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Identification of Markers Associated with Highly Aggressive Metastatic Phenotypes Using Quantitative Comparative Proteomics

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Abstract. *Background: The spread of cancer cells from a primary tumor to form metastases at distant sites is a complex process that remains poorly defined. Certain tumor cells are more aggressive and thus lead to rapid development of multiple distant metastases. Here, we identify proteins associated with these aggressive phenotypes. Materials and Methods: To identify proteins associated with cancer cell aggressiveness, we used comparative, quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteome analysis of a unique metastasis model comprised of three isogenic human breast cancer cell lines that are equally tumorigenic in mice, but display different metastatic potentials ranging from non-metastatic, intermediate-metastatic and highly-metastatic. The altered expression of selected proteins was subsequently confirmed by immunocyto- and immunohistochemistry. Results: The difference in metastatic capabilities was initially confirmed using live animal imaging. Comparative, quantitative proteomics identified 414 proteins, out of which 44 exhibited altered expression between the metastatic and non-metastatic cell lines. The proteins correlating with the aggressiveness of metastasis included leucine-rich repeat containing 59 (LRRC59), while CD59 and chondroitin sulfate proteoglycan 4 (CSPG4) exhibited an inverse correlation with metastatic capability. The altered expression levels of these proteins were biochemically confirmed, as well as demonstrated in xenografts generated from these cell lines. This analysis further demonstrated that the three proteins were associated with the aggressiveness of metastasis rather than metastasis colonization per se. Conclusion: Our study provides novel insights into key proteins associated with the metastatic potential of breast cancer cells and identified LRRC59, CD59 and CSPG4 as candidates that merit further study.*

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Breast cancer is the most common malignant disease among women in Western countries, occurring in approximately one out of fourteen women (1). Malignant breast cancer cells can disseminate to regional lymph nodes and establish distant metastases, most often in the bone, lung, liver and brain, resulting in poor outcome and high mortality (2, 3). The metastatic process is complex, involving local invasion, intravasation, survival in the circulation, extravasation and colonization, and is difficult to be investigated using standard cell lines or patient material. A cell line model developed as a tool to investigate the metastatic process is based on a set of isogenic cell lines, NM2C5, M4A4, M4A4 LM3-2 GFP (LM3) and M4A4 LM3-4 CL16 GFP (CL16), which display different metastatic capacities. The M4A4 and NM2C5 cell lines were derived and selected from among 80 clonal populations resulting from serial dilution of the polyclonal and metastatic breast carcinoma cell line MDA-MB-435S. The M4A4 and NM2C5 cell lines were found to be equally tumorigenic, but while M4A4 cells formed metastases in the lungs and lymph nodes, NM2C5 cells, although capable of disseminating single cells to the lungs, remained dormant (4, 5). The intermediate, metastatic cell line LM3 and the highly-metastatic cell line CL16 were isolated by cyclical cultures and orthotopic re-inoculation of cells from successive generations of metastases (6-8). Thus, this model recapitulates the steps that allow cancer cells to colonize and proliferate at distant sites.

Protein profiling of cancer cell lines with distinct phenotypes is an attractive approach to identify novel markers and profiles of these cancer phenotypes. There are multiple strategies for mass spectrometry-based proteomic comparison of cancer cell lines, including chemical and

metabolic labeling (9). Stable isotopic labeling by amino acids in cell culture (SILAC) is an effective strategy for metabolic labeling of cell line proteins, allowing samples from different cell lines to be quantitatively compared (10). Amino acids containing heavy isotopes are incorporated into proteins synthesized by one of the compared cell lines in culture. The labeled and unlabeled cell lines are subsequently mixed and the proteins are cleaved to create a large number of different peptides that can be quantified by mass spectrometry (MS) in order to distinguish whether they derive from the labeled or unlabeled cell line.

