Biological Pathways Contributing to Organ-Specific Phenotype of Brain Metastatic Cells

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Secondary to the increased survival following chemotherapy, brain metastases have recently become a significant clinical problem for breast cancer patients. The aim of this study was to characterize those functional phenotypes that might enhance brain metastasis in breast cancer cells. We first analyzed by two-dimensional electrophoresis (2DE-DIGE) differences in protein expression between parental MDA-MB 435 cells and the brain metastatic variant 435-Br1, obtaining 19 identified proteins by peptide mass fingerprinting, 11 under-expressed (<2-fold) and 8 overexpressed (>2-fold) in 435-Br1. We created and analyzed protein interaction networks with a bioinformatic program (PIANA) from protein data, and it allowed us to associate 34/67-laminin receptor functionally with HSP 27, through a chaperone glucose-regulated protein GRP 94. Moreover, HSP 27 had the largest amount of direct and indirect protein interactions, forming a cluster of chaperones and cochaperones, associated through kinases to a set of intermediated filament proteins. In addition, functional groups of proteins identified were peptidase, DNA binding transcription factors, ATP synthase complex, anion transporters, and carbohydrate metabolism. Further functional analyses in cells, expression analyses in experimental tissues, and in human brain metastasis were addressed to validate the biological pathways contributing to organ-specific phenotype of brain metastasis.

Keywords: Brain metastasis • Breast cancer • Heat shock proteins • Laminin Receptor • Proteomics

Introduction

Brain metastasis is a major cause of morbidity and mortality in human malignancies. Secondary to greater survival in patients receiving chemotherapy, cerebral metastases have recently become a significant clinical problem for breast cancer patients, particularly those whose tumors overexpress HER2, in which case the incidence of brain metastasis rises to 30–40%.1–3 The current incidence of brain metastasis seems to be the paradoxical result of the effectiveness of drugs that do not cross the blood–brain barrier (BBB).

The propensity to metastasize might be hardwired early during tumor development, even when clinical metastasis appears much later.4 However, whether this propensity is hardwired in all neoplastic cells, or whether they change as metastasis progresses still remains largely unknown. New studies using mRNA expression profiling give us the opportunity to look closer at the molecular networks in cells contributing to metastasis.5 It has been found that breast cancer carrying the gene-expression signature as a predictor of survival were most associated with metastasis and poor clinical outcome, suggesting that the metastatic potential of human tumors is encoded in the bulk of a primary tumor and that tumors likely to metastasize are fundamentally different.6–8 The genes making up the “poor prognosis” signature or “metastatic predisposition” signatures could be genes expressed by the tumor cells themselves or by stromal cells, including vasculature, connective tissue, or immune cells.9

Moreover, microenvironmental factors at the metastatic foci may affect the response of tumors to chemotherapy and condition drug resistance.10 Since different pathways might be connected to the achievement of metastatic activity,11 therapeutic intervention aimed at the stroma may have limited effectiveness. There are diverse growth-promoting factors that redundantly induce tumor–microenvironment interactions.

The aim of this study was to characterize the functional phenotypes that might enhance brain metastasis in breast cancer cells. Two-dimensional electrophoresis (2DE-DIGE) assessed the distinct expression of proteins in a well-known breast cancer cell line MDA-MB 435 (435-P) and in a brain metastatic variant (435-Br1). To describe the proteome network in breast cancer cells that metastasize in brain, we used a
bioinformatics tool (PIANA) that creates and analyzes protein interaction networks. Protein functions were used to cluster proteins within 8 groups: structural proteins, carbohydrate metabolism, protein folding and chaperones, signaling receptors, peptidases, DNA binding and transcription factors, ATP synthase complex, and anion transporters. Given the interactions of known proteins, we propose that HSP activity engages in breast cancer cells through a chaperone glucose-regulated protein, GPR 94. HSP 27 has the largest amount of direct and indirect connections with other HSPs, forming a cluster of associated chaperones and cochaperones.

Materials and Methods

Cells and Experimental Tissues. MDA-MB 435 cell cultures (435-P) and 435-Br1 cells, established from brain metastasis in nude mice, with ability to metastatize in brain,12,13 were maintained under standard conditions.14

We generated orthotopic primary tumors in 7-week-old athymic Nude Balb/c female mice by inoculation of 1 × 10⁶ 435-P cells in 0.05 mL of medium without serum in the right inguinal mammary gland (i.m.f.p.), following previous protocols.14 Metastatic involvement was explored in paraffin sections by hematoxylin-eosin stain. Metastatic variants in lungs (435-L2, 435-L3, and 435-L2/L5) and lymph nodes (435-N1 and 435-N3) were obtained as described.15

Brain metastases were obtained by internal carotid injection (i.a.), using a slight modification of a method already described.12 Briefly, Nude Balb/c female mice were anesthetized by intraperitoneal injection and placed under a dissecting microscope. We used a Hamilton needle to deliver 1 × 10⁶ cells into the internal carotid lumen. When symptoms of brain metastasis appeared 2 months later, mice were put down, and brains were removed and fixed in 4% paraformaldehyde for 48 h. The cell pellets were frozen in N₂ and stored at −80°C until electrophoresis.

