

Her-2 Overexpression Increases the Metastatic Outgrowth of Breast Cancer Cells in the Brain

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Abstract

Retrospective studies of breast cancer patients suggest that primary tumor Her-2 overexpression or trastuzumab therapy is associated with a devastating complication: the development of central nervous system (brain) metastases. Herein, we present Her-2 expression trends from resected human brain metastases and data from an experimental brain metastasis assay, both indicative of a functional contribution of Her-2 to brain metastatic colonization. Of 124 archival resected brain metastases from breast cancer patients, 36.2% overexpressed Her-2, indicating an enrichment in the frequency of tumor Her-2 overexpression at this metastatic site. Using quantitative real-time PCR of laser capture microdissected epithelial cells, Her-2 and epidermal growth factor receptor (EGFR) mRNA levels in a cohort of 12 frozen brain metastases were increased up to 5- and 9-fold, respectively, over those of Her-2-amplified primary tumors. Co-overexpression of Her-2 and EGFR was also observed in a subset of brain metastases. We then tested the hypothesis that overexpression of Her-2 increases the colonization of breast cancer cells in the brain *in vivo*. A subclone of MDA-MB-231 human breast carcinoma cells that selectively metastasizes to brain (231-BR) overexpressed EGFR; 231-BR cells were transfected with low (4- to 8-fold) or high (22- to 28-fold) levels of Her-2. *In vivo*, in a model of brain metastasis, low or high Her-2-overexpressing 231-BR clones produced comparable numbers of micrometastases in the brain as control transfectants; however, the Her-2 transfectants yielded 3-fold greater large metastases (>50 μm^2 ; $P < 0.001$). Our data indicate that Her-2 overexpression increases the outgrowth of metastatic tumor cells in the brain in this model system. [Cancer Res 2007;67(9):4190–8]

Introduction

The Her-2 receptor tyrosine kinase, a member of the Her superfamily, is one of the most studied signal transduction pathways in cancer (reviewed in ref. 1). Amplification or overexpression of Her-2 is detected in ~23% to 26% of primary breast tumors (2) and correlated with poor patient survival (3). Transfection data, genetically engineered mice, and the use of pharmacologic inhibitors revealed effects on tumor cell proliferation, colonization and motility *in vitro*, and tumorigenicity and metastasis to the lung and bone *in vivo* (4–11). Trastuzumab (Herceptin), a recombinant humanized monoclonal antibody to Her-2, has shown clinical activity in combination with chemotherapy in the metastatic and adjuvant settings (12–14). Multiple new Her-2-directed therapeutics are in development and clinical testing.

Historically, symptomatic brain metastases develop in 10% to 20% of patients with metastatic breast cancer, most often following systemic disease at other sites, and are associated with a dismal prognosis (reviewed in refs. 15, 16). Retrospective studies of breast cancer patients who developed brain metastases revealed an enrichment for hormone receptor-negative (17–22) and epidermal growth factor receptor (EGFR)-positive (21) primary tumors, lymph node metastases (17, 22), and young patient age (20, 21, 23).

The relationship of Her-2 overexpression in primary breast tumors and the development of brain metastasis has been the subject of considerable investigation. Bendell et al. reported that 34% of breast cancer patients with Her-2-positive tumors developed brain metastases within 16 months of diagnosis of metastatic disease and 6 months of trastuzumab treatment (24). Fifty percent of the patients were responding to therapy or had stable disease at other sites at the time of brain relapse. Fifty percent of patients died of progressive central nervous system (CNS) disease. Similar results were reported by several other groups (17, 21, 25–28). In a study of isolated brain metastases, patients with Her-2-positive tumors, as assessed by fluorescence *in situ* hybridization (FISH), exhibited a trend ($P = 0.09$) for development of brain metastases (29). Brain metastatic relapses were also observed in the trastuzumab adjuvant trials, indicating that this problem will not abate with earlier treatment (13). In contrast to these reports, a cohort study by Lai et al. (30) and an autopsy study by Tham et al. (31) found no association between Her-2 expression and brain metastasis. In the present study, we avoided correlative studies using primary tumors and determined the incidence of

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Her-2 overexpression in a large cohort of surgically resected brain metastases of breast cancer, showing Her-2 enrichment in the metastatic lesions. Furthermore, we show coordinate EGFR/Her-2 overexpression in a subpopulation of brain metastases.

The possible causes of increased brain metastases among Her-2-positive breast cancer patients are multiple: (a) Her-2 overexpression may affect the natural history of breast cancer to accelerate brain progression. (b) Poor penetration of the blood-brain barrier (BBB) by trastuzumab may render the brain a "sanctuary" site for metastases, and/or (c) the increased life span of patients receiving trastuzumab therapy may permit brain metastases to become evident. Resolution of these issues has been hampered by a lack of model systems for studying the biology of brain metastases. We developed a quantitative experimental metastasis assay using a brain trophic clone of human MDA-MB-231 breast carcinoma cells (231-BR). Transfection of Her-2 into 231-BR cells increased brain metastatic colonization 3-fold, providing the first evidence that Her-2 overexpression affects the natural history of breast cancer brain metastatic growth.

Materials and Methods

Tissue. Samples were coded with no patient identifiers and approved by the National Cancer Institute (NCI) Office of Human Subjects Research. Diagnosis and histopathologic characteristics were confirmed by a single pathologist before use in the study.

Laser capture microdissection, RNA isolation, and amplification. Sections of frozen tissue (8 μ m) were stained using a Histogene Laser Capture Microdissection (LCM) Frozen Section Staining kit (Arcturus) following the manufacturer's instructions. For each sample, using the Pix Cell II LCM System (Arcturus), ~2,500 laser-captured pulses were collected in a 15-min period. Captured cells were lysed in PicoPure RNA Extraction buffer (PicoPure RNA Isolation kit, Arcturus) for 30 min at 55°C, and total RNA was extracted. Quality and quantity of RNA was assessed using the RNA 6000 Pico Assay for the 2100 Bioanalyzer (Agilent Technologies). A minimum of 10 ng RNA was then subjected to two rounds of T7 RNA polymerase-catalyzed amplification using the RiboAmp RNA Amplification kit (Arcturus). Quality and quantity of the amplified RNA was assessed using the RNA 6000 Nano Assay (Agilent Technologies). A reference RNA sample consisting of a pool of six breast cancer cell lines (MCF7, ZR-75, BT-474, BT-549, MDA-MB-231, and T-47-D) was simultaneously amplified.