Proteins and genes from NM2C5 and M4A4 cells in culture have been extensively studied (11-18), and global gene and protein expression analysis of tumors formed by these cell lines, including the host environment, has previously been performed (7, 19, 20). However, little attention has been attributed on the gene and protein expression alterations associated with the aggressiveness of the cancer cells in this model system. Here, we describe a proteomic comparison between the non-metastatic NM2C5, the intermediate-metastatic LM3 and the highly-metastatic CL16 cell lines, revealing a number of candidate proteins associated with the potential metastatic aggressiveness of these cancer cells.

Materials and Methods

Cell culture. The NM2C5 cell line was a kind gift of Dr. D. Tarin, UCSD Medical center, La Jolla, CA, USA. The M4A4 LM3-2 green fluorescence protein (GFP) (LM3) and M4A4 LM3-4 CL16 GFP (CL16) were obtained from the American Type Culture Collection (Boras, Sweden). All cell lines were derived from the triple-negative breast cancer cell line MDA-MB-435S and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Taastrup, Denmark) supplemented with 10% fetal bovine serum (FBS) (Gibco), and 5% penicillin/streptomycin (P/S) (Gibco). Cultures were propagated in a humidified atmosphere of 5% CO_2 and at 37˚C. All analyses were performed on cultures passaged no more than 10 times from frozen stock vials to ensure genetic stability.

Orthotopic transplantation of cancer cells into mice. The NM2C5, LM3 and CL16 cell lines were transfected with luciferase 2 (LUC2)-expressing lentiviral particles (*In Vivo* Imaging Solutions, Fort Collins, CO, USA) using a multiplicity of infection virus particle concentration of 15 according to the manufacturer's guidelines, generating stable luciferase-expressing lines. Subconfluent NM2C5, LM3 and CL16 cells in culture were washed in phosphate buffered saline (PBS) and harvested using a cell scraper. Cells were washed in PBS and 1×10^6 tumor cells for inoculation were resuspended in a 1:1 mixture of extracellular matrix from the Engelbreth-Holm-Swarm sarcoma (Sigma-Aldrich, Brøndby, Denmark) and DMEM. Orthotopic transplantation of tumor cells was performed by injecting the cells into the surgically exposed mammary fat pad of anesthetized eight-week-old female CB-17 SCID mice (n=3 per group) (Taconic, Ry, Denmark). All animal experiments were performed at the Animal Core Facility at University of Southern Denmark and approved by The Experimental Animal Committee, The Danish Ministry of Justice. The animals were housed under specific pathogen-free conditions with *ad libitum* food and drinking water. The mice were euthanized if they showed any adverse signs or symptoms of disease including weight loss, paralysis or general discomfort.

In vivo imaging. Primary tumors were surgically removed five weeks after inoculation when they reached a size of approximately 1.2 cm in diameter and the metastatic burden in these mice was subsequently evaluated weekly for the following three weeks by measuring bioluminescence from spontaneous lung metastases. Relative quantification of metastasis development was performed weekly using whole-body bioluminescent imaging (IVIS-spectrum; Caliper Life Science, Mainz, Germany). Mice were injected with 150 mg D-luciferin/kg body weight (Caliper Life Science) and then anesthetized with isoflurane gas. Images were acquired starting 10 min after luciferin injection. The bioluminescent images were processed using Caliper Life Science Living Image (version 4.0).

Protein labeling using stable isotope labeling by amino acids in cell culture (SILAC). The proteomes of the NM2C5, LM3 and CL16 cells were labeled using SILAC. Cells were cultured in customized DMEM without L-arginine, L-lysine and L-glutamine (Bioconcept, Allschwil, Switzerland) supplemented with 580 mg/l L-glutamine, 28 mg/l L-arginine and 75 mg/l L-lysine, 10% triple 0.1 μm dialyzed FBS (Hyclone, Logan, UT, USA) and 1% P/S (Invitrogen). NM2C5 culture medium was supplemented with $^{13}C_6$-L-lysine (Cambridge Isotope Laboratories, Andover, MA, USA) and $^{13}C_6$-L-arginine (Cambridge Isotope Laboratories), while LM3 and CL16 cells were supplemented with $^{12}C_6$-L-lysine (Sigma Aldrich) and ^{12}-L-arginine (Sigma Aldrich). In order to completely incorporate the stable isotopes, the cells were grown for at least five cell doublings in the supplemented growth medium (14). Cultures were propagated in a humidified atmosphere of 5% CO_2 at 37˚C.