To separate 435-Br1 cells with affinity for laminin adhesion, 1.5 × 10⁶ cells from exponential cultures were resuspended in medium containing 2% BSA and incubated with 40 µg/mL laminin (Invitrogen Corporation, Carlsbad, CA) for 18 h. The cells in the supernatant were collected (435-Br1_NAD), and adherent cells (435-Br1_Ad) were harvested by scraping. In adhesion experiments, we used the peptide YIGSR, corresponding to amino acids 929–933 of the laminin-1 β1 chain, and antilaminin antibody (F-18, Santa Cruz Biotechnology, Santa Cruz, CA) to inhibit specifically the assay.

In some experiments, we used brain metastasis variants from human breast cancer provided through the METABRE consortium: MDA-MB 361 (Laboratoire d’Oncogenetique, Centre Rene Huguenin, Saint-Cloud, France) and SAS2 (Laboratory of Experimental Cancerology, University of Ghent, Belgium).

Five brain metastases from patients surgically treated at the CSUB between 1998 and 2004 have been analyzed by IHC in consecutive slides from a single block of tissue routinely preserved in the Pathology Service. The corresponding breast tumors have been included in the analysis. All of them were invasive ductal carcinomas.

Two-Dimensional Gel Electrophoresis (2DE). For peptidomic analyses, 1 × 10⁶ cells were plated onto 75-mm flask in complete medium for 48 h, starved for 24 h in serum-free medium, and replaced by complete medium for a further 24 h. The cell pellets were frozen in N₂ and stored at −80°C until electrophoresis.

Protein extracts were purified using Ettan 2D CleanUp kit (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer’s instructions. Fifty micrograms each of whole cell extracts of 435-P and 435-Br1 cells were labeled with 400 pmol of Cy3 and Cy5, respectively (EttanTM DIGE, Amersham Biosciences AB). Immobilized pH 4–7 gradient (IPG) 18 cm strips, (Immobiline DryStrip 4–7 NL, Amersham Biosciences AB) were rehydrated overnight in the absence of sample with 350 µL of rehydration solution (8 M urea, 4% CHAPS, 0.5% IPG buffer, and 13 mM DTT). Both protein samples were placed on the Immobiline Strip with the cup-loading technique. Isoelectric focusing was performed using an IPGphor apparatus (Amersham Biosciences AB) for a total of 55 kVh, 50 µA/strip at 20°C, according to manufacturer’s instructions. Immediately after being focused, IPG strips were equilibrated for 15 min in 65 mM DTT, 100 mM Tris, 6 M urea, 30% glycerol, and 2% SDS, followed by 475 mM iodoacetamide, 100 mM Tris, 6 M urea, 30% glycerol, and 2% SDS. Then, the second dimension was carried out on 12.5% polyacrylamide gels of 20 × 20 cm in an electrophoresis tank Protein II (Bio-Rad, Hercules, CA).

For protein identification, preparative 2DE gels loaded with 300 µg of protein were run following the same protocol. After electrophoresis, gels were fixed and stained using a silver-staining low fixation protocol compatible with mass spectrometry.

Analysis of Gel Images. Three gels from three independent experiments were scanned with the Typhoon Variable Mode Imager (Amersham Biosciences AB). The digitalized 2DE gel images were standardized and comparatively analyzed with the DeCyder Differential Analysis Software program, version 5.0 (Amersham Biosciences AB).

Differential expressed proteins were identified with the DIA module of DeCyder (Amersham Biosciences AB). Images were normalized and statistically analyzed, and differentially expressed proteins were identified and quantified with DeCyder-DIA using the Cy dye generated images.

The ratio values were standardized, according to Ri = log10 (V2i/V1i), where the spot volumes (Vi) were in gel sample 1 (V1i) and gel sample 2 (V2i), respectively. Ratios were expressed in the range of 2-fold increase and decrease (2 and −2).

Protein Fingerprinting by MALDI-TOF Mass Spectrometry. Protein spots were selected according to the silver-stain signal without overlap. They were excised from 2DE silver-stained gels and submitted to trypsin in-gel digestion, as described elsewhere.16,17

Peptide mass fingerprinting spectra was recorded in a Voyager STR MALDI-TOF (Applied Biosystems) in positive reflector mode with delayed extraction. The spectra were analyzed using the m/z program (ProteomeTools, New York, NY). Protein was identified against a nonredundant database (NCBI) using the MASCOT program (http://www.matrixscience.com).

Experimental Validation of Specific Proteins Expressed in Metastasis. 1. Western-Blot Analysis (WB). Cells from exponential cultures were lysed in 200 µL of RIPA buffer. The separated proteins in a 12% polyacrylamide gel were transferred to PVDF membranes (Immobilon-™, Millipore Corporation, Bedford, MA). The following antibodies (Ab) were used: casein kinase Iα, clone C-18 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1/100; cathepsin D, clone C-20 (Santa Cruz) at 1:500; galectin 1, clone N-16 (Santa Cruz) at 1/100; GRP 94, clone C-19 (Santa Cruz) at 1/500; GRP 78, clone N-20 (Santa Cruz) at 1/200; HSP 27, clone C-20 (Santa Cruz) at 1:500; keratin 18, clone DC-10 (Santa Cruz) at 1/100; laminin-R, clone F-18 (Santa Cruz) at 1:200 and C-terminal domain (Abcam Ltd., Cambridge, CA)
at 1/300; MMP17, N-terminal domain (Sigma) at 1/500; peroxiredoxin 4, (Laboratory Frontier, Seoul, Korea) at 1/1000; tubulin β, clone H-235 (Santa Cruz) at 1/500; and vimentin, clone RV 202 (Abcam Ltd.) at 1/500, as internal control.