Quantitative real-time PCR. Double-stranded cDNA was prepared using the second-round synthesis protocol from the RiboAmp RNA amplification kit (Arcturus). Real-time quantitative PCR reactions consisted of 1 \times SybrGreen Supermix (Bio-Rad), 0.3 mmol/L forward and reverse primers, and 10 ng cDNA. Cycling conditions consisted of a three-step amplification and melt curve analysis using the iQ5 Real-time PCR Detection System (Bio-Rad). Annealing temperatures and primer sequences are listed in Supplementary Table S1. For generating a standard curve, amplified cDNA from the reference sample detailed above was used in a 5-fold dilution series of 100 to 0.16 ng cDNA per reaction. Relative gene expression was calculated by dividing the specific expression value (starting quantity, ng) by the glyceraldehyde-3-phosphate dehydrogenase expression value.

Assessment of Her-2 amplification in brain metastatic samples. Archival paraffin-embedded tumor samples were obtained at M.D. Anderson Cancer Center (Houston, TX) from patients with a history of breast cancer who presented with metastases to the CNS parenchyma. Sections from these samples were confirmed to have metastatic breast cancer and subjected to immunohistochemistry for Her-2. Sections were processed and stained as previously described (32) using an anti-Her-2 antibody (Ab-8, Calbiochem) at a 1:500 dilution with microwave antigen retrieval in 10 mmol/L citrate buffer. Scoring was done using a four-tiered system (0, 1+, 2+, and 3+) according to standard clinical practice. Tumors scored as 2+ were subjected to FISH for probes at the *Her-2* and *CETP17*

(centromeric region) loci. FISH was done using the PathVysion Her-2 kit (Vysis) according to the manufacturer's specifications. A ratio > 2.0 was considered positive for Her-2 amplification.

Cell culture. The human MDA-MB-231 BR "brain-seeking" (231-BR) cell line and a companion "bone-seeking" line (231-BO) were previously described (33). The retroviral vector pLEGFP-C1 (BD Biosciences) was transfected into murine fibroblast PT67 packaging cells using Effectene reagent (Qiagen). After 24 h, enhanced green fluorescent protein (EGFP)-expressing cells were selected in the presence of 1 mg/mL G418 (Invitrogen), and colonies were expanded. Virus was harvested and used to infect 231-BR cells for 6 h. The following day, 231-BR cells were selected in the presence of 0.8 mg/mL G418, and EGFP expression in 95% to 99% of the cells was confirmed by fluorescent microscopy. EGFP-expressing cells were then cotransfected with pCMV4.ErbB2 full-length human cDNA (kindly provided by Dr. Dihua Yu, M.D. Anderson Cancer Center) and pSVzeo to confer drug resistance. The sequence of the Her-2 insert in pCMV4.ErbB2 was confirmed by sequencing. Stable colonies were selected in the presence of 0.750 mg/mL zeocin (Invitrogen).

Western blotting. Cells were serum starved overnight and stimulated with 100 ng/mL EGF (Peprotech, Inc.) or 10 ng/mL Heregulin (R&D Systems) for 20 min before lysis. Cells were lysed according to standard procedures, and Western blotting was done. For phosphorylated Her-2 (p-Her-2) and phosphorylated Akt (p-Akt), 100 μ g total lysate was used; for all other antibodies, 40 μ g total lysate was used. All primary antibodies were obtained from Cell Signaling, unless otherwise indicated. Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used at dilutions of 1:5,000. Proteins were visualized using enhanced chemiluminescence (Cell Signaling) and autoradiography.

Proliferation assays. Cells were plated at a density of 10,000 per well in 96-well plates and incubated for 3 h to allow cells to adhere. The cells were washed with PBS, and media containing either 1% or 10% fetal bovine serum were added to the cells. After 72 h, 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added, and the cells were incubated for 2 h. Following incubation, MTT was dissolved in DMSO, and absorbance was measured at 570 nm. The absorbance recorded on day 3 was divided by the absorbance recorded on day 0 (day of plating), and results are presented as fold growth compared with day 0. Results are representative of three independent experiments, each done in triplicate.

Soft agar colonization. Cells (10,000 per well) were plated in 1 mL of culture medium containing 0.3% (w/v) top agar in 24-well plates as described previously (34). After 14 days in culture, colonies (>50 cells) were counted. Results are representative of three independent experiments, each done in triplicate.

Transendothelial migration assays. Human brain microvascular endothelial cells (HBMEC) were purchased from Cell Systems and cultured per manufacturer's guidelines. HBMEC (100,000 cells) were plated on fibronectin-coated Transwell inserts with 3- μ m pores (BD Biosciences) and cultured for 5 days to allow a confluent monolayer to form. Medium was replaced daily. On day 5, vector or Her-2-transfected 231-BR cells (25,000 per insert) were added to the top of the insert, and medium containing a chemoattractant was placed in the bottom for 18 h. After incubation, non-migrating cells were removed from the upper surface of the insert. Cells that migrated to the bottom surface were fixed in 4% paraformaldehyde and washed with PBS, and the nuclei were counterstained with Hoechst 33342 (Invitrogen). The membrane was then excised and mounted on a glass slide. Migrating cells were visualized by fluorescent microscope, and the entire bottom surface of the membrane was counted. Data are representative of two experiments conducted using triplicate samples.