Isolation of membrane proteins. The isolation procedure was performed as previously detailed (14). In brief, approximately 2×10^7 cells from each cell line were harvested by scraping, the cells were counted and labeled, and unlabeled cells were mixed at a 1:1 ratio. Following incubation in a hypotonic buffer (10 mM Tris-base, 1.5 mM $MgCl_2$, 10 mM NaCl, pH 6.8) the mixed population of cells was homogenized in gradient buffer (0.25 mM sucrose, 10 mM HEPES, 100 mM succinic acid, 1 mM EDTA, 2 mM $CaCl_2$, 2 mM $MaCl_2$, pH 7.4). The homogenate was centrifuged (1000 ×*g* for 10 min at 4˚C) and the supernatant was collected. The supernatant was centrifuged at 100,000 ×*g* for 30 min and the pellet, containing crude membranes, was washed and resuspended in a gradient buffer. The membranes were mixed with Percoll containing 10% PBS. By displacing the gradient from the bottom with 2 M sucrose, the membranes were separated into 10 fractions according to their density. To exclude mitochondrial membranes, we measured the activity of mitochondrial succinate dehydrogenase (SDH) conversion of *p*-iodonitrotetrazolium violet colorimetrically, as previously described (14). Only early fractions with low SDH activity containing the plasma membranes and endoplasmic reticulum (ER) membranes were included in the following procedure, while fractions with high SDH activity containing the mitochondrial membranes were discarded. Protein concentration was determined in triplicate by using a colorimetric-based assay (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's protocol with a bovine serum albumin preparation used as standard (Pierce, Rockford, IL, USA).

Enzymatic digestion. Proteins (~50 μg) were digested as previously detailed and the tryptic peptides were desalted and concentrated using a modified version of the previously described StageTips (15, 21, 22).

Liquid chromatography – tandem MS. Peptides were separated using an LC-Packings Ultimate 3000 nanoflow system (LC Packings, Amsterdam, the Netherlands). Peptides were loaded at a flow rate of 3 μl/min onto a customized 1-cm precolumn (75 μm inner diameter) of fused silica with kasil-frits retaining Reprosil C18, 3.5-μm reversed-phase particles (Dr. Maisch, GmbH, Ammerbuch-Entringen, Germany). Nanoflow reversed-phase high-performance liquid chromatography (HPLC) was then performed at a flow rate of 0.2 μl/min through a customized 8-12 cm analytical column (50 μm inner diameter), packed with Reprosil C18-AQ, 3-μm reversed-phase particles (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Peptides were eluted directly into the electrospray ionization (ESI) source of a quadrupole time-of-flight (Q-TOF) Premier tandem mass spectrometer (Waters Micromass, Manchester, UK), using a stepped linear gradient (solvent A: 0.1% CH_3COOH, solvent B: 95% acetonitrile, 0.1% CH_3COOH); 0% B for 10 min, 0-35% B 90 min, and 35-100% for 5 min. Mass- and charge-dependent collision energies were used for peptide fragmentation.

MS/MS database search and quantification. Peak list files were created using Masslynx 4.0 (Waters Micromass, Hedehusene, Denmark) with the following processing parameters: background subtraction: polynomial order 10, 10% below curve; smoothing: Savitzky Golay, 3 channels, 2 smooths; centroiding: min peak with at half hight, 4, centroid top, 80%. The data were searched against all human proteins and common contaminants (n=525207) in the SwissProt database as of the 8th of March 2011, using an in-house Mascot server v2.2 (Matrix Science Ltd., London, UK). Search parameters were: 25 ppm precursor ion mass tolerance, 0.1 Da fragment ion mass tolerance, enzyme-specificity: trypsin (C-terminally to R and K, but not PR and PK), 1 missed cleavage, fixed modifications: cystein carbamidomethyl, variable modifications: oxidation (M), label: $^{13}C(6)$ (K and R). The criteria for accepting MS/MS spectra were Mascot scores above 15 and a false discovery rate below 1.5%. Moreover, only peptides with peptide mascot scores above 25, and only peptides that were the best match for the given mass were used for quantification. The false-discovery rate data were assessed against a SwissProt decoy database, essentially as described previously (23), and calculated as follows: false-discovery rate (%) = decoy hits ×100/(SwissProt hits + decoy hits).

