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Biomarkers, Genomics, Proteomics, and Gene Regulation

Expression of Endoplasmic Reticulum Stress Proteins Is a Candidate Marker of Brain Metastasis in both ErbB-2$^+$ and ErbB-2$^-$ Primary Breast Tumors

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The increasing incidence of breast cancer brain metastasis in patients with otherwise well-controlled systemic cancer is a key challenge in cancer research. It is necessary to understand the properties of brain-tropic tumor cells to identify patients at risk for brain metastasis. Here we attempt to identify functional phenotypes that might enhance brain metastasis. To obtain an accurate classification of brain metastasis proteins, we mapped organ-specific brain metastasis gene expression signatures onto an experimental protein-protein interaction network based on brain metastatic cells. Thirty-seven proteins were differentially expressed between brain metastases and non-brain metastases. Analysis of metastatic tissues, the use of bioinformatic approaches, and the characterization of protein expression in tumors with or without metastasis identified candidate markers. A multivariate analysis based on stepwise logistic regression revealed GRP94, FN14, and inhibin as the best combination to discriminate between brain and non-brain metastases (ROC AUC = 0.85, 95% CI = 0.73 to 0.96 for the combination of the three proteins). These markers substantially improve the discrimination of brain metastasis compared with ErbB-2 alone (AUC = 0.76, 95% CI = 0.60 to 0.93). Furthermore, GRP94 was a better negative marker (LR = 0.16) than ErbB-2 (LR = 0.42). We conclude that, in breast carcinomas, certain proteins associated with the endoplasmic reticulum stress phenotype are candidate markers of brain metastasis. ***(Am J Pathol 2011, 179:564–579; DOI: 10.1016/j.ajpath.2011.04.037)***

Brain metastases occur in 10% to 15% of breast cancer patients with advanced disease.[1–3] It can be assumed that up to 30% of metastatic breast cancer patients will undergo brain metastasis during the course of their disease.[4,5] This rate is increasing, which can be linked to greater survival in patients receiving chemotherapy

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and to the fact that it is difficult to cross the blood-brain barrier with current systemic treatments.[6–8] The difficulties in managing brain metastasis therapy result in a median survival of 7 months, with brain metastasis being the cause of death or a major contributing factor in 68% of patients.[9] Thus, there is a need for both prevention and improved treatment of brain metastasis.[2,3]

The association of ErbB-2 overexpression with brain metastasis has been attributed to both the inability of a humanized antibody such as trastuzumab to penetrate the blood-brain barrier[10] and the longer life span of patients receiving therapy that improves visceral disease control.[11] A longer life can lead to the onset of late tumor spread to the central nervous system. The predilection of ErbB-2$^+$ tumor cells for the central nervous system has also been reported.[12] Thus, ErbB-2 may affect the development of breast cancer and increase the potential for brain metastasis.

The development of metastasis in the central nervous system depends on the interaction of tumor cells with host defenses and the brain microenvironment, which, surrounded by the blood-brain barrier and lacking lymphatic drainage, differs from lung, liver, lymph node, or bone microenvironments.[13] Moreover, microenvironmental factors at the metastatic foci may affect the response of tumors to chemotherapy and may condition drug resistance.[14] Unraveling the biological pathways that drive brain metastasis promises insight into how to limit or prevent this deadly aspect of cancer progression.

Our aim was to identify proteins involved in the progression of brain metastasis. Recently, a strategy based on mapping expression profiles with protein interactions has been described.[15] The authors show that it is possible to extract relevant biological information about deregulated functions and the relationship between them, and to identify molecules that could be helpful as metastatic markers or therapeutic targets. We compared data obtained from an experimental protein-protein interaction network (PPIN),[16] which identifies biological pathways contributing to the organ-specific phenotype of brain metastatic cells, with gene expression profile data[17] obtained from published transcriptomic analysis of 23 human breast cancer metastasis samples excised from various anatomical locations, including the brain. To compare the expression and network data sets, we mapped the expression values of each gene onto its corresponding protein in the network and searched for proteins whose activities are highly discriminative of brain metastasis. Protein expression analysis of tissues from metastatic human brain and primary breast tumors provided candidate markers of brain metastasis in both ErbB-2$^+$ and ErbB-2$^-$ breast carcinomas.

Materials and Methods

Sample Collection

The Breast Cancer Committee of the Catalan Institute of Oncology and the University Hospital of Bellvitge supplied samples from patients diagnosed between 1988 and 2006. The series of 122 breast cancers included 71 consecutive primary ductal breast carcinomas at initial diagnosis from metastatic patients in treatment at the time of the study, with one or several organs affected (Table 1), and 51 patients with positive lymph nodes at surgery without metastatic progression after a minimum follow-up duration of 5 years. Three patients had brain as the unique metastasis location and 10 patients had dissemination also at bone (n = 7), lung (n = 6), and liver (n = 4). A total of 48 tumors with bone metastasis, 23 with liver metastasis, and 31 with lung metastasis were included.

To optimize each immunohistochemical analysis, the corresponding control tissues for the expression of each protein were also used. To validate protein expression, we included in the analysis six brain metastasis samples matched with the corresponding ductal breast carcinoma to validate protein expression. As a validation set, we used a series of 295 breast tumors for which the transcriptomic data were publicly available.[18,19]

Identification of Brain Metastasis Candidate Markers

The strategy for identifying novel cancer candidates has been described elsewhere.[20] The general procedure of the study, the steps of the analysis, and the levels of protein expression measured are shown as a flow chart in Figure 1A.

Experimental Proteomic Analysis and Protein Interaction Network Analysis

To identify brain metastasis-associated proteins, we used a prior proteomic analysis that compared differential expression of proteins between 435-P and 435-Br1 cells.[16] Briefly, the proteins differentially expressed by two-dimensional gel electrophoresis (Amersham Ettan DIGE system; GE Healthcare, Little Chalfont, UK) in 435-Br1 cells were identified by peptide mass fingerprinting spectra recorded by a Voyager STR MALDI-TOF system (Applied Biosystems, Foster City, CA) in positive reflector mode with delayed extraction. The spectra were analyzed using the m/z software package (ProteoMetrics, New York, NY). Proteins were identified against a nonredundant database (NCBInr) using online MASCOT search tool (*http://www.matrixscience.com/search_form_select.html*).

The protein network was based on 17 proteins known to be differentially expressed between 435-P breast cancer cells and the brain metastatic variant 435-Br1. We used PIANA[21] to combine data from DIP 2006.01.16, MIPS 2006.01, HPRD 2005.09.13, BIND 2006.01, and the human interactions from two high-throughput experiments. The final PPIN included 628 proteins from 13 known seeds (interacting proteins) identified by MALDI-TOF (Figure 1B).

Table 1. Distribution and Combinations of the Various Metastases from Breast Cancer Tumors Included in the Tissue Array Analysis

Metastatic involvement of organs				
Brain	Bone	Liver	Lung	Total (no.)
In each organ (no.)				
13	48	23	31	
As a unique organ [no. (%)]				
3 (23)	11 (23)	4 (17)	3 (10)	21
Multimetastatic combinations				
×	×			4
×		×		0
×			×	1
×		×	×	2
×	×		×	1
×	×	×		0
	×	×		5
	×		×	5
	×	×	×	4
		×	×	2
×	×	×	×	2
Other multimetastatic combinations*				
				24

Total number of patients with metastasis: 71.
*One or more metastases in combination with other organs (lymph nodes, skin, pleura, esophagus, and vagina).

Human Brain Metastasis Transcriptomic Data

The protein-network approach for identifying markers of brain metastasis was based on results from a previously analyzed microarray hybridization using the GeneChip human genome U133 Plus 2.0 array (Affymetrix, High Wycombe, UK; Santa Clara, CA), which includes more than 47,000 transcripts and variants, according to standard protocols for RNA extraction and probe preparation.[17] Briefly, to process and normalize Affymetrix chips, robust multichip averaging RMA algorithms were used.[22] All these computations were performed with the Bioconductor package version 2.0.[23] Expression profiles were analyzed with BRB Array tools, version 3.3beta3 (Molecular Statistics and Bioinformatics Section, Biometric Research Branch, Division of Cancer Treatment and Diagnosis, NIH-National Cancer Institute, Bethesda, MD).

The univariate *t*-test was used to identify genes differentially expressed in four brain metastases and metastases in organs other than the brain (5 lung, 6 liver, 2 skin, and 6 osteolytic bone metastases) (Figure 1C). Differences were considered significant when $P < 0.001$. This stringent threshold was used to limit the number of false positives. These data sets, under the identification number GSE11078, are freely available from the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (*http://www.ncbi.nlm.nih.gov*).

