against β-catenin
(1:100, BD Biosciences), claudin-5 (1:100, Life Technolo-
gies), GLIT-1 (1:50, SPMM98 clone, Thermo-Fisher),
occludin (1:100, Life Technologies), PECAM-1 (1:50,
Thermo-Fisher), VE-cadherin (1:50, F8 clone, Santa Cruz),
and ZO-1 (1:100, Life Technologies) overnight at 4°C. Anti-
mouse and anti-rabbit conjugated Alexa Fluor secondary
antibodies (1:200, Life Technologies) were incubated with
the monolayers for 1 hour. Nuclei were counterstained with
30 nmol/L 4′,6-diamidino-2-phenylindole (DAPI, Sigma).
Immunolabeled HBMECs were visualized with an inverted
epifluorescence microscope (Olympus) and images
acquired using a 16-bit SPOT camera (Diagnostic Instru-
ments) with Metavue software (Molecular Devices). Images
were visualized and processed using NIH Image software.

β-Crystallin Promotes Brain Metastasis

Twenty-four-well plates were coated with fibronectin,
collagen I, or laminin (20 μg/ml, Invitrogen), washed, and
blocked with 2% bovine serum albumin (BSA) for 1 hour at
37°C. Fifty thousand cells were added to each well, incu-
bated for 20 to 30 minutes at 37°C, washed, and the
attached cells were fixed, stained with crystal violet, and
scored per 10× field.

Transendothelial migration and BBB transmigration

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blocked with 2% bovine serum albumin (BSA) for 1 hour at
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Thermo-Fisher), VE-cadherin (1:50, F8 clone, Santa Cruz),
and ZO-1 (1:100, Life Technologies) overnight at 4°C. Anti-
mouse and anti-rabbit conjugated Alexa Fluor secondary
antibodies (1:200, Life Technologies) were incubated with
the monolayers for 1 hour. Nuclei were counterstained with
30 nmol/L 4′,6-diamidino-2-phenylindole (DAPI, Sigma).
Immunolabeled HBMECs were visualized with an inverted
epifluorescence microscope (Olympus) and images
acquired using a 16-bit SPOT camera (Diagnostic Instru-
ments) with Metavue software (Molecular Devices). Images
were visualized and processed using NIH Image software.

Adhesion to brain endothelium

HBMECs were grown on fibronectin-coated 24-well
plates until confluent. Fifty thousand cancer cells were
added to each well. After 2 hours (231-mCherry) or 4 hours
(GILM2-mCherry), cells were washed, and the attached cells
were fixed in 10% formalin and scored per 10× magnifi-
cation field (Leica MZ10F stereomicroscope). In some
experiments, cells were preincubated for 1 hour with integ-
rin blocking antibodies (Millipore).

Adhesion to extracellular matrix proteins

Twenty-four-well plates were coated with fibronectin,
collagen I, or laminin (20 μg/ml, Invitrogen), washed, and
blocked with 2% bovine serum albumin (BSA) for 1 hour at
37°C. Fifty thousand cells were added to each well, incu-
bated for 20 to 30 minutes at 37°C, washed, and the
attached cells were fixed, stained with crystal violet, and
scored per 10× field.

Transendothelial migration and BBB transmigration

Transendothelial migration was assayed as described (30). Transwell inserts with 8-μm pores (BD Biosciences) were coated with fibronectin (20 μg/ml, Invitrogen), washed, and
blocked with 2% bovine serum albumin (BSA) for 1 hour at
37°C. Fifty thousand cells were added to each well, incu-
bated for 20 to 30 minutes at 37°C, washed, and the
attached cells were fixed, stained with crystal violet, and
scored per 10× field.

Immunocytochemistry of HBMECs

HBMECs (passage #4–5) were grown to confluence on
tissue-culture plastic, fixed with 4% paraformaldehyde (or
100% ice-cold methanol), and permeabilized with 0.1% Triton-X100. Cells were blocked with 10% goat serum and
then incubated with primary antibodies against β-catenin
migrating through the endothelial and astrocyte layers were scored per 10× field at 48 hours (231-mCherry) or 72 hours (GILM2-mCherry).