Mice and imaging. Animal experiments were conducted under an approved Animal Use Agreement with the NCI. Under isoflurane/O₂ anesthesia, twenty 5- to 7-week-old female BALB/c nude mice (Charles River Laboratories) were inoculated with 250,000 cells in 0.1 mL PBS in the left ventricle for each clonal line (see Table 1). Mice were euthanized under CO₂ asphyxiation after 4 weeks, and brains were excised for imaging.

EGFP was detected in whole brains by the Maestro 420 *In vivo* Spectral Imaging System (Cambridge Research and Instrumentation) using software

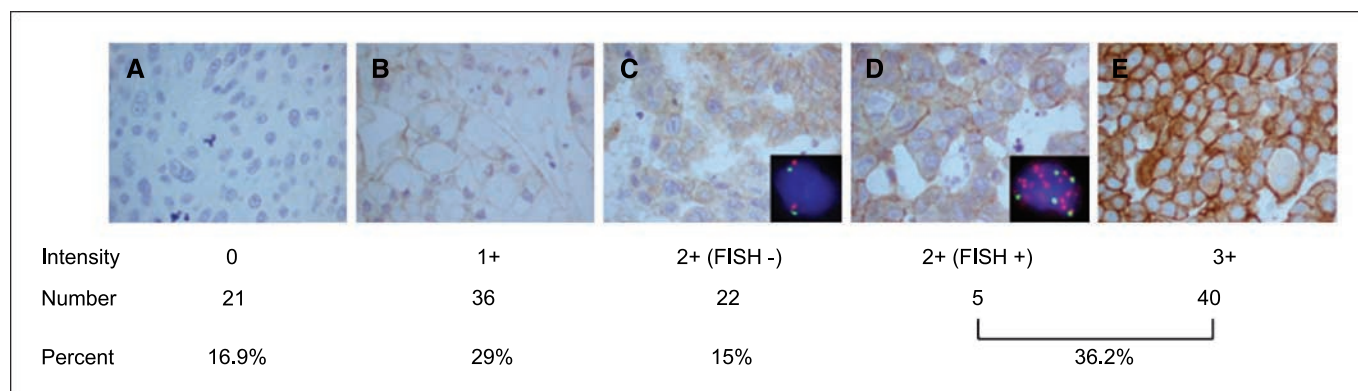


Figure 1. Immunohistochemical and FISH analyses of Her-2 overexpression in a cohort of 124 brain metastases from breast cancer patients. Archival paraffin-embedded tumor samples were subjected to immunohistochemistry for Her-2 using an anti-Her-2 antibody. Scoring was done using a four-tiered system (0, 1+, 2+, and 3+) according to standard practice. Tumors scored as 0 (A) or 1+ (B) were considered negative for Her-2 overexpression. Tumors scored as 2+ (C and D) were considered positive for Her-2 overexpression. Tumors scored as 2+ (C and D) were subject to FISH for probes at the *Her-2* locus and also *CEP17* (centromeric region; inset). A ratio > 2.0 was considered positive for Her-2 amplification. Forty-five of 124 (36.2%) samples were positive for Her-2 overexpression. The comparison of the observed proportion to the historic proportion (2) of primary breast tumors yielded $P = 0.015$ by exact binomial test.

(Nuance Technology) to distinguish or unmix images of fluorescence from multiple sources. After imaging, brains were bisected along the sagittal plane, and the left hemisphere was immediately frozen in ornithine carbamyl transferase compound (for histology). The right hemisphere was fixed in 4% paraformaldehyde for 24 to 48 h at 4°C and then transferred to 20% sucrose overnight at 4°C and frozen (for EGFP detection). Brain sections (10- μ m thick) were serially cut and processed for either EGFP microscopy or histology. To detect EGFP, slides were dried at room temperature, and 25 μ L Vectashield HardSet Mounting Medium with 4',6-diamidino-2-phenylindole was added (Vector Laboratories). Images were captured on a Zeiss Axioskop 2 microscope using OpenLab software (Improvision). For staining with H&E, a standard protocol was followed.

Ten serial sections every 300 μ m through the brain were analyzed using a 5 \times objective on a Zeiss microscope, containing an ocular grid with squares of 0.8 mm². Every micrometastasis or large (>50 μ m²) metastasis in each section was tabulated; counting was terminated at 300 metastases per section. Data are representative of two experiments conducted. All analysis was carried out by two investigators who were blinded to the various groups.

Statistical analysis. ANOVA with post hoc Dunnett's multiple comparison test was used to assess *in vitro* functions of vector and Her-2-overexpressing clones for proliferation and colonization experiments. Student's *t* test was used for transendothelial cell migration experiments, which were conducted in vector and low Her-2 and vector and high Her-2 clone paired experiments, respectively. All *P* values are two tailed. All tests for *in vitro* experiments were done using GraphPad InStat version 3.0 software. For analysis of the Her-2 staining, an exact binomial test was used to compare the observed results with the theoretical (historic) percentage of Her-2 overexpression in primary breast tumors. For *in vivo* experiments, micrometastases and large metastases were evaluated as the primary end points. A general linear model-based ANOVA that adjusted for the effect of clones nested within each group was used to compare vector versus low Her-2 groups and vector versus high Her-2 groups. All *P* values are two tailed.

Results

Frequent Her-2 overexpression in brain metastases. To determine the incidence of Her-2 overexpression in brain metastases of breast cancer, formalin-fixed, paraffin-embedded sections of resected brain metastases from patients with documented primary breast cancer were analyzed for Her-2 expression using immunohistochemistry. The median age of the cohort was 51 years, with a range from 27 to 75 years; 33 of the 124 cases were

estrogen receptor (ER) positive (26.6%). Immunohistochemical scoring by a neuropathologist was based on a 0 to 1+ versus 2 to 3+ intensity point scale (Fig. 1). For the borderline positive (2+) metastases, Her-2 amplification was confirmed by FISH (Fig. 1C and D, inset). Of 124 brain metastases, 45 (36.2%) were Her-2 positive. This is higher than a historical percentage (23–26%) for primary breast tumors (2), statistically significant by the exact binomial test ($P = 0.015$). Estimates of Her-2 overexpression in primary tumors from the metastatic subset of breast cancer patients are rarely reported, but rates of 18% to 30% have been published from small cohort studies using variable detection methodologies (21, 35–37), also lower than the data reported herein.