Identification of Candidate Genes and Pathways

Gene expression levels obtained from the microarray experiments were mapped onto the network proteins, assuming that a protein might be differentially

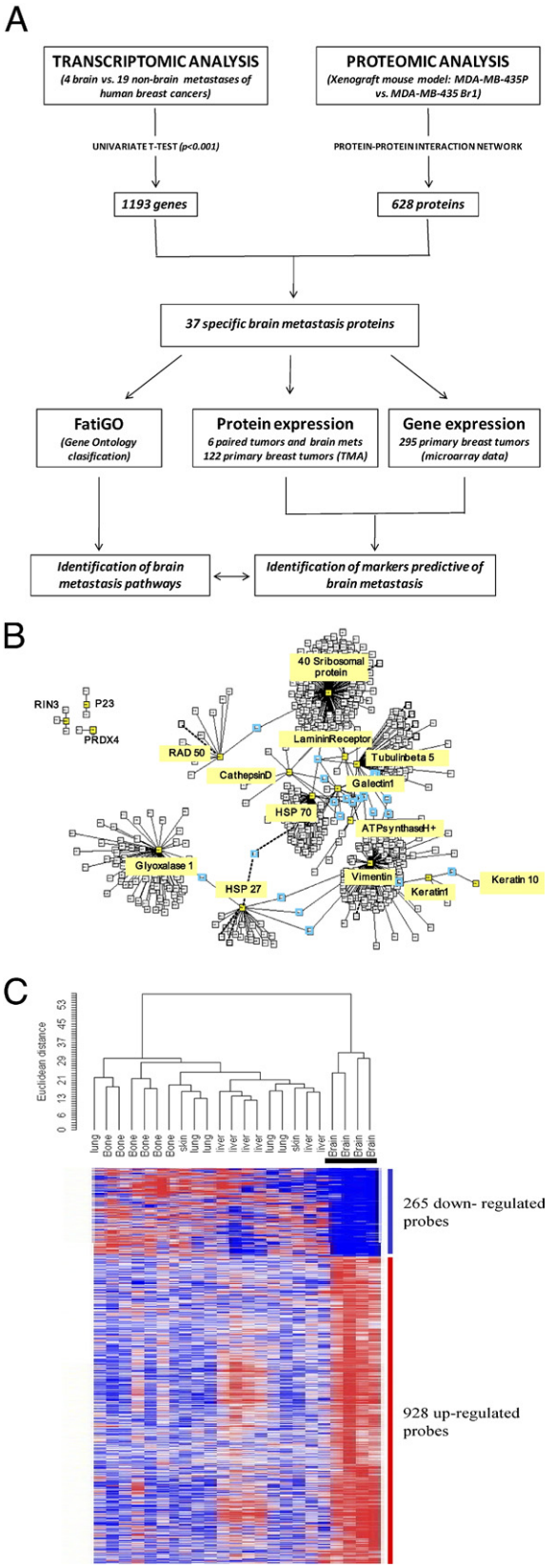

Figure 1. Identification of candidate genes and pathways. **A:** Study design flow chart. **B:** The protein-protein interaction network (PPIN) for interacting proteins identified by mass spectrometry.[16] Root proteins are in **yellow boxes** and linker proteins in **blue boxes**. **C:** Specific signature of brain metastasis. Hierarchical clustering of a series of 23 breast cancer metastases using 1193 genes from the MetaBre brain-specific signature.[17]

expressed if the gene encoding for it was found to be differentially expressed at the RNA level. Differential gene expression was found for 556 of the 658 proteins in the initial PPIN.

To classify proteins by function, we used FatiGO software, an online tool for detecting significant associations between gene ontology terms (GO) and groups of genes.[24]

TMAs and IHC

Tissue microarrays (TMAs) were prepared from three representative areas of the tumor that were carefully selected from H&E-stained sections of 122 donor blocks (S.B. and S.H.). Core cylinders, 2 mm in diameter, were removed from each tumor with a skin-biopsy punch and were deposited into recipient paraffin blocks using a specific arraying device (Beecher Instruments, Sun Prairie, WI), as described elsewhere.[25] Sections (3-μm thick) of the resulting microarray block were cut and used for immunohistochemical (IHC) analysis after being transferred to glass slides.

Experimental conditions, positive control tissues, and the characteristics and source of the antibodies used are listed in Table 2. Staining optimization, evaluation parameters, and analyses were established by two pathologists (P.L.F. and S.B.) who were blinded to the clinical status.

Antigens were retrieved by heating in a pressure cooker for 7 minutes in the appropriate buffer. Primary antibodies were diluted in Dako real antibody diluent buffer (Dako, Glostrup, Denmark; Carpinteria, CA): Tris buffer, pH 7.2, 15 mmol/L NaN_3. LSAB+ system-horseradish peroxidase (Dako) was used, including biotinylated anti-rabbit, anti-mouse, and anti-goat immunoglobulins in PBS; streptavidin conjugated to horseradish peroxidase in PBS; and liquid 3–3′ diaminobenzidine in chromogen solution. A polyclonal antibody anti-ErbB2 (A0485; Dako) was used with an ultraView detection kit in an automatic staining system (Ventana Benchmark XT; Roche, Tucson, AZ).

Statistical Analysis

To evaluate the correlation of protein expression with brain metastasis, immunostained samples were graded on a three-category scale (negative, weak positive, and strong positive). The marker was catalogued as overexpressed in strong-positive samples. The association of brain metastasis for each marker was tested using a two-sided Fisher's exact test and summarized by calculating the sensitivity among tumors that developed metastasis, and calculating the specificity among tumors without metastasis, for strong-positive values. Positive and negative likelihood ratios (LR) were also calculated as integrated predictive indexes, as was the area under the ROC curve (AUC). Markers were assessed using a multivariate logistic regression model in a forward stepwise procedure to identify the best combination to discriminate brain metastasis. Because ErbB-2 is a known metastasis risk factor, an analysis including ErbB-2 as the baseline was also performed, as well as a stratified analysis of each candidate marker within ErbB-2^+ and ErbB-2^- tumors. In all of the analyses, associations were considered significant when $P < 0.05$. No multiple testing correction was done in this analysis, because the search for the best combination of markers started from a very small set of candidates.

Results

Identification of Specific Brain Metastasis Proteins

We mapped human brain metastasis expression profiles with a PPIN to maximize accuracy in the classification of brain metastasis proteins.

The signature of brain genes was catalogued as the organ-specific metastasis signature (BOSMS) with a hierarchical clustering that clearly distinguishes among the different metastases.[17] The BOSMS contained 1193 genes (MetaBre) after the one-versus-all (ONA) class comparisons identified genes differen-

Table 2. Antibodies and Corresponding Conditions for IHC

Antibody	Clone	Supplier*	Protocol	Cellular expression	Control tissue
GRP 94	sc-1794 (C-19)	SCB	1/2000†	Endoplasmic reticulum	Breast carcinoma
TRAF2	SM7106P (clon 33A1293; 205–222 aa)	Acris	1/100 O/N†	Cytoplasm	Breast carcinoma
FN14	sc-27143 (C-13)	SCB	1/3000†	Membrane	Kidney, heart
INHA	MCA951ST (R1)	AbD S	1/50†	Cytoplasm	Testis
TOP1	ab3825 (401–600 aa)	Abcam	1/100‡	Nuclei, cytoplasm	Colorectal tumor
VAV2	sc-20803 (H-200)	SCB	1/1000†	Cytoplasm	Pancreas
GFAP	Z0334	Dako	1/8000†	Cytoplasm	Brain (astrocytes)
TEM 8	ab21270	Abcam	1/2000†	Cytoplasm, membrane	Brain tumor endothelium
ARFGAP	SP1402P	Acris	1/1000†	Cytoplasm	Testis
EIF3s8	ab19359 (N-terminal 1–50 aa)	Abcam	1/1000 O/N†	Cytoplasm	Kidney
BAT 8	G-6919	Sigma	1/250†	Cytoplasm	Lymph node

*Suppliers: Abcam, Cambridge, UK; AbD S, AbD Serotec, MorphoSys UK, Oxford, UK; Acris, Acris Antibodies, Herford, Germany; SCB, Santa Cruz Biotechnology, Santa Cruz, CA; Sigma, Sigma-Aldrich, St. Louis, MO.
†Retrieved in Na-citrate buffer.
‡Retrieved in Tris/EDTA.
O/N, antibody is incubated overnight.