**Immunoblotting**

Immunoblotting was conducted as described (13) with antibodies for αB-crystallin (Enzo Life Sciences/Stressgen) or β-actin (Sigma).

**Cell surface expression of integrins**

The cell surface expression of integrins was determined using an αβ integrin–mediated Cell Adhesion Array kit (CHEMICON). Absorbance at 570 nm was measured using a SpectraMax Plus384 Absorbance MicroPlate Reader (Molecular Devices).

**Orthotopic models of breast cancer brain metastasis**

231-mCherry cells stably expressing vector or αB-crystallin (2.5 × 10^5) or GILM2-mCherry cells stably expressing a nonsilencing construct or shRNA1 targeting αB-crystallin (1 × 10^5) were resuspended in 100% Matrigel (BD Bioscience) and injected bilaterally into the ducts of the fourth mammary glands of 4- to 5-week-old female NOD scid IL2 receptor γ chain knockout (NSG) mice (Jackson Laboratory). Tumor volume was measured weekly as described (32). For GILM2 xenografts, mammary tumors were resected 9 weeks after tumor inoculation to allow sufficient time for metastasis. Mice were euthanized at 7 weeks (231-mCherry) or 12 weeks (GILM2-mCherry). Images of isolated whole brains were obtained (Leica MZ10F fluorescent stereomicroscope) and analyzed with ImageJ software. All animal experiments were approved by the institutional Animal Care and Use Committee.

**Statistical methods**

Statistical significance was assessed as described in "Breast cancer brain metastasis cohort and immunohistochemistry analyses" or by ANOVA with posttests using GraphPad Prism software.

**Results**

**αB-crystallin predicts poor survival in patients with breast cancer brain metastases**

We examined αB-crystallin expression by IHC in primary breast carcinomas and brain metastases in 76 patients with breast cancer who developed brain metastases (Supplementary Table S1). Thirty-seven percent (14 of 38) of primary breast cancers and 47% (23 of 49) of brain metastases expressed αB-crystallin. Representative IHC staining of paired breast tumor and brain metastases from 2 patients is shown (Fig. 1A). Concordance between paired breast tumors and brain metastases was 55% (6 of 11, Supplementary Table S2). It was more common for breast metastases to gain αB-crystallin expression (36%, 4 of 11) than to lose expression of this protein (9%, 1 of 11). αB-crystallin expression in primary breast tumors was associated with breast cancer subtype defined by IHC (P < 0.0001, Supplementary Table S3). Triple-negative (HR+/HER2−) breast cancers were more likely to express αB-crystallin (73%, 11 of 15) compared with HR+/HER2− (10%, 1 of 10) or HER2+ tumors (0%, 0 of 11). These findings indicate that αB-crystallin is commonly expressed in breast cancer brain metastases and confirm the association between αB-crystallin expression and TNBC.

We next examined the relationship between αB-crystallin expression and survival. The median follow-up for survivors was 6.5 years from diagnosis and 1.7 years from time of brain metastases; 79% (60 of 76) of patients had died when these analyses were completed. Overall survival was inferior among patients with αB-crystallin–positive breast tumors compared with αB-crystallin–negative breast tumors [1.4; 95% confidence interval (CI), 0.79–3.01 vs. 4.7; 95% CI, 2.79–9.11 years; P = 0.0002; Fig. 1B]. Similarly, overall survival from the time of brain metastases was inferior among patients with αB-crystallin–positive breast tumors compared with αB-crystallin–negative tumors (0.13; 95% CI, 0.01–0.3 vs. 0.91; 95% CI, 0.13–3.37 years; P = 0.001). Among patients with TNBC, αB-crystallin expression in the primary breast tumors was associated with lower overall survival rates (1.4; 95% CI, 0.79–3.01 vs. 4.7; 95% CI, 1.43–not estimable years; P = 0.01) and survival following brain metastases (0.14; 95% CI, 0–0.3 vs. 1.5; 95% CI, 0.13–not estimable years; P = 0.02) compared with those with αB-crystallin–negative breast tumors. αB-crystallin expression in brain metastases did not predict survival in this cohort (data not shown). These results indicate that αB-crystallin expression in primary breast carcinomas is associated with poor overall survival and poor survival after brain metastasis, even among patients with TNBC.