Her superfamily mRNA expression levels. Her-2 heterodimerizes with other Her superfamily receptors to initiate signal transduction. The relative expression of the Her superfamily of receptors was determined by quantitative real-time PCR of laser capture microdissected epithelial cells from 12 frozen resected brain metastases and an equal number of unlinked primary breast tumors (Fig. 2). Patient and tumor histopathology data for these samples are listed on Supplementary Table S2 and show a general correspondence of patient age, tumor ER status, and tumor-node-metastasis stage.

For Her-2, FISH analysis indicated amplification in 25% (3 of 12) of the primary tumors and 42% (5 of 12) of the brain metastases. The FISH-positive primary tumors expressed increased Her-2 mRNA levels compared with unamplified tumors; however, brain metastasis Her-2 expression levels were increased up to 5-fold over that of any of the primary tumors.

Similar trends were observed for EGFR (Her-1). Variability in primary tumor EGFR mRNA levels was observed. Five brain metastases (42%) expressed higher EGFR mRNA levels than any of the primary tumors; levels of EGFR expression were up to 9-fold higher than any of the primary tumors. Coordinate high expression of Her-2 and EGFR mRNAs was observed in 17% (2 of 12) of the brain metastases.

In contrast, Her-3 mRNA expression levels were variable and were not different between primary tumors and brain metastases. One of 12 tumors (8%) coordinately expressed high Her-2 and Her-3. With the exception of one brain metastasis, Her-4 mRNA levels were higher in primary tumors than in brain metastases. Although based on a limited cohort, the data suggest a selection for

high Her-2 and EGFR mRNA expression in brain metastases. Of the five Her-2-positive (FISH amplified) brain metastases, coordinate high expression of another superfamily member was observed in 60% (3 of 5).

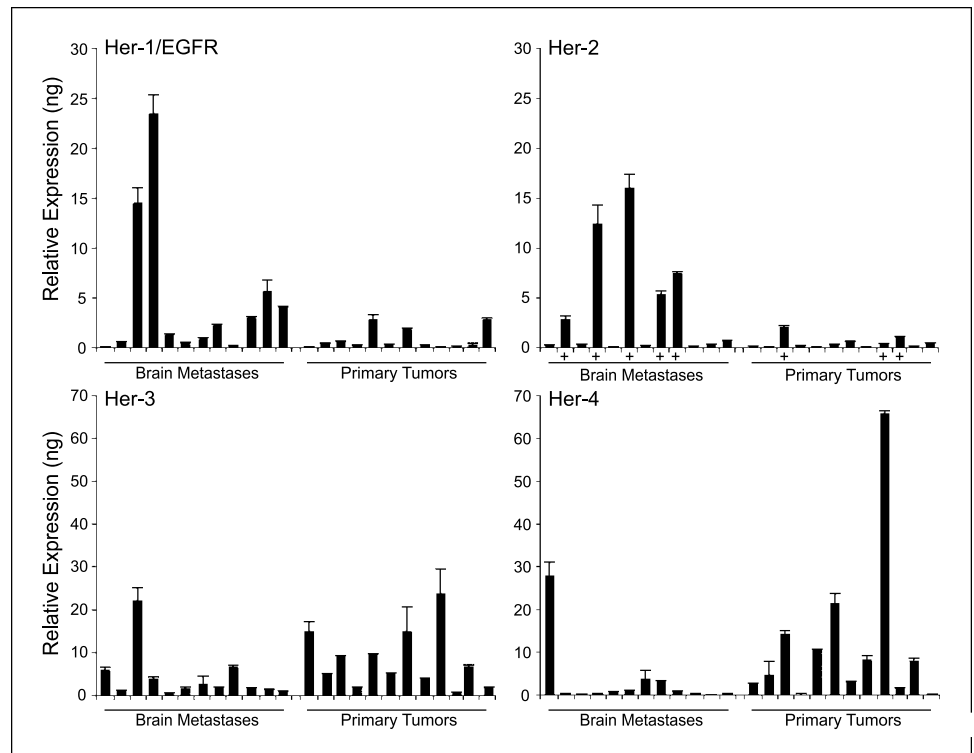
Transfection of Her-2 into a brain-seeking breast cancer cell line augments anchorage-independent colonization. Although an increased incidence of brain metastasis has been correlated with primary tumor Her-2 overexpression and trastuzumab treatment, the lack of breast cancer brain metastasis model systems has hampered our ability to test the functional contribution of Her-2. Two tissue-specific sublines, a brain-seeking (231-BR) and a bone-seeking (231-BO) subline of human MDA-MB-231 breast carcinoma cells, were isolated by successive rounds of *in vivo* selection and *ex vivo* culture (33). Analyses of these lines for several key signaling proteins were assessed under normal growth conditions (Fig. 3A). Acquisition of brain but not bone metastatic activity was accompanied by increased total and p-EGFR expression. Expression of the Her-2, Her-3, and Her-4 proteins was comparable between the lines and at the lower limit of detection (data not shown). Overexpression of EGFR in the 231-BR cell line was accompanied by increased expression of p-Akt; equivalent levels of total and phosphorylated extracellular signal-regulated kinase (p-Erk) were observed in the three lines. Of the many proposed downstream effectors of aggressiveness, we observed elevated p21 levels in both the bone- and brain-selected lines.