Table 3. Identities of 37 Brain Metastasis-Specific Proteins Matched in the Proteomic and Transcriptomic Analyses of Human Brain Metastasis

Gene symbol	UniProtKB ID	Protein name	Function	*P* value	Network position (linked to)
		Up-Regulated			
RPL13	Q3KQT8	60S ribosomal protein L13 (breast basic conserved protein 1)	Protein biosynthesis	0.0008	40S ribosomal protein s12
RPS10	P46783	40S ribosomal protein S10	Protein biosynthesis	0.0005	
RPL5	P46777	60S ribosomal protein L5	Protein biosynthesis	0.0002	
EIF5	P55010	Eukaryotic translation initiation factor 5	Protein biosynthesis	0.0007	
EIF3C (prev. *EIF3S8*)	Q99613	Eukaryotic translation initiation factor 3, subunit 8	Protein biosynthesis	0.00002	
EEF1D	P29692	Eukaryotic translation elongation factor 1-delta, isoform 2	Signal transduction	0.0006	
EEF1D	Q96I38*	Eukaryotic translation elongation factor 1-delta, isoform 1	Signal transduction	0.0006	
PARF (syn. *C9orf86*)	Q8IWK1†	Putative GTP-binding protein Parf [alt.: C9orf86 protein (fragment)]	Signal transduction	0.0001	
INHA	P05111	Inhibin alpha chain	Signal transduction	<0.000001	
CLN3	Q13286	Protein CLN3	Protein folding	0.0008	
FAM3A	P98173	Protein FAM3A precursor (2–19 protein)	No function	0.0009	
PARF (syn. *C9orf86*)	Q9BU21†	Putative GTP-binding protein Parf (alt.: C9orf86 protein)	No function	0.0001	
TUBB2A	P05218	Tubulin beta-2 chain	Structural	0.0004	Root protein
TBCD	Q96E74	Tubulin-specific chaperone D	Structural	0.00005	Tubulin beta-2 chain
MCM4	P33991	DNA replication licensing factor MCM4	DNA binding	0.0004	
ARFGAP1	Q8N6T3	ADP-ribosylation factor GTPase-activating protein 1	Transport	0.0003	
EHMT2 (syn. *BAT8*)	Q96KQ7	Histone-lysine N-methyltransferase EHMT2 (alt.: HLA-B-associated transcript 8)	Methylation	0.0008	
RNF25	Q96BH1	Ring finger protein 25	Ubiquitinization	0.0002	
HMG20B	Q9P0W2	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1-related	DNA binding	0.00001	Vimentin
SIRT6	Q8N6T7	Sirtuin 6	Amino acid metabolism	0.000004	
GFAP	P14136	Glial fibrillary acidic protein	Structural	0.0001	
TOP1	Q9UJN0‡	DNA topoisomerase I	DNA binding	0.00001	
CRAMP1L (syn. *C16orf34*, *KIAA1426*)	Q96RY5	Protein cramped-like (alt.: uncharacterized protein KIAA1426)	DNA binding	0.0003	Glyoxalase I
C9orf84	Q5VXU9	Uncharacterized protein C9orf84	No function	0.0009	
C16orf34	Q9H910	Hematological and neurological expressed 1-like protein	No function	0.0003	
MSH6	P52701	DNA mismatch repair protein MSH6	DNA repair	0.00002	RAD50
TCERG1	O14776	Transcription elongation regulator 1	DNA binding	0.00004	HSP 70
HSP90B1 (prev. *TRA1*; syn. *GRP94*)	P14625	94kDa glucose regulated protein (alt.: GRP94)	Protein folding	0.0009	LINKER (laminin receptor 67 kDa and HSP 27)
TRAF2	Q12933	TNF-receptor associated factor 2	Signal transduction	0.00007	PRDX4
TNFRSF12A (syn. *FN14*)	Q9NP84	TNF-receptor superfamily member 12A (alt.: fibroblast growth factor-inducible immediate-early response protein 14; alt.: FN14)	Receptor	0.0001	TRAF2

(table continues)

Table 3. *Continued*

Gene symbol	UniProtKB ID	Protein name	Function	*P* value	Network position (linked to)
		Down-Regulated			
RPS12	P25398	40S ribosomal protein S12	Protein Biosynthesis	0.0006	Root protein
RPS23	P62266	40S ribosomal protein S23	Protein biosynthesis	0.000001	40S ribosomal protein s12
DNM3	Q6P2G1	Dynamin 3	Protein biosynthesis	0.0008	Tubulin beta-2 chain
SERPINB9	P50453	Serpin B9	Signal transduction	0.0007	Vimentin
CREB1	Q53X93	cAMP responsive element binding protein 1, isoform A	Transcription	0.000005	
CREB1	P16220	cAMP responsive element binding protein 1, isoform B	Transcription	0.00005	
AOC3	Q16853	Vascular adhesion protein-1	Cell adhesion	0.0004	Glyoxalase I

*Q96I38 is a secondary accession number. The primary (citable) accession number is number is P29692.
†Both Q8IWK1 and Q9BU21 link to Q3YEC7 as the main UniProtKB record for the putative GTP-binding protein Parf.
‡Q9UJN0 is a secondary accession number. The primary (citable) accession number is number is P11387.
alt., alternative protein name; prev., previous approved gene symbol; syn., gene symbol synonym appearing in the literature.

tially expressed in the 4 brain metastases versus the 19 metastases to other organs.

Integrating genomic and proteomic analyses, we matched the BOSMS with the PPIN,[16] and obtained 37 organ-specific proteins (Table 3): seven underexpressed and 30 overexpressed. The FatiGO classifier based on GO terms grouped proteins as follows: 13 nucleic acid metabolism proteins (48%), 10 translation proteins (37%), seven cell death proteins (26%), and six modification and folding proteins (22%), as well as a miscellany of metabolic, transport and signaling proteins, some of them with multiple functions (Figure 2). The cellular components of the analysis were as follows: 74% intracellular organelles, 51% cytoplasm, 22% ribonucleoprotein complex proteins, and 15% proteins intrinsic to membrane.

We graphically represented the brain organ-specific metastasis phenotype (Figure 3) in the PPIN-based functional approach from protein interaction databases, providing a novel hypothesis for pathways involved in brain metastasis progression. Indeed, five functions from the PPIN were predominant: i) DNA binding and repair; ii) protein folding and chaperones, which engage one more DNA binding protein (O14776); iii) structural cytoskeleton, which engages four new DNA binding proteins (Q9P0W2, P33991, Q53X93, and Q9UJN0), two new signal transcription factors (P50453 and P16220), one ubiquitinization protein (Q96BH1), one amino acid metabolism protein (Q8N6T7), and one protein involved in methylation (Q96KQ7); iv) protein biosynthesis, which engages four new signal transduction factors (P29692, Q96I38, Q8IWK1, and P05111); and v) vesicle transport, which engages one protein (Q8N6T3).

Additional IHC experiments were performed on six matched breast cancer tumor-brain metastasis samples from patients, to corroborate in human brain metastasis the expression of 11 proteins representative of the functions involved. These proteins were chosen on the basis of commercial availability of antibodies (Table 2 and Figure 4). The IHC analysis validated the expression of GRP94, TRAF2, FN14, TOP1, VAV2, GFAP, TEM8, BAT8, and ARFGAP proteins in brain metastasis. In addition, some of these proteins were also expressed in the corresponding primary breast carcinomas, suggesting their functional involvement from the primary tumor to the brain metastasis.