**αB-crystallin promotes adhesion of TNBC cells to brain endothelium, transendothelial migration, and BBB transmigration in vitro**

The extravasation of breast cancer cells into the brain is a critical stepwise process in brain metastasis that begins with their adhesion to brain microvascular endothelium, followed by migration through the BBB (2, 3). We modeled these metastatic steps in vitro by examining the ability of TNBC cells to adhere to HBMEndCs, migrate through a layer of HBMECs (transendothelial migration), and pass through a model of the BBB composed of HBMEndCs and human astrocytes grown on opposite sides of Transwell inserts (12, 31). HBMEndCs formed a modest restrictive barrier that was enhanced by coculture with astrocytes (Supplementary Fig. S1). In addition, HBMEndC monolayers expressed the vascular markers PECAM-1 and VE-cadherin, the BBB marker GLUT-1, and the BBB cell junction–associated proteins claudin-5, ZO-1, occludin, and β-catenin (Fig. 2A).

To determine the role of αB-crystallin in these steps, we stably overexpressed vector or αB-crystallin in MDA-MB-231-mCherry TNBC cells (abbreviated 231-mCherry-Vector and 231-mCherry-αB cells, respectively). Immunoblot analysis confirmed robust expression of αB-crystallin in 231-mCherry-αB cells, whereas 231-mCherry-Vector cells did not express detectable αB-crystallin protein.
αB-crystallin overexpression increased adhesion to HBMECs (Fig. 2C), transendothelial migration through HBMECs (Fig. 2D), and BBB transmigration in vitro (Fig. 2E). In addition, we stably silenced αB-crystallin in GILM2-mCherry TNBC cells with 2 different shRNAs (sh-αB1 and sh-αB2), which reduced αB-crystallin levels compared with GILM2-mCherry cells stably expressing a nonsilencing (NS) construct (Fig. 3A). To control for potential off-target effects, we coexpressed sh-αB1 with an RNAi-resistant mutant αB-crystallin that restored expression of the wild-type protein (sh1-αBM). Silencing αB-crystallin in GILM2-mCherry cells inhibited adhesion to HBMECs (Fig. 3B), transendothelial migration (Fig. 3C), and BBB transmigration in vitro (Fig. 3D) compared with GILM2-mCherry-NS and GILM2-mCherry-sh1-αBM cells. Notably, altering αB-crystallin levels by overexpression and/or gene silencing did not affect cell viability under standard cell culture conditions (Supplementary Fig. S2A and S2B). Collectively, these
findings indicate that αB-crystallin promotes adhesion of TNBC cells to HBMECs, transendothelial migration, and BBB transmigration in vitro.

**αB-crystallin increases adhesion of TNBC cells to HBMECs, at least in part, through an α3β1 integrin-dependent mechanism and promotes adhesion to extracellular matrix proteins**

Because integrins play an important role in the adhesion of cancer cells to endothelial cells in the microvasculature (33–35), we postulated that the observed effects of αB-crystallin in promoting adhesion to HBMECs might be mediated by one or more integrins. Neither overexpression of αB-crystallin in 231-mCherry cells nor silencing αB-crystallin in GILM2-mCherry cells affected the cell surface expression of several α/β integrin subunits (Fig. 4A). Nevertheless, integrin α3 and β1 blocking antibodies (but not several other integrin-blocking antibodies) inhibited the enhanced adhesion of 231-mCherry-αB cells to HBMECs (Fig. 4B, left). Similarly, integrin α3 and β1 blocking antibodies attenuated the adhesion of GILM2-mCherry-NS cells to HBMECs to a level comparable to that observed in GILM2-mCherry-sh1-αB cells (Fig. 4B, right). In contrast, the integrin β1 blocking antibody had no effect on the adhesion of 231-mCherry-Vector or GILM2-sh-αB cells to HBMECs. The integrin α3 blocking antibody inhibited adhesion of 231-mCherry-Vector cells (albeit to a lesser extent than observed in 231-mCherry-αB cells) but not GILM2-mCherry-sh-αB cells to HBMECs. These results indicate that αB-crystallin promotes adhesion of TNBC cells to HBMECs, at least in part, by an α3β1 integrin-dependent mechanism.