To develop a model system to examine the relevance of Her-2 overexpression in brain metastases, 231-BR cells were first transfected with EGFP and subsequently transfected with either Her-2 cDNA or a control vector. Given the heterogeneity of Her-2 expression levels among brain metastases (Fig. 2), two low and two high Her-2-expressing clones were selected for characterization compared with two vector control clones. To determine whether the overexpressed Her-2 was functional in signal transduction,

clones were serum starved and treated with 100 ng/mL EGF or 10 ng/mL heregulin (HRG), which target Her-2 heterodimerization with either EGFR or Her-3, respectively. Her-2 was overexpressed ~8- and 4-fold (low 1 and low 2, respectively) in the low expressing clones and ~28- and 22-fold (high 1 and high 2, respectively) in the high expressing clones, respectively (Fig. 3B). Her-2 phosphorylation was stimulated by EGF in the low and high Her-2 transfectants and by HRG in the high expressing clones. Expression and ligand-mediated activation of EGFR was observed in all transfectants, consistent with data from the 231-BR line (Fig. 3A). Of the proteins reported to lie downstream of Her-2 signaling, p-Akt was activated by EGF preferentially in the high Her-2-expressing clones, whereas p21 elevation was observed in all Her-2 transfectants; no differences in p-Erk were observed. The data indicate that the overexpressed Her-2 protein was active in signal transduction, with EGFR as a leading heterodimerization partner.

We sought to identify the *in vitro* biological correlates of Her-2 overexpression on metastatic progression (Fig. 4). In 3-day proliferation assays, Her-2-overexpressing clones exhibited a minor (1.4-fold) proliferative advantage compared with controls in low (1.0%) serum ($P < 0.0001$, ANOVA) but no difference at higher (10%) serum levels (Fig. 4A). Anchorage-independent colonization of Her-2 transfectants was statistically significantly elevated over vector transfectants using low (2%) or high (10%) serum levels ($P < 0.0001$ for both, ANOVA; Fig. 4B). Motility of the vector and Her-2 transfectants was comparable in response to serum-free media supplemented with EGF or HRG, conditioned media from brain microvascular endothelial cells, or conditioned media supplemented with EGF (data not shown). To mimic human tumor cells migrating through the BBB, we used HBMEC in an assay that replicates aspects of BBB traversal *in vitro* by measuring the ability of tumor cells to invade through a monolayer of HBMEC. EGF-induced transendothelial cell migration was increased 2-fold

Figure 2. Expression of Her superfamily receptors in unlinked brain metastases of breast cancer and primary breast tumors. Epithelial cells were laser capture microdissected from 12 resected brain metastases and an equal number of infiltrating ductal carcinomas. Real-time quantitative PCR was performed on amplified cDNA. Data are expressed as relative expression in ratio to glyceraldehyde-3-phosphate dehydrogenase levels for each tumor. +, Her-2-amplified tumors as assessed by FISH.



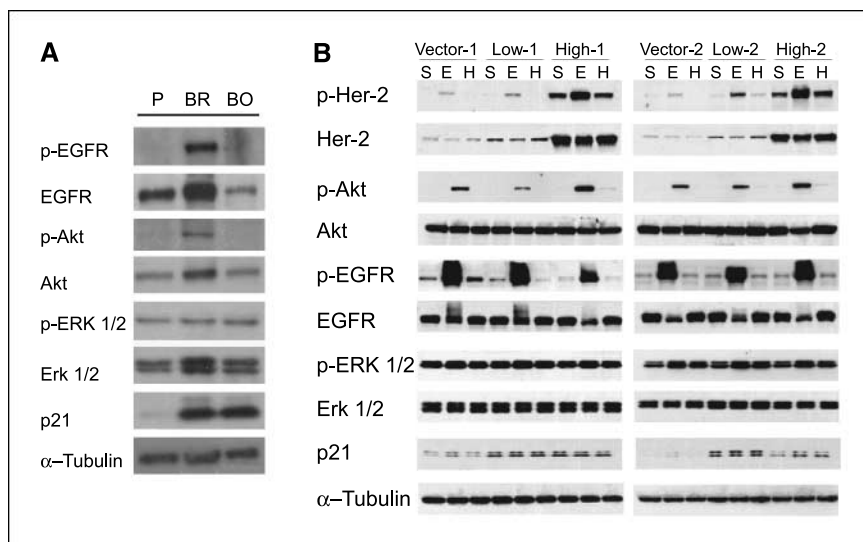


Figure 3. Western blot analysis of the MDA-MB-231BR brain-seeking cell line and clonal transfectants. Western blots of total cell lysates. **A**, acquisition of brain metastatic activity was associated with EGFR overexpression. MDA-MB-231 parental cells (*P*), and sublines selected for brain-seeking (*BR*) and bone-seeking (*BO*) *in vivo* metastatic patterns were previously described (33). **B**, Her-2 overexpression alters downstream signaling pathways in 231-BR brain metastatic breast carcinoma cells. 231-BR human breast carcinoma cells stably transfected to express a control vector (*vector-1* and *vector-2*), low levels of Her-2 (*low-1* and *low-2*), or high levels of Her-2 (*high-1* and *high-2*) were serum starved for 16 h and then stimulated with 100 ng/mL EGF for 10 min or 10 ng/mL heregulin for 20 min, targeting Her-2 heterodimerization with EGFR and Her-3, respectively. α -Tubulin expression was used as a loading control. S, serum starved; E, serum starved then stimulated with EGF; H, serum starved then stimulated with HRG.

($P = 0.0025$ and $P = 0.04$, respectively, t test for paired samples) in high Her-2-expressing cells (Fig. 4C). Transendothelial migration was not statistically significantly increased in the low Her-2-expressing clones, or in any clone in low (1%) serum conditions. The data indicate anchorage-independent colonization as the prime phenotypic effect of Her-2 overexpression in this brain-seeking cell line.

Her-2 overexpression increases brain colonization *in vivo*.

To determine whether Her-2 overexpression is associated with the increased formation of breast cancer brain metastases *in vivo*, vector and low and high Her-2-overexpressing transfectants were injected into the left cardiac ventricle of BALB/c nude mice. The number and size of brain metastases that formed were analyzed after 4 weeks. At necropsy, whole brains were removed, and EGFP imaging was done to identify mice with brain metastases (Fig. 5A). Brains of mice that received diluent only showed nonspecific autofluorescent scatter at either end of the brain, whereas brains of mice that received 231-BR cells showed foci throughout the brain. Although a general trend of increased fluorescent metastases with Her-2 overexpression was noted in the whole-brain imaging, quantification was difficult because of frequent "carpeting" of the brain by overlapping lesions.