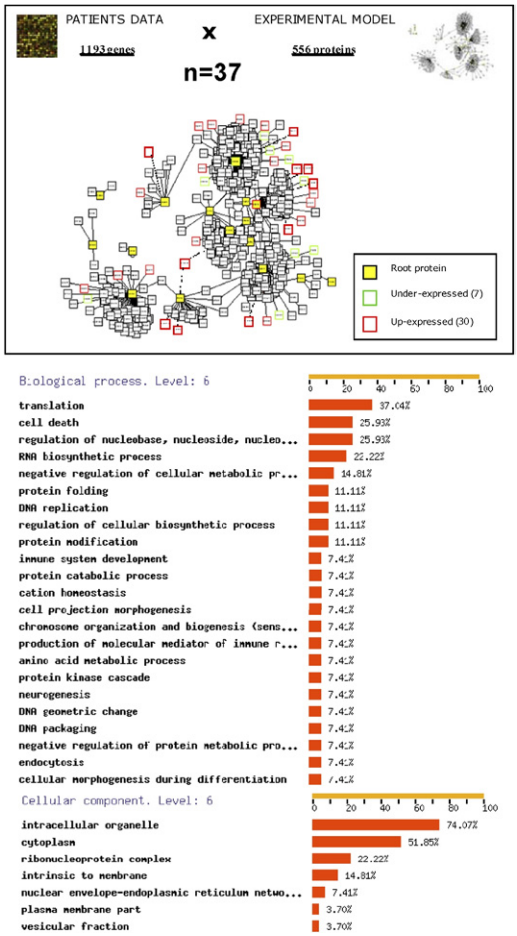

Figure 2. The PPIN analysis was performed for 556 proteins matched with 1193 differentially expressed brain metastasis genes (transcriptomic comparison of brain metastases versus other metastases), yielding 37 pairs corresponding to 7 underexpressed and 30 overexpressed organ-specific proteins. FatiGO, an online tool for finding significant associations of gene ontology-terms with groups of genes,[24] shows the preponderant functions of significant proteins in clusters of coexpression. The classification by function was performed using GO level 6.

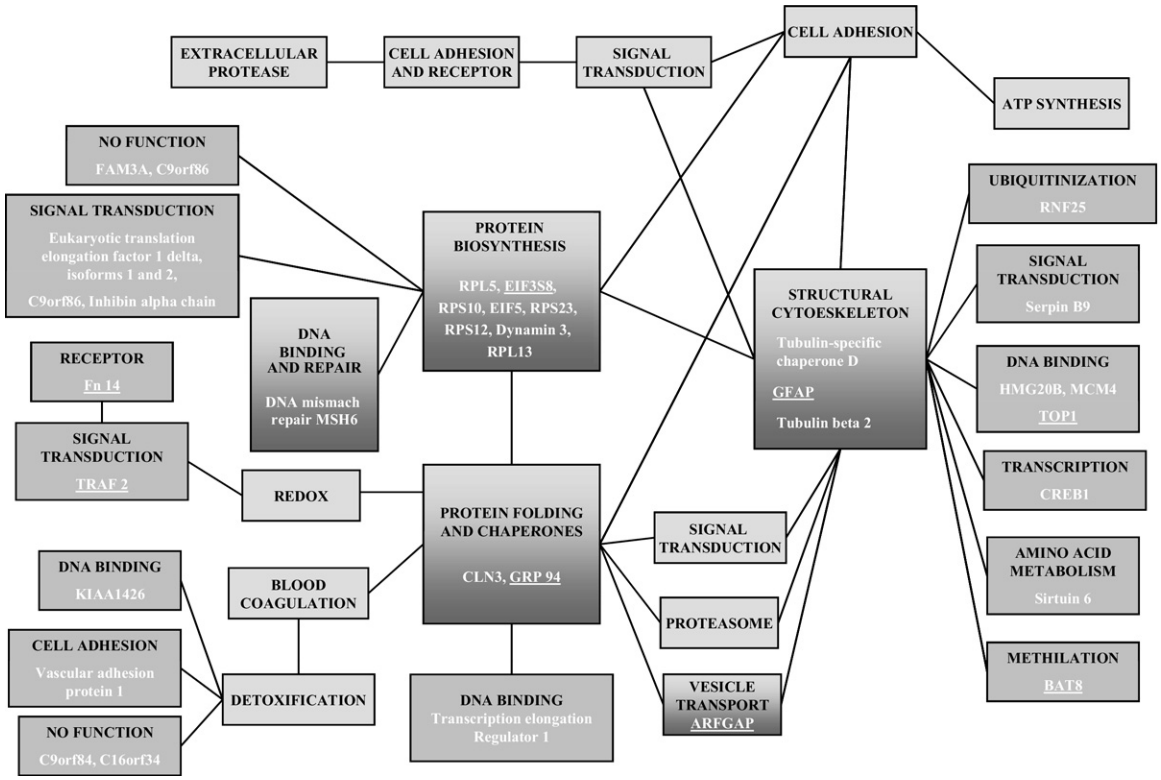

Figure 3. Functional classification of the PPIN. Proteins in functional clusters are grouped within a single box containing root and linker proteins. Functions are indicated in black type; the 37 brain metastasis proteins are indicated in white type. Boxes in light gray indicate the previous network[16] of brain metastatic cells; boxes in dark gray indicate new functions added from the transcriptomic analysis; boxes shaded from light to dark represent redundant functions identified in proteomics and transcriptomic analysis. Proteins that were validated by IHC are underlined.

We searched for references to brain metastasis signatures in published genomic data from experimental and clinical breast cancer and metastasis analysis. From our list of genes, only seven appeared in previous lists of gene expression profiling predicting clinical outcomes of breast cancer[26–35] (Table 4): *EEF1D*, *MCM4*, *RPL5*, *RPS12*, and *CLN3*[26] and also *FAM3A* and *TBCD*.[27] *GFAP*, encoding a ubiquitous protein in the central nervous system, also appeared in a list of genes differentially expressed between brain and bone breast cancer metastasis.[35]

These findings indicate that cells metastasizing in brain were enriched in cell structure, chaperones, stress and redox regulation, and intracellular transport proteins. The organ-specific character of this functional signature was also found in the transcriptomic data from breast cancer brain metastasis (Figure 5 and Table 5). From these, the most differentially expressed in brain metastasis, compared with metastases in other organs, were GRP94 ($P = 0.002$), FN14 ($P = 0.002$), ARFGAP1 ($P = 0.003$), TRAF2 ($P = 0.003$), and PDGFRA (0.002) genes. In contrast, other functions had no relevant expression in brain; for example, amino acid metabolism genes were overexpressed only in liver.

After mapping transcriptomic into proteomic analyses, we concluded that molecules involved in protein folding and chaperones might connect different functions and presumably act by rescuing cells from endoplasmic reticulum stress responses.

Expression of Endoplasmic Reticulum Stress Phenotype in Breast Cancer Primary Tumors Is Associated with Brain Metastasis Progression

Because proteins were also expressed in primary tumors, to estimate the probability of specific brain metastasis outcomes we further analyzed the proteins in a series of primary breast carcinomas using TMA technology. We considered a marker to be positive when strong expression was detected, to avoid false positives, and taking into account the known expression in a control tissue (Figure 6).

Statistical analysis of the data showed significant associations between brain metastasis progression and high expression of GRP94 ($P < 0.0001$), TRAF2 ($P < 0.001$), and FN14 ($P < 0.0001$). As expected, ErbB-2 expression was associated with brain metastasis with a high significance ($P < 0.0001$): 8/13 (62%) breast cancers that progressed to brain metastasis were positive, versus 12% and 5% of breast carcinomas that relapsed in other locations or without metastasis (7/57 and 2/42, respectively). ErbB-2 expression was also associated with the absence of hormone receptors: ER, 55% versus 30% (6/11 and 29/98, respectively, $P = 0.016$); PR, 73% versus 39% (8/11 versus 37/95, respectively, $P = 0.009$). A slight association with histological grade (HG) was also observed: HG III 58% versus 47% (7/12 versus 45/96, $P = 0.105$). In addition, we did not find correlation be-