We next examined whether αB-crystallin regulated adhesion of TNBC cells to extracellular matrix (ECM) proteins, which are present in subendothelial microvasculature and on the exterior of microvessels in organs (6, 36).
231-mCherry-αB cells adhered more robustly to laminin, fibronectin, and collagen than 231-mCherry-Vector cells, whereas GILM2-sh1-αB cells exhibited reduced adhesion to laminin and collagen compared with control GILM2-mCherry-NS cells (Fig. 4C). These results indicate that αB-crystallin promotes adhesion of TNBC cells to multiple ECM proteins, including laminin, a key step in both extravasation and perivascular growth of micrometastases.

αB-crystallin overexpression increases brain metastases in an orthotopic TNBC model

To explore the potential role of αB-crystallin in brain metastasis in vivo, we injected 231-mCherry-αB or 231-mCherry-Vector cells intraductally into the fourth mammary glands of NSG mice. αB-crystallin overexpression did not affect mammary tumor growth in this model (Fig. 5A). αB-crystallin overexpression in mammary tumors was confirmed by immunoblotting and IHC (Fig. 5B). Mice were euthanized 7 weeks after tumor inoculation and mCherry-fluorescent metastatic lesions were identified at autopsy. Both groups of mice had widespread metastases to many organs, including the brain, lungs, liver, lymph nodes, and other tissues (Supplementary Table S4). NSG mice with 231-mCherry-αB tumors had a greater number of mCherry-positive brain metastatic lesions and more extensive tumor burden as determined by the percentage of the surface area of the brain occupied by metastases compared with mice with 231-mCherry-Vector tumors (Fig. 5C and D). In addition, αB-crystallin overexpression resulted in increased metastatic tumor burden in the liver and bone (Supplementary Table S4 and Supplementary Fig. S3). αB-crystallin expression in brain metastases was observed by IHC in mice with 231-mCherry-αB tumors but not vector controls (Fig. 5B, bottom). Intriguingly, αB-crystallin overexpression did not affect proliferation as determined by Ki67 IHC or apoptosis as determined by active caspase-3 IHC of mammary tumors or brain metastases analyzed at 7 weeks (Supplementary Fig. S5A). These observations indicate that...
αB-crystallin promotes brain metastases in an orthotopic TNBC model with widespread metastatic dissemination.

**Silencing αB-crystallin inhibits brain metastases in an orthotopic TNBC model**

We also examined the effect of silencing αB-crystallin in TNBC cells on brain metastases in vivo. GILM2-mCherry-sh1-αB and GILM2-mCherry-NS cells were injected intradermally into the fourth mammary glands of NSG mice. Silencing αB-crystallin did not alter mammary tumor growth (Fig. 6A), despite robust reduction of αB-crystallin levels (Fig. 6B). Mammary tumors were resected at 9 weeks to allow additional time for metastatic mCherry-positive metastases were identified at autopsy 3 weeks later. Both

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Figure 4. αB-crystallin promotes adhesion to HBMECs, at least in part, by an α3β1 integrin-dependent mechanism and enhances adhesion to ECM proteins. A, the cell surface expression of various integrins in 231-mCherry breast cancer cells stably expressing vector or αB-crystallin (top) and GILM2-mCherry cells stably expressing NS or sh-αB (bottom) was determined using an α3β1 integrin-mediated cell adhesion array. Data are expressed as absorbance at 570 nm (mean ± SEM, n = 3). A negative control (c) was included. B, 231-mCherry-Vector, 231-mCherry-αB, GILM2-mCherry-NS, and GILM2-sh-αB cells were preincubated with integrin-blocking antibodies for 1 hour, seeded onto confluent HBMECs for 2 hours (231-mCherry cells) or 4 hours (GILM2-mCherry cells), washed, and attached mCherry-positive cells were scored (mean ± SEM, n = 3). C, adhesion of breast cancer cells to different ECM proteins. Cells were plated on fibronectin-, collagen-, or laminin-coated 24-well plates for 20 minutes (231-mCherry cells) or 30 minutes (GILM2-mCherry cells) at 37°C, washed, and attached cells were counted (mean ± SEM, n = 3). (A–C): ***P < 0.001; **P < 0.01; *P < 0.05; /C: /P < 0.01; /C/C: /P < 0.001.
groups of mice had metastases to many organs, including the brain, lungs, liver, lymph nodes, and other organs (Supplementary Table S4). Mice with GILM2-mCherry-sh-αB tumors had fewer brain metastases as determined by mCherry-fluorescence (Fig. 6C) and hematoxylin and eosin (H&E) staining (Fig. 6D). GILM2-mCherry-sh-αB brain metastases had reduced expression of αB-crystallin compared with NS controls (Fig. 6B). Moreover, silencing αB-crystallin reduced metastases in other organs, including the liver (Supplementary Table S4 and Supplementary Fig. S4). Silencing αB-crystallin did not affect proliferation or apoptosis as determined by IHC of mammary tumors or brain metastases analyzed at 12 weeks (Supplementary Fig. S5B). These findings provide additional evidence that αB-crystallin promotes brain metastasis in vivo in a second orthotopic TNBC model.