After imaging, the brain was bisected, and 10 sagittal sections were prepared at 300- μ m intervals through one hemisphere. The sections were processed for either EGFP visualization or H&E stained for histology. The presence of brain metastases, which was confirmed by a neuropathologist, occurred in both the parenchyma and leptomeninges. Most of the metastases in each section could be classified as micrometastases, comprising few tumor cells (Fig. 5B, top). For the control transfectants, micrometastases represented 98% of the lesions identified. Variable numbers of micrometastases were formed by the clonal control transfectants, which may reflect heterogeneity in tumor cell survival in this site. To distinguish micrometastases from larger metastases that are diagnosed clinically, each lesion was viewed through an ocular micrometer, and those with an area $> 50 \mu\text{m}^2$ were designated as "large" (Fig. 5B, bottom). This is similar to the size of a clinically detectable metastasis in the human brain by magnetic resonance imaging.

A statistically significant difference was noted in the number of large metastatic lesions formed by the Her-2-overexpressing 231-BR cells (Table 1). The number of total metastases did not vary between vector control and Her-2-overexpressing clones, indicating that the tumor cells arrested, extravasated into the brain, and

Table 1. *In vivo* analysis of control and Her-2-overexpressing MDA-MB-231 BR clones

Clone	No. mice	Mean no. total metastases (95% confidence interval)*	Mean no. large metastases (95% confidence interval) [†]	P^{\ddagger}
Vector 1	8	255.6 (211.4-299.7)	5.1 (3.7-6.6)	
Vector 2	5	182.4 (93.8-271.1)	2.9 (2.0-3.8)	
Low Her-2 1	7	156.8 (110.3-203.2)	11.3 (8.3-14.4)	0.0001
Low Her-2 2	11	211.1 (17.0-248.1)	16.6 (15.1-18.1)	
High Her-2 1	9	193.8 (149.7-237.8)	10.9 (8.9-12.9)	0.0001
High Her-2 2	5	269.0 (185.6-352.3)	14.0 (11.6-16.4)	

NOTE: Data shown are representative of one experiment. Two experiments were conducted.

*Mean number of metastases counted in 10-step sections from one hemisphere of the brain.

[†]Size of metastases determined by 16-mm² ocular grid (large metastases $>50 \mu\text{m}^2$).

[‡]Linear model-based ANOVA used to determine significance for large metastases (total metastases not significantly different).

formed micrometastases with equal efficiency. However, both the low and high Her-2-overexpressing clones formed 3-fold greater large metastases compared with the vector transfectants ($P < 0.0001$ for both), a trend consistent with the high rate of clinical detection of brain metastases in patients with Her-2-amplified tumors.

Discussion

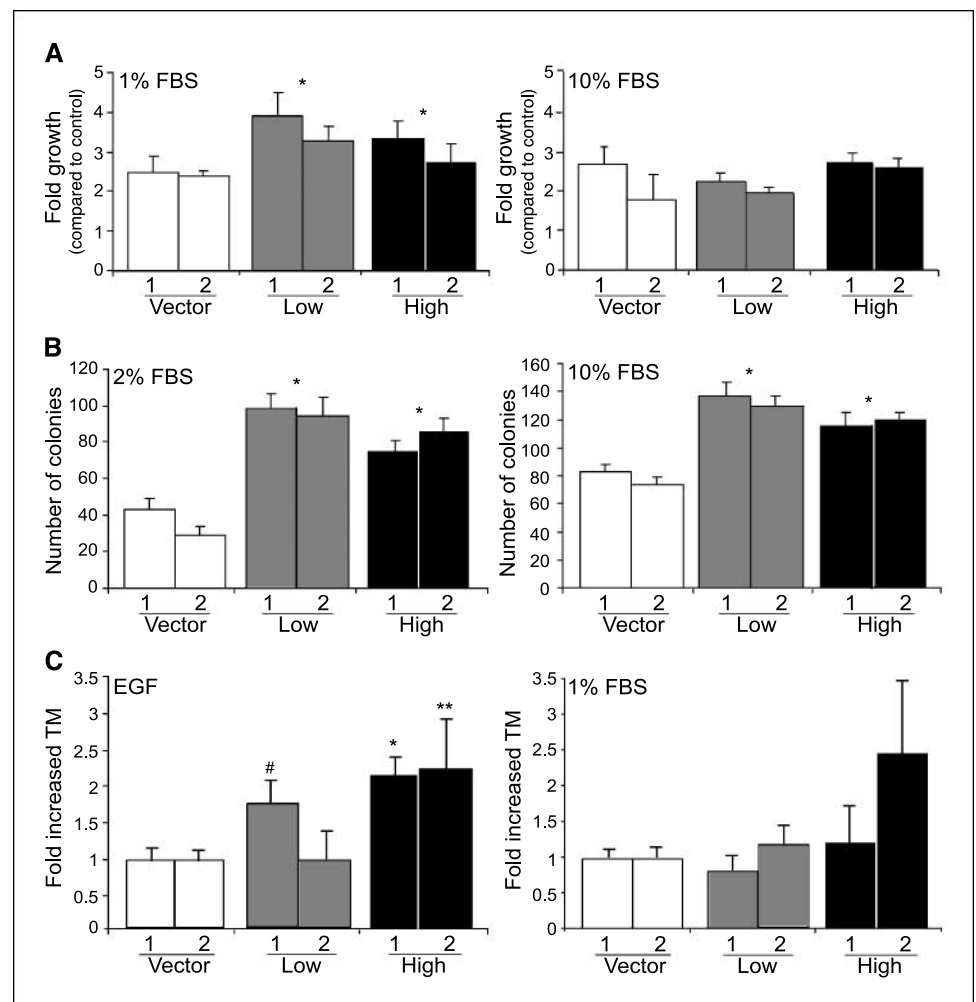
Brain metastases of breast and other cancers seem to be increasing in incidence. For breast cancer patients with Her-2-positive tumors, metastases to the brain are also reportedly occurring in patients responding to treatment or with stable disease at other sites. Given the dire cognitive and physical consequences of the development of brain metastases and their treatment, reflected in the dismal 1-year patient survival rate (20%; ref. 15), it is important that we obtain a molecular understanding of metastasis to this site. The data reported herein support three conclusions: (a) Her-2 overexpression is enriched in brain metastases of breast cancer, confirming Her-2 as a molecular target for metastasis to this site. (b) Coordinate high expression of Her-2 and EGFR occurs in a subset of brain metastases. (c) Overexpression of Her-2 elevated the number of large brain metastases 3-fold in an EGFR-overexpressing 231-BR experimental metastasis model sys-