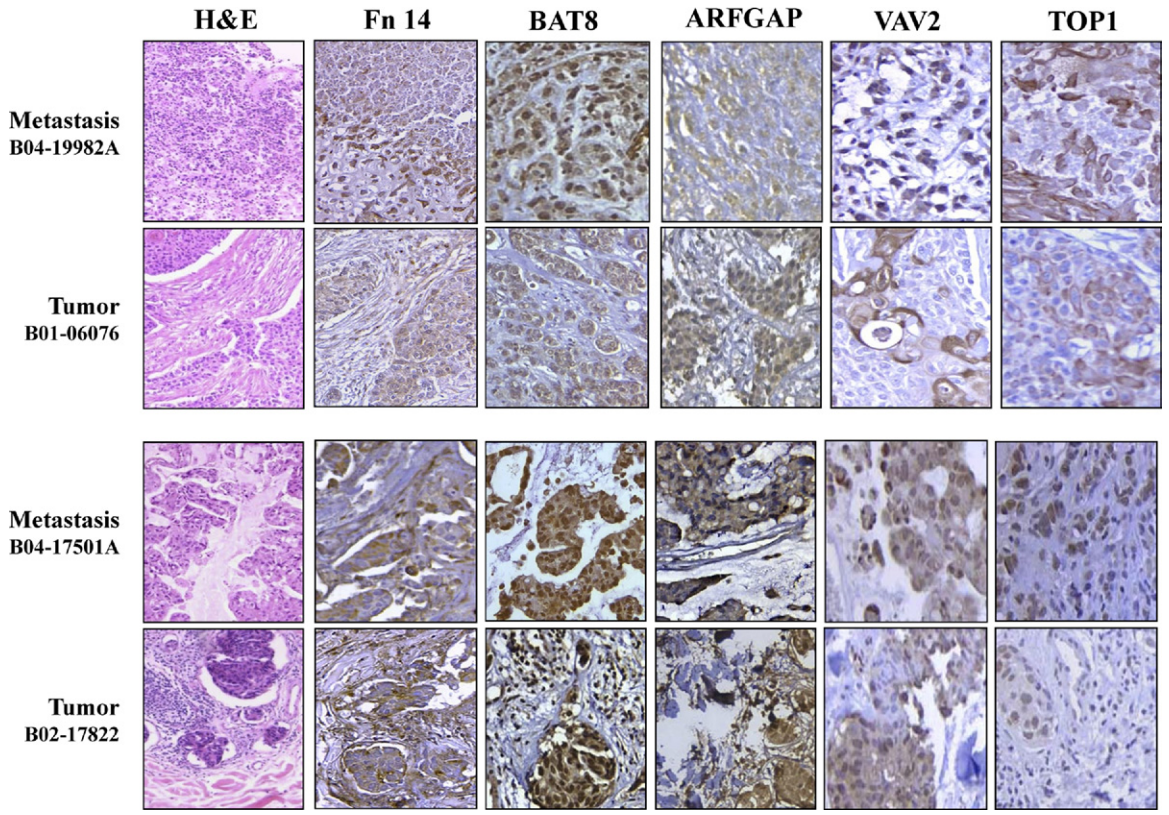

Figure 4. Validation at the protein expression level (brown) in matched tumor-brain metastasis samples by means of IHC analysis to identify representative functional-type proteins in representative paraffin-embedded tumor-brain metastasis pairs. H&E staining of each tissue is shown as viewed by light microscopy. Original magnification: ×10 (H&E stain); ×20 (all others).

tween lung, bone, or liver metastasis and high expression of these proteins.

We calculated the positive and negative likelihood ratios (LR) to assess the predictive accuracy of each molecule as a brain metastasis marker, considering the sensitivity and the specificity of each (Table 6). The highest predictive value for the presence of metastasis was ErbB-2 expression (positive LR = 6.7, $P < 0.0001$), followed by FN14 (positive LR = 3.01, $P = 0.001$), GRP94 (positive LR = 1.89 $P = 0.003$), and TRAF2 (positive LR = 1.67, $P = 0.055$). Furthermore, GRP94 was the best negative predictive marker (negative LR = 0.16), followed by TRAF2 (negative LR = 0.35), FN14 (negative LR = 0.40), and ErbB-2 (negative LR = 0.42). Thus, the absence of the endoplasmic reticulum stress (ERS) response phenotype in tumors predicted the absence of brain metastasis.

For a validation set, we used a series of 295 breast tumors for which the transcriptomic data were publicly available.[18,19] As expected, the highest predictive value was ErbB-2 expression (positive LR = 8.27, $P < 0.00001$). Moreover, TRAF2 (positive LR = 2.36, $P = 0.026$), GRP94 (positive LR = 1.72 $P = 0.028$), and FN14 (positive LR = 1.78, $P = 0.044$) were associated with brain metastasis.

A multivariate analysis based on stepwise logistic regression retained GRP94, FN14, and inhibin as the best combination to discriminate brain metastasis. The AUC value for this combination was 0.85 (95% CI = 0.73 to 0.96), substantially better than that for ErbB-2, which was the variable more strongly associated with brain metastasis (AUC = 0.76, 95% CI = 0.58 to 0.93). The model that combined ErbB-2, GRP94, FN14, and inhibin expression further increased the discrimination of metastatic disease in brain (AUC = 0.91, 95% CI = 0.77 to 1.00). The ROC curves for the three models are shown in Figure 7.

We also performed a stratified analysis to check the relationship between ErbB-2 positivity and ERS response phenotype in binary combinations (Table 7). In ErbB-2$^-$ tumors, FN14 had a high negative likelihood ratio to predict the absence of brain metastasis progression (LR = 0.26, sensitivity = 0.8, $P = 0.015$).

Discussion

Fewer than 10% of breast cancer patients have detectable distant metastasis at diagnosis.[36] Thus, it is necessary to understand the properties of brain-tropic tumor cells to identify patients with risk of brain metastasis and to effectively prevent it. Because we assume that metastasis colonization could already be underway at the time of diagnosis, the ERS response phenotype might be a predictive tool to help decide on treatment under the risk of brain metastasis progression.

Table 4. *In silico* Validation of the Endoplasmic Reticulum Stress Phenotype, Taking Into Account Previous Experimental And Clinical Reports

Reference	Array platform	Description of sample	Gene signature	Match to present study
26	Agilent 24479 60-mer oligos	97 Samples from LN^- patients	231 Prognosis reporters (risk of distant metastasis)	0
			430 Brca1 reporters	3 (*EEF1D*, *MCM4*, *RPL5*)
			2460 ER^- reporters	3 (*CLN3*, *MCM4*, *RPS12*)
30	Rosetta inkjet (24479 genes; breast adenocarcinoma) oligonucleotide microarray	279 Primary tumors of diverse types (lung, breast, prostate)	128 Genes able to distinguish patients with good versus poor prognosis	0
27	Multiple gene expression signatures "metagenes"	86 LN^+ breast cancer patients	143 Predictors of lymph node metastasis	0
			165 Predictors of breast cancer recurrence	2 (*FAM3A*, *TBCD*)
28	Affymetrix U133A 25-mer oligos	LN^- and LN^+ patients with invasive breast cancer	76-Gene signature to distinguish LN^- primary breast cancer to develop distant metastasis within 5 years	0
33	Affymetrix U133A	82 Breast cancer patients (primary tumors)	95 Genes predictors of lung metastasis	0
31	Agilent 24479 60-mer oligos	161 Patients in stage I and II breast cancer with age <53 years	70-Gene predictor of local recurrence	0
29	Agilent 22575 60-mer oligos	135 Tumor samples (no criteria for selection)	70-Gene prognostic signature (risk of metastasis)	0
32	Operon 70-mer two-color 21239 probes	35 Patients: primary tumor and lymph node metastasis paired samples	79 Differentially expressed genes between primary samples and metastasis samples	0
35	Affymetrix U133A	8 Bone metastases, 18 brain metastases and 3 primary tumors	51 Brain metastasis specific genes (versus bone metastasis)	1 (*GFAP*)
19	Affymetrix U133A	CN34-BrM2 and MDA231-BrM2 brain metastatic cell lines.	17 Genes whose expression was correlated with brain relapse	0
		368 Breast cancer primary tumors	26 Genes whose expression was increased in brain metastatic cell lines but not in bone or lung metastatic cell lines	0

LN, lymph node.

The phenotype includes the overexpression of GRP94, FN14, and TRAF2, which are well correlated with brain metastasis in breast cancer patients. Our search for a multivariate panel of markers to predict brain metastasis revealed that the combination of GRP94, FN14, and inhibin together has a better discriminate accuracy than ErbB-2 alone, and that the best accuracy is obtained combining all four markers. Although all variables in these models were significantly associated with brain metastasis in the multivariate models, the increase in predictive accuracy measured by the difference in AUC was not (because of the small sample size in the present study). Indeed, TRAF2, which was associated with brain metastasis, had many missing values and could not be included in the multivariate analysis. The ERS response phenotype is indicative of a new tool to discriminate the risk of brain metastasis in both ErbB-2^+ and ErbB-2^- breast cancers. Moreover, the absence of the ERS response phenotype in tumors might predict the absence of metastasis.