Peroxidase conjugated goat anti-rabbit secondary antibody 1/2000 (Amersham), antihuman secondary antibody 1/2000 (Pierce, Perbio Science Ltd., Cheshire, U.K.), or antigoat secondary antibody 1/3000 (Santa Cruz) were used as appropriate in each case.

Immunoreactive bands were viewed on VersaDoc (Bio-Rad) Imaging System, using the Super Signal west-Pico (Pierce). MWs were established with See Blue Plus2 prestained Standford (Invitrogen, San Diego, CA).

An antihuman Actin monoclonal antibody 1/2000 (Sigma, St. Louis, MO) and antihuman tubulin α, clone B-5-1-2 (Sigma) at 1/10 000 were also used as an internal standard for densitometric analysis, which was evaluated using the quantity of a band with the Quantity One program, as the sum of the intensities of all the pixels within the band boundary multiplied by the area of each pixel.

2. Immunohistochemistry (IHC). IHC analyses of tumor, node, lung, and brain metastases were performed on OCT-embedded tissue sections of 6-µm. We incubated directly the following primary Abs: laminin-R, clone F-18 (Santa Cruz) at 1:100; HSP 27, clone C-20 (Santa Cruz) at 1:100; cathepsin D, clone C-20 (Santa Cruz) at 1:100; galectin 1, clone 1/100 (Santa Cruz); and peroxiredoxin 4 (Laboratory Frontier) at 1/200; GRP 94, clone C-19 (Santa Cruz) at 1/100; GRP 78, clone N-20 (Santa Cruz) at 1/100.

Bound antibody was viewed with appropriate biotinylated anti-IgG: antimouse, 1/250 (Pierce), antirabbit 1/250 (Vector Laboratories, Burlingame, CA), antigoat 1/250 (Vector Laboratories), following DAB staining and counterstaining with hematoxylin in an Olympus BX60 (Olympus Optical Co., LTD, Japan) and digitalized in a Spot digital camera using the Spot 4.2 software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Semiquantitative analysis was assessed from five different areas of the tumor.

3. Immunoprecipitation (IP). Cells were lysed in Privalsky buffer and lysates were incubated with protein A-Sepharose (Amersham Biosciences). We carried out immunoprecipitation of 34/67-LMR and HSP 27 with the corresponding antibody. Bound proteins were solubilized, separated by electrophoresis, and immunoblotted with antibodies anti-37/67-LMR, HSP 27, cathepsin D, GRP 78, GRP 94, and peroxiredoxin 4, as described.

Bioinformatics. Protein–protein interactions can be represented as a protein interaction network, where the nodes are proteins and the edges between the nodes are interactions between proteins. PIANA is a software framework that creates, manages, and analyzes protein interaction networks. PIANA can also be used to predict new interactions for proteins, as well as identifying proteins relevant to the pathway being studied by combining data from interaction databases and electrophoresis experiments. For this work, PIANA contained 2 378 113 interactions from the database of Interacting Proteins, the MIPS Mammalian Protein–protein Interaction database, the STRING database, and interactions predicted by looking at structural similarities between proteins.

The method of prediction based on structural similarities is similar to other methods also used to predict novel interactions involved in cancer. However, it has the advantage to include different levels of accuracy. At the lowest level, it may add novel interactions inferred without homology (i.e., between proteins with other folds different than those of the known interaction initially used for the prediction). We used PIANA to (1) create the protein–protein interaction network for 18 proteins identified by mass spectrometry (hereafter referred to as root proteins), (2) predict new interactions for root proteins using the concept of interologs, and (3) identify proteins that connect the root proteins in the network (hereafter referred to as linker proteins). The results were analyzed by clustering the nodes of the network (proteins) according to their functions and identifying their main connectivity and their relationships with external elements (i.e., receptors) or its signaling transfer (i.e., kinases and transcription factors).

Results

Protein Expression Changes in Brain Metastasis and Organ Specificity. We detected an average of 2053 spots in the 2DE from three independent experiments. Of these spots, 550 were further analyzed to assess differential expression of proteins between parental cells (435-P) and brain metastatic variant 435-Br1 cells. The range of 2-fold increase and decrease (2 and –2) was admitted as differential expression. As a result, 21 proteins were differentially expressed in the three experiments. We picked 23 by MALDI-TOF peptide fingerprint analysis, of which we identified 19 (enlarged with the boxes in Figure 1A). Known proteins are listed in Table 1, with their corresponding MW, pl, and recognized function (according to Swiss-Prot database), as well as references to their relevance to breast cancer, largely derived from experiments using array technologies. The majority of them belong to cell structure, chaperones, stress and redox regulation, and intracellular transport.