tem, providing the first evidence that Her-2 can affect the natural history of breast cancer metastasis to the brain.

We determined Her-2 overexpression in a large cohort of resected brain metastases of breast cancer. With the classification for Her-2 positivity defined as 3+ immunohistochemical staining or 2+ immunohistochemical staining and gene amplification by FISH, 36.2% of resected brain metastases of breast cancer were Her-2 positive. A comparable technical approach to defining Her-2 positivity was used for entry into trastuzumab clinical trials (13). This increase in percentage of Her-2 overexpression represents a 42% to 61% enrichment over reported primary tumor Her-2 positivity (23–26%; ref. 2) and supports a predilection of Her-2-positive tumors for brain metastasis. These data stand in agreement with observational studies reporting a correlation of primary tumor Her-2 positivity, or trastuzumab treatment with the development of brain metastases (21, 24–28). Where such correlations were not observed, several explanations may be germane. For instance, methods of detecting Her-2 overexpression vary among studies. Protein-based methods, such as Western blotting, can add dilutional artifacts and have been reported to be inferior to FISH (2).

Not only is the incidence of Her-2 positivity enriched in brain metastases, but based on a limited cohort of unlinked primary tumors and brain metastases, Her-2 mRNA expression levels may be higher than in primary tumors. LCM of epithelial cells from each

Figure 4. *In vitro* phenotypes of Her-2 overexpression in 231-BR cells. **A**, proliferation of control and low and high Her-2-expressing transfectants over 3 d using the MTT microtiter plate assay. $P < 0.0001$ by ANOVA for 1% fetal bovine serum (FBS) with *, $P < 0.01$ for Dunnett multiple comparison test. **B**, soft agar colonization in 2% and 10% fetal bovine serum. Colonies were counted after 14 d in culture. $P < 0.0001$ by ANOVA for 2% and 10% fetal bovine serum with *, $P < 0.01$ for Dunnett multiple comparison test. **C**, transendothelial cell migration of control and low and high Her-2-expressing 231-BR cells toward EGF. HMVEC were plated on fibronectin-coated Transwell inserts and cultured for 5 d to establish a monolayer with characteristics of a BBB. EGFP-transduced 231BR cells were added to the top of the insert on day 5 and allowed to migrate for 18 h. GFP-positive tumor cells were counted on the entire bottom surface of the insert. Data are expressed as fold increase in transendothelial migration (TM) compared with vector controls. *, $P = 0.0025$; **, $P = 0.04$ by Student's *t* test for vector and high Her-2-expressing clones, respectively, in paired experiments. #, $P = 0.03$ by Student's *t* test for vector and low Her-2-expressing clones, respectively, in paired experiments.