These biomarkers can help in selection of treatment strategies, furthering the current ambitious aim of identifying treatment strategies that will cure patients with ErbB-2^+ disease while ensuring minimal toxicity for each individual patient.[37] Hicks et al[38] reported that EGFR expression, like ErbB-2, predicted the develop-

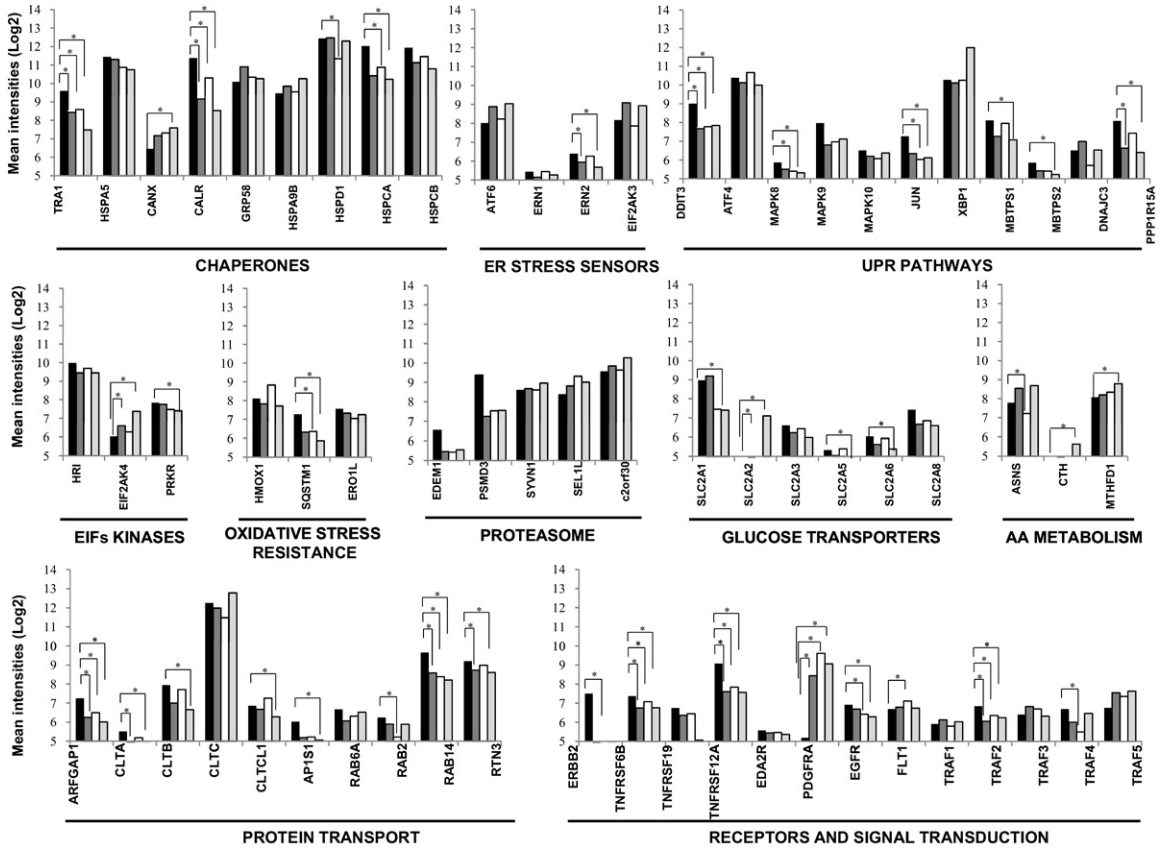

Figure 5. Differentially expressed genes in brain metastasis (black), compared with metastases in other organs: lung (dark gray), bone (white), and liver (light gray), based on a Mann-Whitney test calculated for each gene using the normalized log intensities (see further in Table 5). $*P < 0.05$, statistically significant different expression between bracketed organs.

ment of brain metastasis. The incidence of brain metastasis in patients with breast cancer overexpressing ErbB-2 who are being treated with trastuzumab is double that in other breast cancer patients; one-third will develop central nervous system metastasis, and this often occurs when they are responding to therapy at other sites or have a stable disease.[39] One of the clinical questions is whether this receptor remains a viable target after disease progression,[40] and whether trastuzumab treatment can prevent brain metastasis or whether it encourages the development of metastatic cells that have crossed the blood-brain barrier.

GRP94 overexpression in brain metastasis might modulate ERS responses through activation of PERK, ATF6, and IRE1.[41] Downstream from GRP94 activation, transcription of chaperones and protein degradation might increase in brain metastatic cells. (We are currently exploring these pathways in our laboratory.) Because therapy decisions should depend on the tumor phenotype, the known close correlation between brain metastasis potential and ERS response phenotype in primary tumors suggests that HSP90 and proteasome inhibitors might be alternatives for treatment of breast cancer patients with a high risk of brain metastasis.[42] It has been reported that inhibition of HSP90, which helps expression and folding of its client proteins, such as ErbB-2, could simultaneously inhibit the expression of viable receptors.[40] Furthermore, the expression of GRP94 has been associated with poor prognosis in gastric carcinomas,[43] and with chemotherapy resistance of lung cancer cells and ovarian carcinoma cells.[44]

The switch from dormancy to growth of cancer cells in the brain may be dependent on stress response mechanisms, subsequent coordination of detoxification and redox pathways,[16] and cytokines produced by glial cells, which may contribute, in a paracrine manner, to the final development of brain metastasis. We identified overexpression of the FN14 gene, a member of the tumor necrosis factor (TNF) superfamily of receptors.[45] FN14 is an immediate early response gene whose expression is directly activated after exposure to growth factors in fibroblasts; it is up-regulated in migration-stimulated glioma cells *in vitro*, and it has been associated with high-grade tumors.[46] Through activation of MAPK8/JNK and NF-κB, the TRAF proteins mediate signal transduction of the TNF receptor superfamily members[47]; they could connect ERS responses and FN14 signaling pathway activation. Because FN14 and TRAF2 are overexpressed in breast cancer tumors that develop brain metastasis, and in brain metastatic cells, the disruption of the TWEAK/FN14 feedback loop also emerges as a molecular targeting strategy.

Table 5. Differentially Expressed Genes in Brain Metastasis versus Non-Brain Metastases

UniProtKB ID	Protein name	Gene symbol	Metastasis*		*P* value†
			Brain	Non-brain	
		Chaperones			
P14625	94kDa glucose-regulated protein (alt.: GRP94)	*HSP90B1* (prev. *TRA1*)	9.59	8.10	**0.002**
P11021	78kDa glucose-regulated protein	*HSPA5*	11.44	10.95	0.168
P27824	Calnexin	*CANX*	6.45	7.35	**0.035**
P27797	Calreticulin	*CALR*	11.38	9.22	**0.006**
P30101	58kDa glucose-regulated protein (alt.: p58; ERp57; ERp60)	*PDIA3* (prev. *GRP58*)	7.82	4.05	**0.003**
P38646	75kDa glucose-regulated protein	*HSPA9* (prev. *HSPA9B*; syn. *GRP75*)	9.46	9.91	0.465
P10809	60kDa heat shock protein, mitochondrial	*HSPD1*	10.47	9.71	0.144
P07900	Heat shock protein 90kDa alpha (cytosolic), class A member 1	*HSP90AA1* (prev. *HSPCA*)	12.03	10.52	**0.006**
P08238	Heat shock protein 90kDa alpha (cytosolic), class B member 1	*HSP90AB1* (prev. *HSPCB*)	11.93	11.06	0.168
		Endoplasmic reticulum stress sensors			
P18850	Activating transcription factor 6	*ATF6*	8.01	8.70	0.256
O75460	Serine/threonine-protein kinase/endoribonuclease IRE1 (alt.: inositol-requiring protein 1; IRE1a)	*ERN1*	5.44	5.27	0.441
Q76MJ5	Serine/threonine-protein kinase/endoribonuclease IRE2 (alt.: inositol-requiring protein 2; IRE1b)	*ERN2*	6.39	5.93	**0.038**
Q9NZJ5	Eukaryotic translation initiation factor 2-alpha kinase 3 (alt.: PRKR-like endoplasmic reticulum kinase)	*EIF2AK3* (syn. *PERK*)	5.02	4.85	0.155
		UPR pathways			
P35638	DNA damage-inducible transcript 3 protein	*DDIT3* (syn. *CHOP*, *GADD153*)	9.01	7.69	**0.006**
P18848	cAMP-dependent transcription factor ATF-4	*ATF4*	10.38	10.27	0.626
P45983	Mitogen-activated protein kinase 8 (alt.: c-Jun N-terminal kinase 1)	*MAPK8*	5.86	5.38	**0.009**
P45984	Mitogen-activated protein kinase 9 (alt.: c-Jun N-terminal kinase 2)	*MAPK9*	7.96	6.95	0.144
P53779	Mitogen-activated protein kinase 10 (alt.: c-Jun N-terminal kinase 3)	*MAPK10*	6.50	6.26	0.441
P05412	Transcription factor AP-1 (alt.: proto-oncogene c-Jun)	*JUN*	7.27	6.08	**0.006**
P17861	X-box-binding protein 1	*XBP1*	10.27	10.85	0.417
Q14703	Membrane-bound transcription factor site-1 protease (alt.: endopeptidase S1P)	*MBTPS1*	8.10	7.35	0.062
O43462	Membrane-bound transcription factor site-2 protease (alt.: endopeptidase S2P)	*MBTPS2*	5.85	5.31	0.155
Q13217	DnaJ homolog subfamily C member 3 (alt.: Protein kinase inhibitor p58)	*DNAJC3*	4.87	4.43	**0.006**
O75807	Protein phosphatase 1 regulatory subunit 15A (alt.: growth arrest and DNA damage-inducible protein GADD34)	*PPP1R15A* (syn. *GADD34*)	8.08	6.74	**0.009**
		EIF kinases			
Q9BQI3	Heme-regulated inhibitor	*HRI*	9.98	9.51	0.33
Q9P2K8	Eukaryotic translation initiation factor 2-alpha kinase 4 (alt.: GCN2-like protein)	*EIF2AK4*	6.02	6.73	0.052
P19525	Interferon-induced, double-stranded RNA-activated protein kinase (alt.: protein kinase RNA-activated)	*EIF2A2* (syn. *PKR*, *PRKR*)	7.84	7.52	0.061
		Oxidative stress resistance			
P09601	Heme oxygenase 1	*HMOX1*	8.10	8.02	0.516
Q13501	Sequestosome-1	*SQSTM1*	7.27	6.15	**0.004**
Q96HE7	ERO1-like protein alpha	*ERO1L*	7.56	7.19	0.18