One of the spots located at an over-40 kDa MW level in the 2DE was identified in Swiss-Prot as a 40S ribosomal protein (P08865), the synonym of 34/67 kDa laminin-receptor. We assume that this molecule corresponds to 67 kDa laminin-receptor 1, RAD 50-splice isoform 3, RIN3, and 40S ribosomal precursor were overexpressed in 435-Br1 cells. The range of 2-fold increase and decrease (2 and –2) was admitted as differential expression. As a result, 21 proteins were differentially expressed in the three experiments. We picked 23 by MALDI-TOF peptide fingerprint analysis, of which we identified 19 (enlarged with the boxes in Figure 1A). Known proteins are listed in Table 1, with their corresponding MW, pl, and recognized function (according to Swiss-Prot database), as well as references to their relevance to breast cancer, largely derived from experiments using array technologies. The majority of them belong to cell structure, chaperones, stress and redox regulation, and intracellular transport.

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Moreover, since degradation of keratins can occur, the corresponding identified spots were localized in 2DE-gels below 30 kDa.

To assess the different expression of proteins and their specificity in metastasis from different organs, we carried out WB (Figure 2A). We included in the analyses metastatic variants of bone (435-B1), lung (435-L2, 435-L2/L5), lymph node (435-N1 and 435-N3), and a different variant of human brain metastasis from a human breast carcinoma (MDA-MB 361). WB confirmed with an alternative technique the overexpression of 34-LMR (2.15 ratio) in 435-Br1, similar to 361 brain metastatic cells, different from the others metastatic variants; and the greater under-expression of galectin 1 (0.36 ratio), Prx IV (0.48 ratio), cathepsin D precursor (0.29 ratio), and cathepsin D processed form (0.54 ratio) in 435-Br1 cells than in 435-P cells (Supporting Information Table 2). Since the HSP 27 expression was similar in all variants in the WB analysis, we did not validate these experiments the low expression that had been found in the 2DE suggesting a labile expression of this molecule under culture conditions.

Conversely, lung, bone and lymph node metastasis had greater expression of galectin 1 (ratios 1.82 to 2.58), without changes in 34-LMR, Prx IV, and processed form of cathepsin D, than 435-P cells. As a result, we concluded that 34/67-LMR overexpression and galectin 1 and Prx IV under-expression were changes associated with brain metastatic tropism.

Since the growth of cells arrested in an organ is dictated by molecular interactions of the cells with the organ environment,27 we evaluated by IHC protein expression in tissues from experimental metastasis how to differentiate protein expression changes due to culture conditions without relevance for metastatic growth. Furthermore, we checked the brain microenvironment influences and evaluated organ-specificity comparing expression in brain metastasis with the expression in metastasis from other organs (Figure 2B). Semiquantitative analysis was assessed from five different areas of the tumor. 34-LMR was located in membranes and cytoplasms of 80% of cells in brain metastasis, but only cytoplasmic staining was found in lung, lymph node metastasis, and tumor cells. Galectin 1, Prx IV, and cathepsin D were under-expressed, with less than 50% of cells with low expression in cellular membranes of brain metastasis, as against the nuclear and cytoplasmic expression in lung and lymph node metastasis and tumor (70–80%). HSP 27 was expressed in 80% of cells in brain metastasis,

**Figure 1.** Differential expression of proteins in 435-Br1 cells. (A) Image of 2DE-DIGE carried out with 50 µg of total protein from 435-P and 435-Br1 cells labeled with Cy3 (green) and Cy5 (red), respectively, separated on immobilized pH 4–7 gradient strips, and run on 12.5% polyacrylamide gels. Gels from three different experiments were scanned using Typhoon Variable Mode Imager. The gel in the picture had 2253 spots, of which 658 were further analyzed with the DeCyder software program version 5.0 to assess differential expression of proteins between 435-P and 435-Br1 cells. Proteins identified by mass spectrometry are enlarged with the boxes. (B) Western-blot analyses of 2DE from 435-P and 435-Br1 metastatic variants. A total of 50 µg of protein extracts was applied to immobilized pH 4–7 gradient, and isoelectric focusing was performed according to manufacturer’s instructions. The second dimension was carried out on 12.5% polyacrylamide gels and blotted to PVDF membranes. 34-LMR was detected using specific primary antibodies anti-34-LMR and viewed with HRP-conjugated secondary antibodies.
The root protein HSP 27 (with chaperone function, located between root proteins and the rest of the original proteins). Among them, five were found in the predicted linkers in proliferation, apoptosis, cell death, or the prefix onco*.

Within the set of proposed linkers between root proteins, we also found three transcription factors, TEF-5, the Zinc Finger protein ZNF76, and GTFII-I. The first two are associating information.