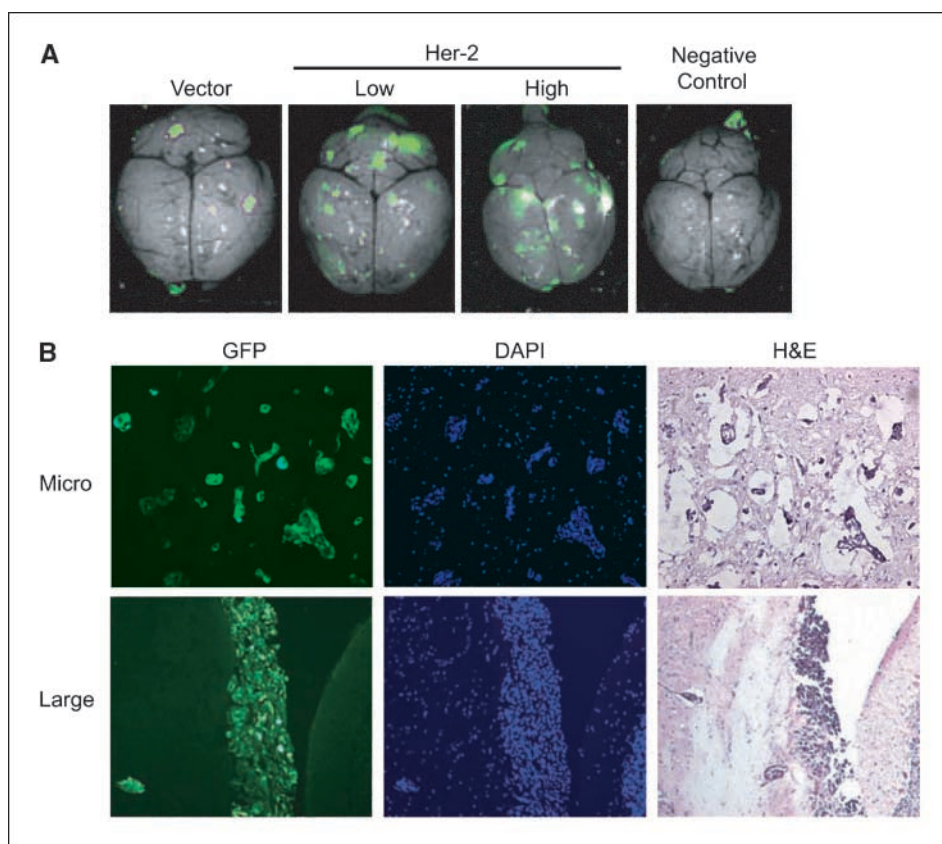


Figure 5. 231-BR *in vivo* model system for quantification of brain metastases. EGFP-labeled 231-BR brain-seeking cells were injected into the left cardiac ventricle of BALB/c nude mice, and brain metastases were determined 4 wks later. *A*, *ex vivo* whole-brain images were captured using the Maestro 420 *In vivo* Spectral Imaging System. Representative images from each group. Negative control is a representative image from a mouse that did not receive an injection of tumor cells. Autofluorescence is routinely noted on the periphery of the tissue. *B*, histologic images representative of micrometastases and large brain metastases. *Left*, EGFP fluorescence; *middle*, 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei; *right*, H&E-stained images in a successive section. Magnification, $\times 200$.

tumor permitted quantification of Her-2 mRNA levels without dilution artifacts associated with varying numbers of stromal cells. Each of the five Her-2 FISH-amplified brain metastases had higher Her-2 mRNA levels than the FISH-amplified primary tumors (i.e., up to 5-fold higher). A similar trend was observed for EGFR. Increased EGFR expression was also found in the brain-seeking but not the bone-seeking sublines of our MDA-MB-231 model system, suggesting its potential importance. No trend was observed for Her-3 mRNA levels, providing evidence of specificity in expression patterns. Taken together, the data suggest a selection for high EGFR and Her-2 expression in brain metastases that merits further study in larger cohorts.

Combined high EGFR and Her-2 expression was observed in two of five Her-2-positive brain metastases. Coordinate overexpression of Her-2 and EGFR is seldom observed in breast cell lines (38). In tumor cohorts, coordinate overexpression measured by immunohistochemistry to total or phosphorylated receptors, or gene amplification, was found in 2% to 13% of all breast carcinomas (39, 40) and 13% of Her-2-positive tumors (41). Where measured, combined overexpression correlated with poor disease-free survival (40, 41). Grupka et al. (42) used immunohistochemistry to determine Her-2 and EGFR status in a limited brain metastatic cohort and reported that 18% of the lesions were coordinately positive. These data support our mRNA coexpression conclusions at the protein level. Zhan et al. (43) recently reported that EGFR/Her-2 heterodimers in MCF10A cells, compared with Her-2 homodimers, preferentially activated the phosphatidylinositol 3-kinase and phospholipase $\gamma 1$ pathways and induced invasion in three-dimensional cultures. These data suggest that coordinate expression of EGFR and Her-2 may endow breast tumors with added invasive properties. The data suggest that the coordinate

EGFR/Her-2-positive overexpressing tumors may represent an interesting subset. This subset of breast tumors with Her-2/EGFR overexpression is represented by our MDA-MB-231-BR Her-2 transfectants, which exhibit both high EGFR and Her-2 expression.

An *in vivo* model system to quantify brain metastases was developed using intracardiac injection of 231-BR cells. Initial experiments confirmed that brain metastatic potential directly associated with the number of tumor cells injected and the time after injection. Although imaging is convenient, a careful cross-comparison with histology indicated the latter as a superior method to quantify brain metastases. In our experience, an imageable brain metastasis could be a single large metastasis or could result from a closely spaced collection of micrometastases. A histologic approach was therefore employed for quantification of our *in vivo* data and was also used in a similar model (44).

Transfection of Her-2 into 231-BR cells resulted in a 3-fold increase in large ($>50 \mu\text{m}^2$) brain metastases, providing the first evidence that Her-2 affects the natural history of breast cancer progression to the brain. Surprisingly, both low (4- to 8-fold) and high (22- to 28-fold) Her-2-overexpressing clones exhibited increased brain colonization. Many of the large brain metastases observed in the Her-2 transfectants were parenchymal (data not shown).

In other reported model systems, Her-2 transfection increased metastasis to the lung or bones (5, 7, 8, 45–47). The brain may not constitute an obvious extension of these trends. Brain tissue is protected by a BBB consisting of endothelial cells linked by continuous tight junctions, pericytes, a basement membrane, and the feet of astrocytes. Intravasation of this complex barrier cannot be assumed to occur at rates equivalent to arrest by size restriction in a lung capillary. The brain microenvironment is poorly described

but seems distinct from that of other organs and may differentially influence the outgrowth of metastases (15). One of two of these theoretical factors seemed to be important in our model system. Her-2-positive and Her-2-negative tumor cells intravasated into the brain and formed micrometastases at comparable frequencies; the outgrowth of Her-2-positive tumor cells to form large metastases in the brain microenvironment was augmented. These *in vivo* data closely compare with the *in vitro* analysis; colonization in soft agar was the only assay in which Her-2 transfectants outperformed the vector controls under all conditions tested.

A host of signaling pathways may mediate Her-2 aggressiveness. Both Her-2 phosphorylation and increased p21 expression were associated with increased brain metastatic potential in this system. p21^{Waf1/Cip1} was classically described as an inhibitor of cell cycle progression but has also been linked to both innate tumor cell aggressiveness and chemotherapeutic resistance. In a breast cancer cohort, phosphorylation or cytoplasmic localization of p21 was associated with Her-2 overexpression (48); the subcellular distri-

bution and influence of p21 on tumor cell sensitivity to chemotherapeutic compounds was modulated by the Her-2 pathway (49). The role of p21 in this model system, as well as other potential signaling mechanisms, remains under study. It will be of interest to determine the role of Her-2 overexpression in brain metastasis in the absence of tumor cell EGFR overexpression and to compare the signaling pathways involved. Given the causal relationship of Her-2 overexpression and brain colonization showed herein, *in vitro* and *in vivo*, the efficacy of small BBB-permeable inhibitors of Her-2 will be of great interest.

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