Changes in BAI1 and Nestin Expression Are Prognostic Indicators for Survival and Metastases in Breast Cancer and Provide Opportunities for Dual Targeted Therapies

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Abstract

The 2-year survival rate of patients with breast cancer brain metastases is less than 2%. Treatment options for breast cancer brain metastases are limited, and there is an unmet need to identify novel therapies for this disease. Brain angiogenesis inhibitor 1 (BAI1) is a GPCR involved in tumor angiogenesis, invasion, phagocytosis, and synaptogenesis. For the first time, we identify that BAI1 expression is significantly reduced in breast cancer and higher expression is associated with better patient survival. Nestin is an intermediate filament whose expression is upregulated in several cancers. We found that higher Nestin expression significantly correlated with breast cancer lung and brain metastases, suggesting both BAI1 and Nestin can be therapeutic targets for this disease. Here, we demonstrate the ability of an oncolytic virus, 34.5ENVE, to target and kill high Nestin-expressing cells and deliver Vstat120 (extracellular fragment of BAI1). Finally, we created two orthotopic immune-competent murine models of breast cancer brain metastases and demonstrated 34.5ENVE extended the survival of immune-competent mice bearing intracranial breast cancer tumors.

Introduction

Breast cancer is one of the leading causes of brain metastases. The 2-year survival rate of patients with breast cancer brain metastases (BCBM) is less than 2% (1). Treatment options for patients refractory to standard surgery and radiotherapy are limited. These tumors are frequently resistant to conventional chemotherapeutic drugs and antibody-based therapies, which poorly penetrate the blood brain barrier (2, 3). There is an unmet need to identify novel, targeted strategies to treat this disease.

Vstat120 is a cleaved and secreted fragment of brain angiogenesis inhibitor 1 (BAI1). The loss of BAI1 expression is observed in several cancers, including glioblastoma, colorectal cancer, gastric cancer, and renal cell carcinoma (4). The reexpression of BAI1 or Vstat120 exerts potent antiangiogenic and antitumor effects in animal models of glioblastoma and renal cell carcinoma (5, 6). Surprisingly, its expression and function has not been examined in breast cancer. Nestin is an intermediate filament whose expression is upregulated in several cancers (7–9). Nestin is also expressed in cancer stem cells, which are known to promote cancer resistance and progression (10). Here, we determined the roles of BAI1 and Nestin gene expression in breast cancer metastases and patient survival. We found that lower BAI1 expression correlates with poorer patient survival, and high Nestin expression is associated with an increased probability of metastases. 34.5ENVE is an oncolytic herpes simplex virus that expresses Vstat120, and its replication is driven by a cancer stem cell–specific Nestin promoter (11). 34.5ENVE has demonstrated unparalleled antitumor efficacy in murine models of glioblastoma (11). Given the roles of Nestin and BAI1 in BCBMs, we tested the ability of 34.5ENVE to target breast cancer cells in vitro. In these studies, 34.5ENVE killed breast cancer cells of varying molecular subtypes, including those known to frequently metastasize to the brain. To test the therapeutic efficacy of a Vstat120 expressing oncolytic virus (OV) in vivo, we created three new, orthotropic models of BCBM in immune-competent FVB/NJ mice. Two of these models recapitulated the biology of human intracranial BCBM. In both of these models, we found that 34.5ENVE treatment significantly improved the survival of mice with established BCBM.
Materials and Methods

Cell lines and viruses

Vero, DB-7, Met-1, U251-T3, MCF7, MDA-MB-231, and MDA-MB-468 cells were maintained in DMEM supplemented with 10% FBS. SKBR3 cells were maintained in McCoy’s 5A Medium supplemented with 10% FBS. All cells were incubated at 37°C in an atmosphere with 5% carbon dioxide and maintained with 100 units of penicillin/mL and 0.1 mg of streptomycin/mL. U251 cells were obtained from Dr. Erwin G. Van Meir (Emory University, Atlanta, GA) and authenticated by us through the University of Arizona Genetics Core in July 2013. U251-T3 cells were created in our laboratory (May 2009) as a tumorigenic clone of U251 cells by serially passaging these cells three times in mice (these cells have not been separately authenticated). DB-7, Met-1 (murine breast cancer), MCF7, MDA-MB-231, SKBR3, and MDA-MB-468 (human breast cancer) cells were obtained in December 2012 from Dr. Michael C. Ostrowski (Ohio State University, Columbus, OH) and have not been authenticated since receipt (12, 13). Monkey kidney epithelial derived Vero cells were obtained in April 2005 from Dr. E. Antonio Chiocca (Ohio State University, Columbus, OH). These cells have not been authenticated since receipt. All cells are routinely monitored for changes in morphology and growth rate. All cells were negative for mycoplasma. RAMBO and 34.5ENVE viruses were prepared and titered as previously described (11).