**Table 1. Identities of Differentially Expressed Proteins in 435-Br1 Metastatic Cells**

<table>
<thead>
<tr>
<th>protein identity</th>
<th>access no.</th>
<th>pI</th>
<th>MW (kDa)</th>
<th>function</th>
<th>localization</th>
<th>in silico validation</th>
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<tr>
<td>ATP synthase β chain</td>
<td>P06576</td>
<td>5.26</td>
<td>56.53</td>
<td>Catalytic subunit</td>
<td>Mitochondrial</td>
<td>Ross et al.</td>
</tr>
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<td>Cathepsin D</td>
<td>P07339</td>
<td>5.31</td>
<td>26.46</td>
<td>Protease</td>
<td>Lysosomal</td>
<td>Hurlimann et al.</td>
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<td>CGI-38 brain specific Galectin 1</td>
<td>Q9B3W0</td>
<td>9.15</td>
<td>19.15</td>
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<td>Cytoskeletal</td>
<td>Ross et al.</td>
</tr>
<tr>
<td>Glyoxalase I</td>
<td>Q04268</td>
<td>5.34</td>
<td>14.92</td>
<td>Apoptosis/Cell differentiation</td>
<td>Cytoplasmic</td>
<td>Su et al.</td>
</tr>
<tr>
<td>Heat shock 27</td>
<td>Q04760</td>
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<td>20.86</td>
<td>Detoxification</td>
<td>Cytoplasmic</td>
<td>Liu and Rabinovich</td>
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<td>Heat shock 70</td>
<td>P11142</td>
<td>5.37</td>
<td>71.08</td>
<td>Stress/Structural</td>
<td>Cytoplasmic</td>
<td>Perou et al.</td>
</tr>
<tr>
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<td>7.90</td>
<td>80.97</td>
<td>Immune response</td>
<td>Extracellular</td>
<td>Corrona et al.</td>
</tr>
<tr>
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<td>31.89</td>
<td>Adhesion/Transduction</td>
<td>Cytoplasmic</td>
<td>Ross et al.</td>
</tr>
<tr>
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<td>30.75</td>
<td>Redox</td>
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<td>Perou et al.</td>
</tr>
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<td>P23</td>
<td>P13693</td>
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<td>19.60</td>
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<td>Su et al.</td>
</tr>
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<td>Q92878-3</td>
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<td>13.90</td>
<td>DNA repair</td>
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<td>Perou et al.</td>
</tr>
<tr>
<td>40S ribosomal s12</td>
<td>P25398</td>
<td>6.30</td>
<td>14.86</td>
<td>Structural/RNA binding</td>
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*Spots were excised from preparative 2DE gel. Peptide mass spectrum from gel trypsin digestion was obtained by MALDI-TOF mass spectrometry. In silico validation took into account previous experimental and clinical results referring to breast cancer and metastasis expression.

showing strong membrane expression, but only 50% of cells in lymph node, lung, and tumor had HSP 27 in cytoplasm. Therefore, the expression and location of proteins in vivo changed, influenced by the microenvironment in metastatic foci. The increased expression of HSP 27 and 34/67-LMR in metastasis makes it plausible that both proteins were associated functionally.

**Functional Analysis of the Proteome Network in Brain Metastasis.** The protein interaction network between the 19 identified proteins was performed with a bioinformatics tool (PIANA) that creates and analyzes protein interaction. Most edges of the graph were found by protein–protein relationships from STRING, including predictions and text-mined data. Only two interactions were predicted by interologs from two experimentally proved interactions in DIP (between two root nodes: HSP 27 and galectin 1; and between a root node and a nonlinker protein, which was not studied any further: cathepsin D and presenilin 1). Several links were also predicted from sequence patches with structural conservation of interfaces: HSP 70, cathepsin D; and for the ATP-synthase complex.

Protein functions as defined by UniProt were used to cluster proteins within 8 groups (Table 2). A simplified network containing only root and linker proteins was obtained (Supporting Information Table 3), and after further analysis, the network was shown in Figure 3.

A total of nine proteins contained the terms tumor, cancer, proliferation, apoptosis, cell death, or the prefix onco*. Among them, five were found in the predicted linkers between root proteins and the rest in the original proteins. The root protein HSP 27 (with chaperone function, located in cytoplasm and involved in stress resistance) had the largest amount of direct and indirect connections with other root proteins, most of them with other heat shock proteins forming a cluster of associated chaperones and chaperones. It also connected with 34/67-LMR through a chaperone glucose-regulated protein GRP 94 (glucose-regulated protein of 94 kDa, located in endoplasmic reticulum) containing the term “tumor” in the synonym list of gene-names, with GRP 78, with a set of protein kinases (casein kinase I-γ 2 isoform and N1), and with a DNA binding protein, p79PIF, which is also known as trans-acting factor GMEB-2, which associates the cluster of chaperones and the tubulin β 5.

The root protein galectin 1, which is a putative MAPK that may regulate “apoptosis”, is associated with HSP 27 in the cluster of chaperones by a direct link, and also indirectly through the peptidyl prolyl cis–trans isomerase. Galectin 1 is connected to p23, a histamine-releasing factor, through cytokine A2 acting as linker. Galectin 1 is also connected to the ATP-synthase β chain in the ATP synthase complex by the ATP synthase δ chain.

It is noteworthy that several proteins on the graph, either root or linker proteins, are membrane receptors or signal transducers: (i) GTPase potential effector of RAS; (ii) mitogen-activated protein kinase 14; (iii) protein kinase C, which is activated by diacylglycerol; (iv) periaxin; and (v) the glucocorticoid modulatory element binding protein 2, GMEB-2.

Within the set of proposed linkers between root proteins, we also found three transcription factors, TEF-5, the Zinc Finger protein ZNF76, and GTFII-I. The first two are associ-
ated with the 40S ribosomal protein S12 and the last factor with the DNA repair RAD50. TEF-5 connects on the graph the root protein p23 of the family of the translationally controlled tumor proteins (TCTP), and cytokine A2. ZNF76 is a linker between the 40S ribosomal protein S12 and the glyoxalase I (see above). On the other hand, GTFII-I connects RAD50 and tubulin β 5 chain.