(table continues)

Table 5. *Continued*

UniProtKB ID	Protein name	Gene symbol	Metastasis*		*P* value†
			Brain	Non-brain	
		Proteasome			
Q92611	ER degradation-enhancing alpha-mannosidase-like 1	*EDEM1*	6.53	5.41	0.088
O43242	26S proteasome regulatory subunit S3	*PSMD3*	9.38	7.45	**0.043**
Q86TM6	E3 ubiquitin-protein ligase synoviolin	*SYVN1*	8.59	8.77	0.441
Q9UBV2	Protein sel-1 homolog 1 (alt.: suppressor of lin-12-like protein 1)	*SEL1L*	8.36	9.05	0.871
Q96DZ1	Endoplasmic reticulum lectin 1 (alt.: XTP3-transactivated gene B protein; XTP-3)	*ERLEC1* (prev. *C2orf30*)	9.54	9.93	0.57
		Glucose transporters			
P11166	Solute carrier family 2, facilitated glucose transporter member 1 (alt.: GLUT-1)	*SLC2A1*	8.96	7.97	0.081
P11168	Solute carrier family 2, facilitated glucose transporter member 2 (alt.: GLUT-2) (liver)	*SLC2A2*	3.43	4.64	0.123
P11169	Solute carrier family 2, facilitated glucose transporter member 3 (alt.: GLUT-3) (brain)	*SLC2A3*	6.60	6.13	0.871
P22732	Solute carrier family 2, facilitated glucose transporter member 5 (alt.: GLUT-5)	*SLC2A5*	5.33	5.07	0.074
Q9UGQ3	Solute carrier family 2, facilitated glucose transporter member 6 (alt.: GLUT-6)	*SLC2A6*	6.03	5.60	0.074
Q9NY64	Solute carrier family 2, facilitated glucose transporter member 8 (alt.: GLUT-9)	*SLC2A8*	7.43	6.72	**0.035**
		Amino acid metabolism			
P48067	Sodium- and chloride-dependent glycine transporter 1 (alt.: GlyT-1)	*SLC6A9*	5.06	4.78	**0.035**
P08243	Asparagine synthetase	*ASNS*	7.78	8.15	0.49
P32929	Cystathionine gamma-lyase	*CTH*	4.06	4.75	0.144
P11586	Methylenetetrahydrofolate dehydrogenase	*MTHFD1*	8.07	8.48	**0.035**
		Protein transport			
Q8N6T3	ADP-ribosylation factor GTPase-activating protein 1	*ARFGAP1*	7.25	6.21	**0.003**
P09496	Clathrin light chain A	*CLTA*	5.51	4.80	**0.009**
P09497	Clathrin light chain B	*CLTB*	4.66	4.11	**0.015**
Q00610	Clathrin heavy chain 1	*CLTC*	12.25	12.07	0.685
P53675	Clathrin heavy chain 2	*CLTCL1*	6.86	6.72	0.33
P61966	AP-1 complex subunit sigma-1A (alt.: sigma-adaptin 1A)	*AP1S1* (prev. *CLAPS1*)	6.02	5.12	**0.009**
P20340	Ras-related protein Rab-6A	*RAB6A*	6.66	6.30	0.074
P61019	Ras-related protein Rab-2A	*RAB2A* (prev. *RAB2*)	9.67	8.73	**0.043**
P61106	Ras-related protein Rab-14	*RAB14*	9.65	8.38	**0.002**
O95197	Reticulon-3	*RTN3*	9.19	8.73	**0.019**
		Receptors and signal transductors			
P04626	Receptor tyrosine-protein kinase erbB-2	*ERBB2*	7.50	4.17	**0.009**
O95407	Tumor necrosis factor receptor superfamily member 6B	*TNFRSF6B*	7.37	6.85	**0.003**
Q9NS68	Tumor necrosis factor receptor superfamily member 19	*TNFRSF19*	5.14	4.53	**0.006**
Q9NP84	Tumor necrosis factor receptor superfamily member 12A (alt.: FN14)	*TNFRSF12A* (syn. *FN14*)	9.07	7.68	**0.002**
Q9HAV5	Ectodysplasin-A2 receptor (alt.: tumor necrosis factor receptor superfamily member 27)	*EDA2R* (syn. *TNFRSF27*)	5.57	5.41	0.516
P16234	Alpha-type platelet-derived growth factor receptor	*PDGFRA*	5.19	9.09	**0.002**
P00533	Epidermal growth factor receptor	*EGFR*	6.92	6.42	**0.012**
P17948	Vascular endothelial growth factor receptor 1	*FLT1*	7.09	7.47	0.088
Q13077	TNF receptor-associated factor 1	*TRAF1*	5.91	5.99	0.655
Q12933	TNF receptor-associated factor 2	*TRAF2*	6.85	6.20	**0.003**
Q13114	TNF receptor-associated factor 3	*TRAF3*	6.40	6.63	0.417
Q9BUZ4	TNF receptor-associated factor 4	*TRAF4*	6.60	5.39	**0.035**
O00463	TNF receptor-associated factor 5	*TRAF5*	6.75	7.55	0.330

*Based on a Mann-Whitney *U*-test calculated for each gene using normalized log-intensities.
†Statistically significant differences are highlighted in bold face type.
alt., alternative name (proteins); prev., previously approved symbol (genes).