Cell viability assays

Cells were plated in 96-well plates and infected simultaneously with 2% FBS in DMEM containing 34.5ENVE at the indicated multiplicity of infection (MOI). Viability was assessed as described previously using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (14).

Virus replication assay

Cells (500,000) plated in 6-well plates were infected with 34.5ENVE at an MOI of 0.005. Three days later, cells and supernatants were harvested, and the viral titers were determined via a standard plaque-forming unit (pfu) assay.

Animal surgery

All animal experiments were performed in accordance with the Subcommittee on Research Animal Care of The Ohio State University guidelines and were approved by the Institutional Review Board. Female FVB/N mice (6–8-week-old; The Jackson Laboratory) were used for in vivo tumor studies. Intracranial surgeries were performed as previously described (15, 16).
were performed as previously described with stereotactic implantation of 100,000 DB-7, Met-1, or Mvt1 cells (11). Tumors were treated with Hank’s Balanced Salt Solution (HBSS) or 34.5ENVE virus at the location of tumor implantation. Animals were euthanized when they showed signs of morbidity.

Immunohistochemistry/immuno fluorescence
Mouse BCBM tumors were fixed in zinc formalin (Anatech Ltd.) and parafﬁn embedded. Tumors were sectioned at 5 µm and stained using the following antibodies: anti-MECA32 (TROMA-1), anti-F4/80 (Invitrogen; MF48000), Alexa Fluor 594 (Invitrogen). Human tumor immunohistochemistry was performed using antigen retrieval at pH 6.0. Tumors were stained with anti-CD163 (Leica Microsystems Novocastra), anti-CD31 (Dako; M0823), and a horseradish peroxidase–linked secondary antibody. Specimens were visualized with 3,3′-diaminobenzidine.

Image acquisition
Immunofluorescent images were acquired using a Nikon Eclipse E800 epifluorescence microscope equipped with a Photometrics Coolsnap camera and Nikon Plan Fluor objectives. MetaVue software (Molecular Devices) was used for image acquisition. Immunohistochemical staining was imaged using a Nikon Eclipse 50i microscope equipped with an Axiocam high resolution channel camera (Zeiss) and Nikon Eclipse Ci microscope equipped with a digital sight Fi2 camera system.

MRI analysis
Mice bearing intracranial tumors were treated with HBBS or virus. Anatomic imaging was performed on the days indicated using a Gadolinium enhanced T1-weighted imaging sequence. For data analysis, a region-of-interest (ROI) that included the tumor was manually outlined. Tumor volumes were calculated from ROIs as previously described (11).

Table 1. Description of breast cancer molecular subtypes and Nestin expression in a panel of human breast cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Estrogen receptor</th>
<th>Progesterone receptor</th>
<th>HER2</th>
<th>Nestin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>MCF7</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>SKBR3</td>
<td>+</td>
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</table>

Figure 2.
Increased Nestin expression is associated with breast cancer metastases. A, the Bos et al. cohort was stratified by median NES expression (83 NES-high and 83 NES-low patients) to examine the probability of brain and lung metastases in patients with breast cancer (P = 0.02). B, NES expression correlates with TNBC status in the Curtis et al. dataset. A total of 1,975 samples in the Curtis et al. breast cancer patient cohort were stratified by TNBC status. There are 250 TNBC and 1,725 patients with other biomarker status in this cohort. Mean NES expression is signiﬁcantly higher (P = 2.79E−37) in TNBCs. C, analysis of NES expression in the Neve et al. breast cancer cell line microarray database. Expression levels are normalized to the nontumorigenic, epithelial cell line MCF10A.
Statistical analysis
The Student t test was used to analyze changes in cell killing, viral plaque-forming assays, and tumor volume measurements. A P value of <0.05 was considered statistically significant. In survival assays, Kaplan–Meier curves were plotted, and the log-rank test was utilized to determine statistical significance. All statistical analyses were performed with the use of Graph Pad Prism software (version 5.01).