Finally, a large set of proteins structurally involved with the cytoskeleton yields a cluster containing three root proteins: keratin 1, keratin 10, and vimentin. The class-III intermediate filament, vimentin, is connected to the chaperone root protein HSP 70 through Actin α 1, to tubulin β 5 chain through casein kinase II, and to keratin 10 through two anion transporters (anion exchange protein 3 and 1) and two cytokeratins 18, type I and II.

**Experimental Validation of the Proteome Network in Brain Metastasis.** To avoid false positives in protein–protein interaction network results, we analyzed known molecules for which specific human antibodies are commercial and the validation tests were possible. Since in vivo a diverse spectrum of extracellular and intracellular stimuli leading to alterations in gene expression and cellular function might be expected, we explored differences between laminin adherent and non-adherent cells in their expression of some linkers (GRP 78, GRP

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Figure 2. Specific expression of proteins in vitro and in experimental metastasis obtained by injecting 435-P cells i.m.f.p. (lung and lymph node metastasis) and, in brain metastasis, obtained by injection of intracranial 435-Br1 cells. (A) Western-blot analyses from metastatic variants. Whole-cell lysates containing 50 µg of total protein from 435-P cells, brain metastatic cells (435-Br1, 361), and variants which metastasize in bone (435-B1), lung (435-L2, 435-L2/L5), and lymph nodes (435-N1, 435-N3) were loaded, separated by PAGE, and blotted to PVDF membranes. The proteins indicated were detected by specific primary antibodies and viewed by HRP-conjugated secondary antibodies. Antihuman Actin monoclonal antibody was used as an internal standard. (B) Immunohistochemistry (IHC) of OCT-embedded tissues obtained from mice, in which breast cancer cells were injected i.m.f.p. Hematoxylin-eosin staining (H & E) of each tissue is shown (panels a, g, m, s) and viewed by light microscopy (×20). Immunolocalization of cathepsin D, peroxiredoxin 4, laminin-R, galectin 1, and HSP 27 expression, respectively, are shown: in breast tumors from mice injected with 435-P cells (panels b, c, d, e, f); in lymph node metastasis (panels h, i, j, k, l); and lung metastasis (panels n, o, p, q, r) from these mice; and in brain metastasis from 435-Br1 cells injected in carotid (panels t, u, v, x, y), all viewed at ×40.
To characterize the organ-specific character of these proteins in brain metastasis, we checked binding to laminin and protein expression in metastatic variants (bone, lymph node, and lung) from 435 cells, two more brain metastatic cells from human breast cancer (361 and SA52), and in 435-Br1 cells from which we separated the laminin-adherent subpopulation, 435-Br1AD, and the non-adherent 435-Br1NAD cells.

Adhesion to laminin (Figure 4A) increased in brain metastatic cells with regards to parental cells \((p = 0.005)\) and cells which metastasize in lung \((p = 0.01)\). This adhesion could be partially inhibited with specific antibodies antilaminin receptor \((p = 0.005)\) and with the YIGSR peptide \((p = 0.01)\). Thus, the increased expression of 34/67-LMR might have functional activity in brain metastatic cells (Figure 4B).

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### Table 2. Classification by Functional Groups of Root and Linker Proteins

<table>
<thead>
<tr>
<th>functional groups</th>
<th>root proteins</th>
<th>linker proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural Proteins</td>
<td>Cytokeratin 1 Cytokeratin 10</td>
<td>Cytokeratin 19, Cytokeratin 14, Cytokeratin 4, Cytokeratin 8, Cytokeratin 18, Alpha-Actin-1, Kinesin family member 21A Phosphoacetylglucosamine mutase</td>
</tr>
<tr>
<td>Protein Folding and Chaperones</td>
<td>40S ribosomal protein S12, Heat shock protein 70, Heat shock protein 27,</td>
<td></td>
</tr>
<tr>
<td>Signaling and Receptors</td>
<td>RIN3 Galectin-1 67 kDa laminin receptor</td>
<td></td>
</tr>
<tr>
<td>Peptidases</td>
<td>Cathepsin D</td>
<td>Casein kinase II, Protein kinase C, DNA-dependent protein kinase catalytic subunit, Casein kinase I, Protein kinase N1, DNA binding protein p79P1F, Mitogen-activated protein kinase 14, Periavin, Acetylcholine receptor protein, epsilin subunit</td>
</tr>
<tr>
<td>DNA-binding and Transcription Factors</td>
<td>RAD50</td>
<td>Xaa-Pro dipeptidase, Carboxypeptidase B DNA binding protein p79P1F, TFII-I, Transcriptional enhancer factor TEF-5, Zinc finger protein 76</td>
</tr>
<tr>
<td>ATP Synthase complex</td>
<td>ATP synthase beta chain, mitochondrial</td>
<td>ATP synthase delta chain, mitochondrial, ATP synthase alpha chain, mitochondrial</td>
</tr>
<tr>
<td>Anion Transporters</td>
<td></td>
<td>Anion exchange protein 3, Band 3 anion transport protein</td>
</tr>
</tbody>
</table>

### Figure 3.

Abstraction of the protein–protein interaction network for the proteins identified by mass spectrometry. Proteins in functional clusters are grouped within a single box containing root and linker proteins (color green). Root proteins are in yellow boxes and linker proteins in cyan, showing transcription factors (TF), the translationally controlled tumor protein (TCTP), peroxiredoxin 4 (Prx IV), and glucocorticoid modulatory element binding 2 (GMEB-2), among others. The inset at bottom right shows the original protein–protein interaction network.
Figure 4C shows that expression of 37/67-LMR, casein kinase II α chain and keratin 18 was greater in 435-Br1AD than in 435-Br1 and 435-Br1NAD (Supporting Information Table 4), which shows that the expression of both protein linkers may be...
regulated by the interaction of 37/67-LMR with laminin, validating their linker character. In addition, keratin 18 was increased in 361 and SA52 brain metastatic cells.