Discussion

The molecular pathogenesis of breast cancer brain metastasis remains poorly understood due to limited access to brain metastases from patients, a dearth of clinical trials, and the lack of animal models that recapitulate the entire metastatic cascade (37). The vast majority of animals models reported rely on intracardiac or carotid artery injection of breast tumor cells (38). We have shown that αB-crystallin, a molecular chaperone previously linked to an aggressive tumor phenotype in TNBC, glioblastoma multiforme (GBM), and other neoplasms (13, 19, 20, 22, 39), is a novel regulator of breast cancer brain metastasis. Specifically, we have shown that αB-crystallin is commonly expressed in clinical breast cancer brain metastases, including some brain metastases that developed from breast tumors that did not express αB-crystallin. αB-crystallin expression in breast carcinomas was associated with triple-negative IHC status and with poor overall survival and poor survival after brain metastasis in a cohort of breast cancer cases across all subtypes and also within the subset of TNBC cases. We have also shown a direct causal role for αB-crystallin in promoting breast cancer brain metastasis in vivo in 2 orthotopic models in which fluorescently labeled TNBC cells metastasize from the mammary gland to the brain in NSG mice. These models recapitulate the entire metastatic cascade and several clinical aspects of breast cancer brain metastasis, including triple-negative status and αB-crystallin expression by tumors, widespread metastatic disease, and late-onset brain metastases. Using overexpression and gene silencing to alter αB-crystallin levels in mammary tumors, we showed that αB-crystallin promotes brain metastases in vivo without accelerating mammary tumor growth. Collectively, our results point to a previously unrecognized role for αB-crystallin in breast cancer brain metastasis and suggest that αB-crystallin may be a useful biomarker to identify poor-prognosis patients with breast cancer who might be enrolled in clinical trials for early detection or prevention/treatment of brain metastases. Moreover, our observation that silencing αB-crystallin inhibits brain metastases suggests that αB-crystallin may be a promising drug target.
We have shown that αB-crystallin promotes several of the earliest steps in the extravasation of circulating tumor cells across the BBB, including cell adhesion to HBMECs, transendothelial migration, and transmigration across a model of the BBB in vitro. Of note, our in vitro BBB model mimics some features of the BBB, but like most in vitro BBB models, the barrier is not as tight as that observed in vivo (31). Nevertheless, our observation that αB-crystallin enhances transmigration through our in vitro BBB model and breast cancer brain metastasis in vivo strongly suggests that αB-crystallin promotes BBB penetration. Moreover, αB-crystallin promotes adhesion to several ECM proteins including laminin, which are present in the subendothelial vessel wall and mediate adhesion to intraluminal circulating tumor cells and support perivascular growth of newly extravasated tumor cells (6, 36). Notably, the initial adhesion of TNBC cells to HBMECs is dependent, at least in part, on α3β1 integrin, which has been broadly implicated in metastasis, including brain metastasis. Specifically, α3β1 integrin has been reported to mediate arrest of circulating tumor cells in the pulmonary vasculature by engaging its ligand laminin-5 in exposed regions of the vessel wall; pulmonary arrest in vivo was inhibited by an integrin β1 blocking antibody (36). α3β1 integrin is also robustly expressed in a non-small cell lung cancer cell line highly metastatic to the brain and mediates adhesion to brain slices and invasion in vitro; an α3 integrin blocking antibody dramatically suppressed brain metastases when these cells were injected into the left ventricle of mice (40, 41). In addition, β1 integrin plays a key role in the perivascular growth of early brain micrometastases upon intracardiac injection of breast and other tumor cells by mediating “vascular cooption,” the adhesion of metastatic tumor cells to the exterior surface of the vascular basement membrane of preexisting vessels and subsequent expansion of micrometastases in the perivascular niche (6). Intriguingly, deletion of β1 integrin in the MMTV-activated ErbB2 model did not affect mammary tumor induction but suppressed lung metastases in this model (42). Although we have yet to determine the nature of the interaction between αB-crystallin and α3β1 integrin, these studies suggest that inhibition of α3β1 integrin might be a promising therapeutic strategy against brain metastases arising from αB-crystallin–positive TNBCs.