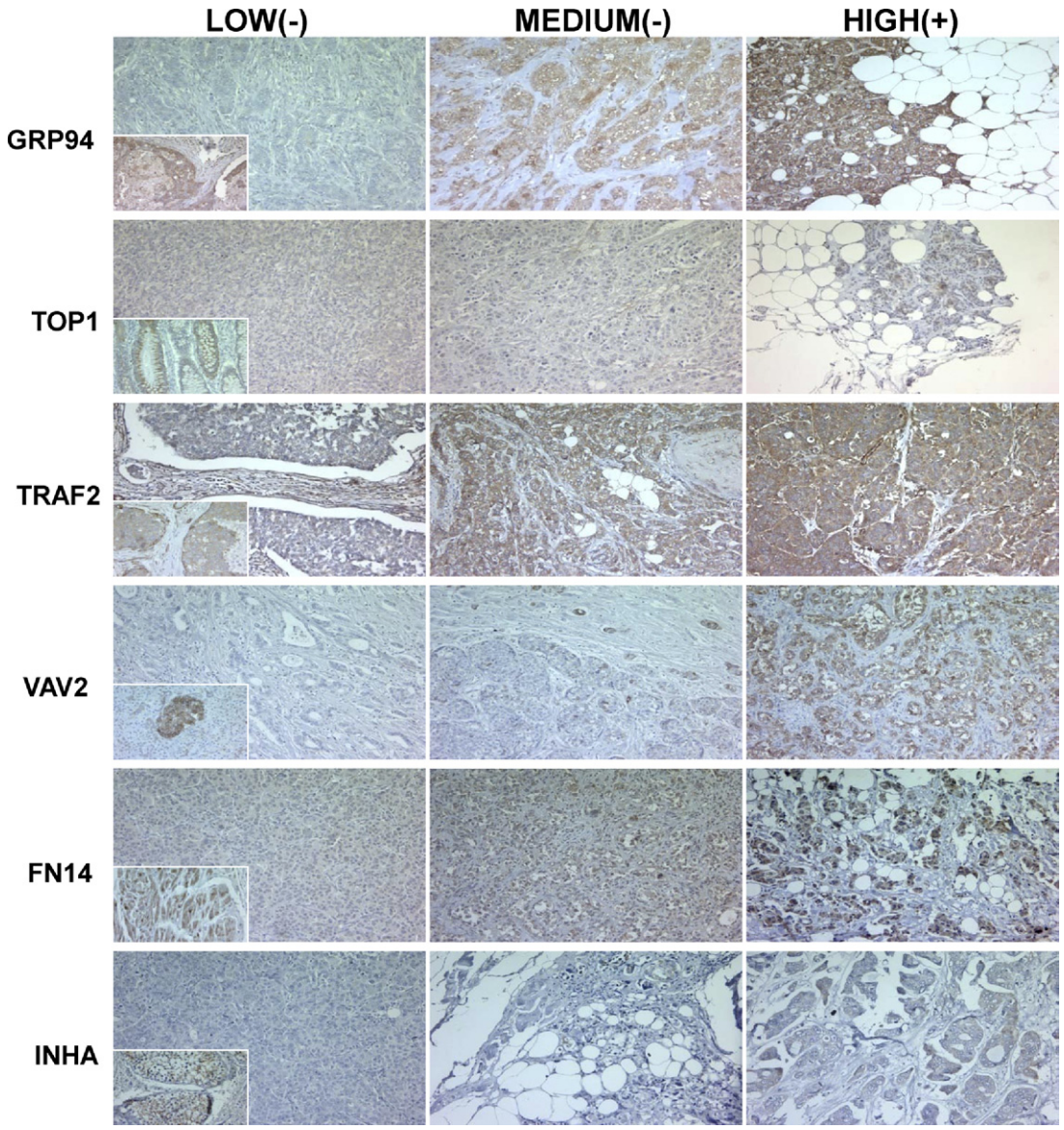

Figure 6. ERS response phenotype in breast cancer at first diagnosis. Representative tabulation of protein expression in breast cancer tissues. Tissues are shown as viewed by light microscopy. Low and medium intensities of staining were considered negative for semiquantitative purposes, and only tumors with high intensity staining were considered positive. **Insets:** Standard positive control tissue sample used in each determination. Original magnification, ×10.

Table 6. Risk of Brain Metastasis Associated with Each Marker in Breast Cancer

Brain metastasis marker	UniProtKB ID	Sensitivity*	Specificity*	LR		*P* value†
				Pos	Neg	
ErbB-2	P04626	8/13 (61.5)	90/99 (90.9)	6.70	0.42	<0.0001
GRP94	P14625	12/13 (92.0)	55/107 (51.4)	1.89	0.16	0.003
FN14	Q9NP84	9/13 (69.2)	80/104 (77.0)	3.01	0.40	0.001
TRAF2	Q12933	9/11 (81.8)	45/88 (51.1)	1.67	0.35	0.055
VAV2	P52735	2/13 (15,4)	95/107 (88.8)	1.38	0.95	0.65
TOP1	Q9UJN0‡	4/13 (30.8)	91/105 (86.6)	2.30	0.80	0.11
Inhibin	P05111)	0/13 (0)	97/107 (90.7)	0	1.10	0.60

*Variation in denominators derives from missing values in the IHC (eg, tissue lost, unviable staining, or background).
†Fisher's exact test, 2-sided.
‡Secondary accession number. The primary (citable) accession number is number is P11387.
LR, likelihood ratio; Neg, negative LR; Pos, positive LR.

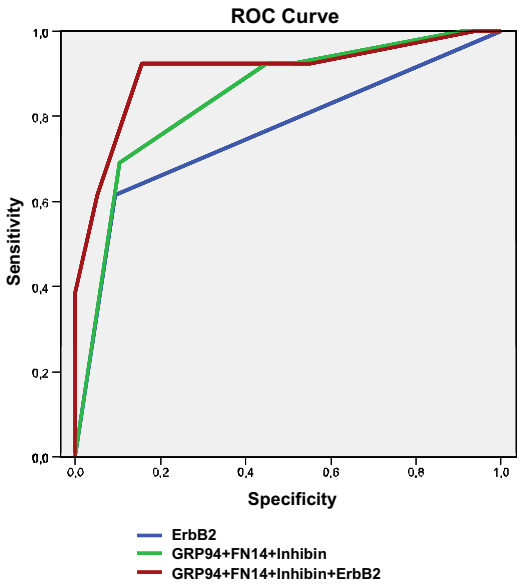

Figure 7. The area under the ROC curve (AUC) obtained with the integrated predictive indexes. Markers were assessed in a multivariate logistic regression model using a forward stepwise procedure to identify the best combination to predict brain metastasis. For ErbB-2 alone, AUC = 0.76; for GRP94, FN14, and inhibin in combination, AUC = 0.85; and for ErbB-2, GRP94, FN14, and inhibin in combination, AUC = 0.91.

FN14 overexpression can stimulate survival through interaction with the inhibitor-of-apoptosis proteins (IAPs).[48] TNF-like weak inducer of apoptosis (TWEAK)/FN14 signaling recruits cIAP1-TRAF2 complex, which is then targeted for lysosomal degradation. Cell sensitivity to TWEAK correlates with sensitivity to synthetic IAP antagonist. Studies with FN14-overexpressing tumor cells that could be selectively destroyed using a TWEAK-cytotoxin protein fusion suggest that FN14 could be a new molecular target for treating metastasis.[49] Indeed, the ERS response phenotype might indicate new opportunities in anticancer strategies for sanctuary sites and micrometastatic disease. Evidence of such phenotypes can be used to develop more specifically addressed therapies.

Microarray-based gene studies are difficult to interpret, because of the huge amount of data involved, and it is therefore a challenge to derive biological insights. We applied a PPIN-based approach that identifies markers not as individual genes but as subnetworks extracted from PPINs, thus providing a systemic view of the interactome. This method serves to filter information by picking out key protein functions as metastasis markers. Indeed, we have delineated a pathogenic mechanism of metastasis to the brain based on the information from a proteomic study of brain metastasis cellular proteins. Further work is needed to confirm the prognostic value of the ERS response phenotype in a second validation step that includes a large independent group to increase the statistic power of the study and to assess the usefulness of the ERS response phenotype as a predictive tool at first diagnosis.

To validate these markers, the main objective is to obtain a large collection of retrospective samples, far in excess of the typical numbers required to obtain statistical significance in the data. This could also lead to preventive therapy for brain metastases at initial diagnosis, not only in breast cancer patients, but also for other carcinomas with brain tropism.

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Table 7. Risk of Brain Metastasis Associated with Each Marker in Breast Cancer with Regard to ErbB-2 Expression

	ErbB-2$^+$			ErbB-2$^-$		
Brain metastasis marker*	Sensitivity†	Specificity†	*P* value‡	Sensitivity†	Specificity†	*P* value‡
Novel markers						
GRP94	8/8 (100)	2/9 (22.2)	0.47	4/5 (80)	48/89 (53.9)	0.19
FN14	5/8 (62.5)	6/9 (66.7)	0.35	4/5 (80)	68/88 (77.3)	0.015
TRAF2	6/7 (85.7)	5/9 (55.6)	0.15	3/4 (75)	36/71 (50.7)	0.62
Traditional markers						
ER	3/7 (42.9)	6/8 (75)	0.61	2/4 (50)	21/83 (25.3)	0.28
PR	2/7 (28.6)	6/7 (85.7)	1.0	1/4 (25)	29/81 (35.8)	0.15
Histologic grade III	6/8 (75.0)	3/9 (33.3)	1.0	1/4 (25)	42/45 (93.3)	0.62

*For novel markers, UniProtKB identifiers are as follows: GRP94, P14625; FN14, Q9NP84; TRAF2, Q12933. For traditional markers: estrogen receptor ER, P03372; progesterone receptor PR, P06401.
†Variation in denominators derives from missing values in the IHC (eg, tissue lost, unviable staining, or background).
‡Fisher's test.

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