We included in these experiments the analyses of MMP17, a metaloprotease associated with the laminin receptor, which exhibited low expression in all cells, particularly in 435-Br1AD.

The linker proteins GRP 78 and 94 were present in metastatic cells and expression was similar in all variants. Thus, we could not see differences secondary to laminin adhesion.

The plausibility of functional interaction between 34-LMR and HSP 27 was shown with the identification of HSP 27 in immunoprecipitated 34-LMR. Cathepsin D was also detected in the precipitate, though Prx IV was not. Moreover, immunoprecipitated HSP 27 allowed the identification of GRP 94, though GRP 78 was not precipitated (Figure 4D). Thus, the association with Prx IV and GRP 78 was not by direct interaction.

**Plausibility of a Functional Phenotype in Human Brain Metastasis.** The expression of 34/67-LMR is up-regulated in neoplastic cells, in which exogenous laminin-induced mitogen-activated protein kinases directly correlate with invasion and metastatic potential (14). Taking into account known proteins and their interactions, we posit that HSPs are also engaged by 34/67-LMR in brain metastatic cells with a protective function involving glucose-regulated proteins. To analyze this hypothesis, we checked the activation of HSP 27, GRP 78, and GRP 94 proteins in cells in which 34/67-LMR was stimulated (Figure 5). Under stress conditions, HSP 27 and GRP 78 increased more than 2-fold in brain metastatic cells, in contrast to parental cells, associated to a decreased expression of GRP 94. We did not find different expression of these proteins between parental and brain metastatic cells in conventional cultures. These experiments confirmed a different functional axis 34/67-LMR/HSP in brain metastatic cells with regard to parental cells.

To validate the presence of these experimental proteins in human brain metastasis, we explored the expression of them by IHC in five patients with brain metastasis matched with the primary tumor (Figure 6). Semiquantitative analysis was assessed from five different areas of the tumor. 34/67-LMR were...
located in 80–100% of cell cytoplasms and some membranes in brain metastasis; neurons and glia were also positive. In primary tumors, the stained cells were 20–100%, that decreased in the intraductal carcinoma. Only few normal glands were immunoreactive for the antibody.

We confirmed the low expression of galectin 1 in human brain metastasis and primary tumors. Only in two metastasis, we found low and focal cytoplasmic staining.

Cathepsin D had strong cytoplasmic staining in 80–100% metastatic cells and reactive astrocytes. Similar staining was found in primary tumors, either in the infiltrate and in the intraductal component, with negative normal glands. Moreover, we found stromal staining compatible with excreted protein in two cases.

HSP 27 was expressed with medium intensity in 50–100% of cell cytoplasms in brain metastasis, showing strong expression in reactive astrocytes. Similar distribution and intensity was found in primary tumors, including intraductal component and normal glands.

The linker proteins GRP 94 and GRP 78 had strong cytoplasmic staining in 100% of cells in metastasis and in reactive astrocytes, similarly than cells in primary tumors.

**Discussion**

Metastasis to different organs is determined by the tumor cell phenotype and interactions between the tumor cell and the organ environment. Indeed, successful metastasis in the central nervous system (CNS) depends upon the interaction of tumor cells with the host defenses and the brain microenvironment, which, surrounded by the BBB and lacking lymphatic drainage, differs from lung, lymph node, or bone microenvironment. Among other differences in protein expression, we found 34/67-LMR overexpressed in brain metastatic cells and in vivo brain experimental metastasis, suggesting that the lodging and growth of metastatic cells in the CNS is mediated by this nonintegrin receptor. The validation in human brain metastasis, as well as in matched primary tumors, reinforce the importance of this molecule from the primary tumor to the brain metastatic state.

67-LMR recognizes various binding sites on laminin, mediating cellular attachment, migration, angiogenesis, growth, invasion, and metastasis. The exact manner by which the 67-LMR configures its mature form is not clear. It has been shown that a cell–laminin interaction via the 67-LMR is an important step in signal transduction pathways inducing...
mitogen-activated protein kinase cascades, probably in association with the integrin receptor. Therefore, in vivo, a diverse spectrum of extracellular and intracellular stimuli leading to alterations in gene expression and cellular function might be expected.

The plausibility of functional interaction between 34-LMR and HSP 27 was shown with the identification of HSP 27 in immunoprecipitated 34-LMR. The highly conserved heat shock proteins act as molecular chaperones with antiapoptotic activities under physiological conditions, usurping Apaf-1 and cytochrome c, preventing maturation of caspase-9 by the apoptosome, and inhibiting downstream activation of the caspase cascade. Regulated by Stat3, HSP 27 in human breast cancer cells increases anchorage-independent growth, invasiveness, metastatic potential, and resistance to chemotherapeutic drugs, and decreases motility. Expression of HSP 27 in cancer cells increases anchorage-independent growth, invasion, and motility. 