In our orthotopic TNBC models, αB-crystallin increased brain metastases without affecting mammary tumor growth, suggesting that the enhanced adhesion to HBMECs and BBB transmigration may be the principal mechanisms by which αB-crystallin promotes brain metastases. However,
we cannot exclude other potential mechanisms, including increased intravasation and survival of circulating tumor cells, increased perivascular growth of micrometastases and/or diminished tumor dormancy. Although we did not observe differences in apoptosis or proliferation of mammary tumors or brain metastases at the conclusion of the experiments, αB-crystallin might affect proliferation or apoptosis during the initial perivascular expansion of micrometastases. Furthermore, we have previously reported that ectopic expression of high levels of αB-crystallin in a single clone of MDA-MB-231 cells promoted mammary tumor growth (43). No such mammary tumor growth advantage was observed in these experiments using a polyclonal population of MDA-MB-231 cells with more modest levels of αB-crystallin, suggesting that these effects may be dose-dependent. In addition, the prometastatic activity of αB-crystallin is not limited to the brain. In both orthotopic models, αB-crystallin promoted liver metastases, indicating that αB-crystallin may also regulate cell adhesion and/or extravasation in the liver microvasculature. Consistent with this idea, integrin β1 has been implicated in tumor cell extravasation and hepatic colonization (44). Although we did not observe a difference in lung metastatic tumor burden at autopsy by altering αB-crystallin levels, the lungs in all animals had extensive tumor burden by the time brain metastases became apparent, suggesting the need for more detailed analyses at earlier time points or different tumor models to determine whether αB-crystallin may affect lung metastasis as well.

In summary, our results underscore a previously unrecognized role for αB-crystallin in brain metastasis in TNBC and point to αB-crystallin and α3β1 integrin as potential drug targets for this devastating disease. We are currently using our orthotopic TNBC models to further delineate the prometastatic mechanisms of αB-crystallin and to evaluate novel therapies, including neutralizing α3β1 integrin antibodies. Moreover, our findings point to αB-crystallin as a potential biomarker to help identify patients with breast cancer who might benefit from additional diagnostic or therapeutic interventions.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: D. Malin, E.V. Shustoa, C.K. Anders, V.L. Cryns
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Petrovic, A. Al Ahmad, B. Adamo, C.R. Miller,
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Malin, V. Petrovic, A.M. Deal, A. Al
Writing, review, and/or revision of the manuscript: D. Malin, E. Stre-
kalova, V. Petrovic, A.M. Deal, A. Al Ahmad, B. Adamo, C.R. Miller, A.
Ugilkov, L. Livasy, E. Hamilton, J. Geradis, M. Fwenda, L. Carey, E.V. Shustoa,
C.K. Anders, V.L. Cryns
Administrative, technical, or material support (i.e., reporting or orga-
nizing data, constructing databases): D. Malin, K. Blackwell, J. Geradis,
L. Carey, V.L. Cryns
Study supervision: V.L. Cryns

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Dmitry Malin, Elena Strekalova, Vladimir Petrovic, et al.


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