See Supplementary Materials and Methods for details.

Results

BAI1 expression is reduced in breast cancer and is associated with patient survival
To determine the relevance of BAI1/Vstat120 in breast cancer, we analyzed patient-derived gene expression data from The Cancer Genome Atlas (TCGA). We observed a 52% reduction in BAI1 expression in invasive ductal breast carcinomas (n = 389) compared with normal breast tissue (n = 61; P < 0.0001; Fig. 1A; ref 15). Further analysis revealed that low BAI1 expression was also associated with decreased disease-free survival (DFS; n = 324; P < 0.03; Fig. 1B). An examination of BAI1 expression in 50 breast cancer cell lines from the Neve and colleagues dataset showed that BAI1 mRNA levels were reduced in 38% of breast cancer cell lines compared with the MCF10A breast epithelial cell line (19 of 50 cell lines; Fig. 1C; ref. 16). These results suggest that the loss of BAI1 promotes breast cancer tumorigenesis and the restoration of BAI1/Vstat120 may have therapeutic effects in breast cancer.

Nestin expression is upregulated in breast cancer and is associated with metastases
Nestin is upregulated in several metastatic cancers, and its high expression correlates with reduced breast cancer patient survival (7–9). Nestin is also expressed in cancer stem cells known to promote cancer resistance and progression (10). Analysis of a cohort of 166 patients stratified by median Nestin expression revealed a significant association between Nestin expression and incidence of brain and lung metastases (n = 164; P < 0.02; Fig. 2A; 17). Of the breast cancer subtypes, we found that high Nestin expression was most strongly associated with triple-negative breast cancers (TNBC), which are highly prone to brain metastases (Fig. 2B; ref. 8). Additional analysis of the Neve and colleagues microarray dataset showed that Nestin was upregulated in 100% of the breast cancer cell lines examined (50 of 50; Fig. 2C; ref. 16). These results suggest that Nestin may be a strong therapeutic target for aggressive and metastatic breast cancers.

Oncolytic virus is cytotoxic in multiple human breast cancer subtypes
34.5ENVE expresses Vstat120 and its replication is driven by a Nestin promoter; thus, we hypothesized 34.5ENVE might be
therapeutically relevant for BCBMs. To test this hypothesis, we tested the infection, replication, and cytotoxicity of 34.5ENVE in a variety of human breast cancer cell subtypes (Table 1). Over the course of 3 days, 34.5ENVE infected and replicated in human breast cancer cells, as determined by increasing virus encoded GFP expression (Fig. 3A). In vitro cytotoxicity of human breast cancer cells to 34.5ENVE infection was dose dependent and increased with time (Fig. 3B and C). Four days after infection at an MOI of 0.05, we observed 83.4%, 75.7%, 80.6%, and 90.1% cell death in MDA-MB-231, SKBR3, MCF7, and MDA-MB-468 cells, respectively. Most breast cancer treatments are targeted to particular subtypes, but 34.5ENVE killed breast cancer cells across multiple subtypes. Importantly, we observed significant killing in the Her2⁺ and TNBC subtypes. TNBCs are notoriously resistant to conventional therapies, and Her2⁻ targeted antibody treatments poorly penetrate the blood–brain barrier. As a result, 25% to 55% of these patients with breast cancer will develop brain metastases (18). These results highlight the therapeutic potential of 34.5ENVE to treat BCBMs.

HSV-1 replication and cytotoxicity is enhanced by the viral neurovirulence gene ICP34.5 (11). To improve the safety and targeting of the 34.5ENVE virus, the expression of ICP34.5 is driven by a Nestin promoter. Nestin expression was increased in all 50 of the breast cancer cell lines examined, suggesting that it is a relevant therapeutic target for breast cancer (Fig. 2C). To determine the efficacy of Nestin-driven ICP34.5 on tumor cell killing, we compared the cytotoxicity of 34.5ENVE with a similar virus lacking Nestin-driven ICP34.5 (RAMBO; refs. 11, 19). We observed 54.14% increased killing in the TNBC MDA-MB-468 cells in the Nestin-driven 34.5ENVE virus, as compared with a virus without ICP34.5 (P < 0.001; Fig. 3D). These results further support the use of 34.5ENVE for the treatment of this disease. This is the first study to specifically use Nestin expression to target breast cancer.