HSP 27 is an ATP-independent molecular chaperone that influences the assembly, transport, and folding of other proteins, and leads to stress resistance against the CNS microenvironment, in which the ATP-dependent chaperon HSP 70 is also implicated. The protective function of these chaperones may involve glucose-regulated proteins, GRP 78 and GRP 94, and an ATP-synthase complex linked with glyoxalase I, on the glyoxal pathway, in a chaperone cluster, and with peroxiredoxins. Several interactions with intermediate-filament polymers might occur by dynamic binding to them, resulting in cell motility, survival, and invasion during metabolic or therapeutic stress. The activity of these pathways might allow tumor–microenvironment cross-talk leading to the achievement of brain metastatic growth.

A set of adaptive pathways known collectively as the endoplasmic reticulum (ER) stress response could be implicated in brain metastasis. In fact, the regulatory role of GRP 94, linkers molecule between 34-LMR and HSP 27 interaction, suggest that brain metastatic cells might respond to environmental stress through the induction of specific ER-proteins, including heat shock proteins and glucose-regulated proteins.

Because of the importance of this function in cancer cells, novel therapeutic approaches targeting molecules implicated in this mechanism are being explored. Thus, in addition to description of functional molecules in brain metastasis, we provide evidence of potential targets for treatment to reduce resistance to chemotherapy. Indeed, inhibition of experimental metastasis using antirecombinant 37-kDa laminin-binding protein antibody has demonstrated that 37LBP is the functional domain of 67-LMR with potential to prevent metastasis.

The root protein galectin 1, which is a putative MAPK that may regulate “apoptosis”, is associated with HSP 27 in the cluster of chaperones by a direct link. Galectins are present both inside and outside cells, and function both intracellularly and extracellularly. Galectin 1 may be exported to the cell surface, affecting the stroma laminin-binding dependent adhesion and growth of carcinoma and melanoma cells, and contributes to immune evasion by inducing apoptosis in effector T cells. Indeed, galectin 1 was under-expressed in brain metastatic cells and was found extracellularly in some brain metastasis. Moreover, galectins may regulate the advanced glycosylation end products-binding proteins (AGE-receptor) by modifying the function of the complex. Low levels of galectin has been associated with increase AGE levels and signaling, and altered AGE-receptor pattern. Thus, the under-expression of this protein also in human brain metastasis suggest an important role in progression.

The principal route for methylglyoxal catabolism is the glyoxalase pathway, and HSP 27 is a major methylglyoxal-modified protein in cells, which in turns contributes to protect from caspase-dependent cell death. Interestingly, glyoxalase I, under-expressed in brain metastasis, is also associated with an unusual level of AGE. Furthermore, glyoxalase I might belong to detoxification pathways altered in breast cancer tumors. A proteomic study comparing HER-2/neu-positive and negative breast cancer cells found enhanced activation of various metabolic, stress-responsive, antioxidant, and detoxification pathways in HER-2/neu-positive cells, and in tumors with Herceptin resistance, including HSP 27 and glyoxalase I.

Peroxiredoxin IV secretable form located on the endoplasmic reticulum has peroxidase activity, and may exert its protective function against oxidative damage by scavenging reactive oxygen species in the extracellular space, whereas the precursor form is enzymatically inactive. The low expression of Prx IV in brain metastatic cells could be attributable to an underestimation due to extracellular production, where it may control cytokine-induced peroxide levels, which mediate signaling cascades leading to cell proliferation, differentiation, and apoptosis. Its different expression among the metastatic variants suggests that brain metastasis has metabolic detoxification pathways different from those of lung metastasis.

The switch from dormancy to the growth of cancer cells in brain may be dependent on stress response mechanisms, and a subsequent coordination of detoxification and redox pathways. Cytokines produced by glial cells may contribute, in a paracrine manner, to the final development of brain metastasis. The functional role of 34/67-LMR, HSP 27, and GRP 94 proteins in stress response mechanisms and the successful metastatic growth remain under study. Their involvement in cell vehiculization and its interactions with the vascular system and brain parenchyma need further investigation, since a taxonomy built on functional response to a diverse set of cellular perturbations could be highly informative on how best to match the functional behavior of cancer cells to the optimal therapy.

**Abbreviations:** BBB, blood–brain barrier; BSA, bovine albumin; i.a., carotid injection; GRP, glucose-regulated protein; HSP, heat shock protein; IHC, immunohistochemistry; IP, immunoprecipitation; 34/67-LMR, 34–67 laminin-receptor; PLA-ANA, bioinformatic program for protein interaction network analyses; Lm.f.p., right inguinal mammary gland; 2DE, two-dimensional electrophoresis; WB, Western-blot analysis.

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Supporting Information Available: Tables listing the expression of identified proteins in 435-B1 metastatic cells, the ratio of protein expression in metastatic variants, using the Quatity One program to analyze the WB, the root and linker expression of identified proteins in 435-Br1 metastatic cells, grants from Ministry of Education and Science (MEC and Science SAF2004-0188-E. B.O. and R.A. acknowledge No. LSHC-CT-2004-506049, and by the Ministry of Education Consumer Affairs FIS/PI041937, by the EC MetaBre contract Williger (Cancer Tek Pharmaceuticals Ltd, Israel). This study (Wroclaw Agriculture University, Poland), G. van der Pluijm Biological Pathways of Brain Metastasis

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