Figure 4.
Characterization of three murine models of BCBM for preclinical evaluation of oncolytic HSV-1-derived therapeutics. A, representative panel of DB-7, Met-1, Mvt1, and human BCBM tumors. Human biopsy sample was stained for H&E, macrophages (CD163), endothelial cells (CD31; ×100 magnification). Murine specimens were stained for H&E, macrophages (F4/80), and endothelial cells (MECA-32; ×20 magnification). B, 72-hour viral titers of DB-7, Met-1, and U251-T3 cells were infected with 34.5ENVE (0.005 MOI). Data shown are mean viral titers ± SD [U251-T3 to DB7 (**, P < 0.01); U251-T3 to Met-1 (***, P < 0.001)]. C, 48-hour cell viability of breast cancer cells treated with 34.5ENVE at the indicated MOIs. Data shown are mean cell viability ± SD. D, temporal response of murine breast cancer cells treated with 34.5ENVE at 0.01 MOI for 3 days. Data shown are mean cell viability ± SD.
Syngeneic model of BCBMs in an HSV-1-sensitive strain

While there are several excellent models to study the biology of brain metastasis development in immune-compromised mice, there are currently few immune-competent models to test the safety and efficacy of its potential therapies (1). Cody and colleagues (20) previously described an immune-competent model of BCBM to test OVs, but in these murine breast cancer cells the virus had limited antitumor efficacy in vitro and in vivo, suggesting that it was not an optimal model to evaluate oncolytic HSV-derived therapeutics. For these studies, we characterized three novel, murine breast cancer (DB-7, Met-1, and Mvt1) models. The DB-7 and Met-1 cells are derived from transgenic FVB/N mice expressing polyoma virus middle T oncogene (PyVmT) under the control of a mammary epithelium promoter (12). PyVmT serves as a surrogate for activated receptor tyrosine kinase signaling pathways, such as Her2, commonly activated in breast cancer (18). Mvt1 cells are derived from tumors of MMTV-c-myc/VEGF bitransgenic mice (21). In these studies, DB-7, Met-1, and Mvt1 breast cancer cells were implanted intracranially into the brains of FVB/NJ mice. DB-7 and Met-1 tumor borders were generally demarcated from the normal brain parenchyma with localized invasion in the Met-1 tumors. Similar to patient specimens, these tumors contained significant tumoral vascularization as well as tumor-associated microglia/macrophages (Fig. 4A). Mvt1 tumors were highly invasive and infiltrated into distant brain structures, including the ventricles and meninges. This phenotype was characteristic of rare leptomingeal metastases and did not resemble the parenchymal brain metastases commonly observed in patients (22). Histologic comparisons of patient BCBMs with all three murine tumors indicated that DB-7 and Met-1 derived tumors most closely recapitulated the human CNS metastases.

Table 2. 34.5ENVE treatment reduces tumor volumes in Met-1 BCBM tumors by MRI

<table>
<thead>
<tr>
<th>Days after implantation</th>
<th>HBSS (mm$^3$)</th>
<th>34.5ENVE (mm$^3$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>6.16</td>
<td>3.72</td>
<td>0.51</td>
</tr>
<tr>
<td>20</td>
<td>25.60</td>
<td>8.66</td>
<td>0.14</td>
</tr>
<tr>
<td>24</td>
<td>59.01</td>
<td>3.43</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NOTE: Data shown are mean tumor volumes (mm$^3$) of Met-1 BCBM tumors treated with HBSS or 34.5ENVE from Fig. 5A-C ($n = 6$ mice/group).
34.5ENVE is cytotoxic to murine breast cancer cells in vitro

Human tropic viruses often replicate poorly in murine cells, so there are very few immune-competent models of cancer to study OVs derived from HSV-1. To determine whether we could evaluate the therapeutic effects of 34.5ENVE in this murine BCBM model, we examined the ability of the virus to infect and replicate in the DB-7 and Met-1 tumor-derived cell lines. For these assays, human glioma cells were used as a positive control. We observed an increase in virus-encoded GFP expression over 48 hours following infection at a low MOI, consistent with virus replication and spread in these cells (Supplementary Fig. S1A). Quantification of virus replication revealed that DB-7 and Met-1 murine cells supported replication at a similar rate compared with human glioma cells (Fig. 4B). 34.5ENVE killed tumor cells in a dose- and time-dependent manner at levels comparable to human glioma cells (Fig. 4C and D). Three days following infection at an MOI of 0.01, we observed 91.5%, 82.5%, and 88.2% cell death in DB-7, Met-1, and human glioma cells, respectively. We also verified these cells secreted virally expressed Vstat120 and demonstrated improved cytotoxicity of the ICP34.5-expressing 34.5ENVE virus (Supplementary Fig. S1B–C). The characterization of this HSV-1–sensitive murine model will aid in the future evaluation of preclinical toxicity and efficacy of novel, HSV-1–derived therapeutics.

34.5ENVE treatment extends survival in vivo

We utilized MRI to noninvasively evaluate the antitumor response of 34.5ENVE in mice with established Met-1 brain tumors. Mice were treated intratumorally with a single dose of HBSS or 34.5ENVE (n = 6/group) 14 days after tumor cell implantation (average initial tumor volume, 4.94 mm³). Figure 5A shows representative coronal T1-weighted MRI images from mice treated with PBS or 34.5ENVE 1 day before treatment and on days 6 and 10 after treatment. The tumor volumes in mice treated with HBSS grew rapidly and obtained an average tumor volume of 59.01 mm³ within 10 days of treatment (Fig. 5B and Table 2). Significantly, 34.5ENVE-treated tumors showed substantial decreases in tumor volume (3.43-mm³ average tumor volume 10 days after viral therapy; P < 0.02). Interestingly, we observed initial pseudoprogression of tumors (by volume) in 34.5ENVE-treated mice before tumor regression, possibly due to tumor destruction and immune cell infiltration. Following these mice over time, we observed that 34.5ENVE treatment significantly enhanced the survival of mice bearing Met-1 breast cancer brain tumors. Control-treated mice had a median survival of only 13 days, whereas mice receiving 34.5ENVE therapy survived significantly longer (median survival, 52 days; P < 0.038; Fig. 5C). We next tested the antitumor effects of 34.5ENVE in the DB-7 BCBM model. Mice treated with 34.5ENVE showed a 100% increase in median survival compared with control mice with DB-7 tumors. HBSS- (n = 5) and 34.5ENVE (n = 7)-treated mice showed median survival times of 17 and 34 days, respectively (P < 0.0004; Fig. 5D).

Disclosures

BCBMs continue to present a significant therapeutic challenge. A recent BCBM clinical trial with lapatinib and capcitabine noted that nearly a third of patients experienced at least one severe adverse event due to toxicity (24). Conversely, OV therapies, which are currently in clinical trials for a variety of solid tumor malignancies, including breast cancer and brain tumors (NCT01656538, NCT02031965, NCT01174537, and NCT00794131), have proven to be safe and well tolerated. In this study, we identified BAI1/Vstat120 and Nestin as novel therapeutic targets for BCBMs. We demonstrated that an OV, 34.5ENVE, expressing antiangiogenic Vstat120 and ICP34.5 under a Nestin promoter had significant cytotoxic effects in breast cancer cells of varying molecular subtypes, including Her2⁺ and TNBC. Significantly, we also described two novel, immune-competent murine models of BCBMs that closely recapitulate the human disease. Finally, we demonstrated that a single, intratumoral dose of 34.5ENVE virus significantly enhanced the survival of mice with established metastatic breast cancer brain tumors. The results of this study warrant further investigation of BA11 and Nestin dual-targeted therapies to treat established BCBMs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.H. Meisen, S. Dubin, S.T. Sizemore, H. Mathsyaraja, K. Thies, N.L. Lehman, P. Boyer, A.C. Jaime-Ramirez, J.B. Elder, A. Chakravarti
Writing, review, and/or revision of the manuscript: W.H. Meisen, S. Dubin, S.T. Sizemore, K. Thies, N.L. Lehman, A.C. Jaime-Ramirez, J.B. Elder, A. Chakravarti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.H. Meisen
Study supervision: W.H. Meisen, M.C. Ostrowski, B. Kaur

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