Peak list files were recalibrated using the in-house Perl script MSRecal, and relative quantification of stable isotope-labeled peptides was performed using MSQuant v2.0 (24). MSQuant uses extracted ion current (XIC) for quantification, and all peptides fulfilling the criteria mentioned above for MS/MS spectra were quantified, no outlier points were removed. The quantifications were normalized to their collective median and imported *via* a comma-separated value format to ProteinCenter v2.8.1 (Proxeon A/S, Odense, Denmark), in order to identify for differentially expressed proteins. All protein identifications from the technical replicates from each biological sample were merged (12 LC-MS/MS analyses were merged into four biological samples), and proteins that were 98%, or more, similar were clustered into groups to reduce redundancy. Proteins that were identified in two or more technical repeats were selected for further evaluation.

Differentially expressed proteins had to meet the following criteria: Identification and quantification in two technical replicates by two or more different peptides in NM2C5 *vs*. CL16 or NM2C5 *vs*. LM3, and differential expression of >1.5-fold. The quantitative and qualitative data of the differentially expressed proteins were manually inspected using the Mascot search results and MSQuant.

Supplementary data 1 provide the accession number, number of unique peptides and the quantification for each identified protein. The mean and standard deviation for all quantified proteins and the peptides used for quantification are provided in Supplementary data 2. Gene ontology annotations are provided in Supplementary data 3.

Immunocytochemistry and immunohistochemistry. The cell line-derived tumor tissue from mice was established as described above in Orthotopic transplantation of cancer cells into mice; the generation of tissue and cell microarrays and the staining procedures are described elsewhere (15). The following primary antibodies from Sigma Aldrich were used: CD59 (HPA026494, 1:500), chondroitin sulfate proteoglycan 4 (CSPG4) (HPA002951, 1:250) and Leucine-rich repeat-containing 59 (LRRC59) (HPA030827, 1:500). Immunostaining was performed using the PowerVision™ horse radish peroxidase detection system (ImmunoVision Technologies, Burlingame, CA, USA) on a TechMate™ 500 instrument (Dako, Glostrup, Denmark).

Results

Evaluation of the metastatic potential of different isogenic cancer cell lines. The metastatic potential of the isogenic human breast cancer cell lines NM2C5, LM3 and CL16 were investigated by live animal bioimaging. Mice inoculated with CL16 cells developed metastases the fastest, and these were visible as early as the first week after removal of the primary tumor. Mice inoculated with LM3 developed later and fewer metastases, and mice inoculated with NM2C5 did not develop metastases (Figure 1).

Proteomic comparison of isogenic cancer cell lines that exhibit different metastatic capabilities using quantitative LC-MS/MS analysis. To identify proteins associated with the potential aggressiveness of breast cancer cells to establish distant metastases, we used a cell line-based metastasis model comprising of three isogenic human breast cancer cell lines with varying metastatic potential: The non-metastatic cell line NM2C5, the intermediate-metastatic cell line LM3 and the highly-metastatic cell line CL16. These cell lines were analyzed in biological duplicates, each of which was analyzed in technical triplicates. Comparison of protein expression levels in NM2C5, LM3 and CL16 cells using quantitative LC-MS/MS proteomics identified a total of 414 protein entries: 195 and 244 proteins in NM2C5 *vs*. LM3 and NM2C5 *vs*. CL16, respectively. LC-MS/MS data were processed in MSQuant. Restricting the list of proteins to those identified by at least two peptides and present in at least two technical replicates reduced the identified protein entries to 188. Forty-four proteins exhibited significantly

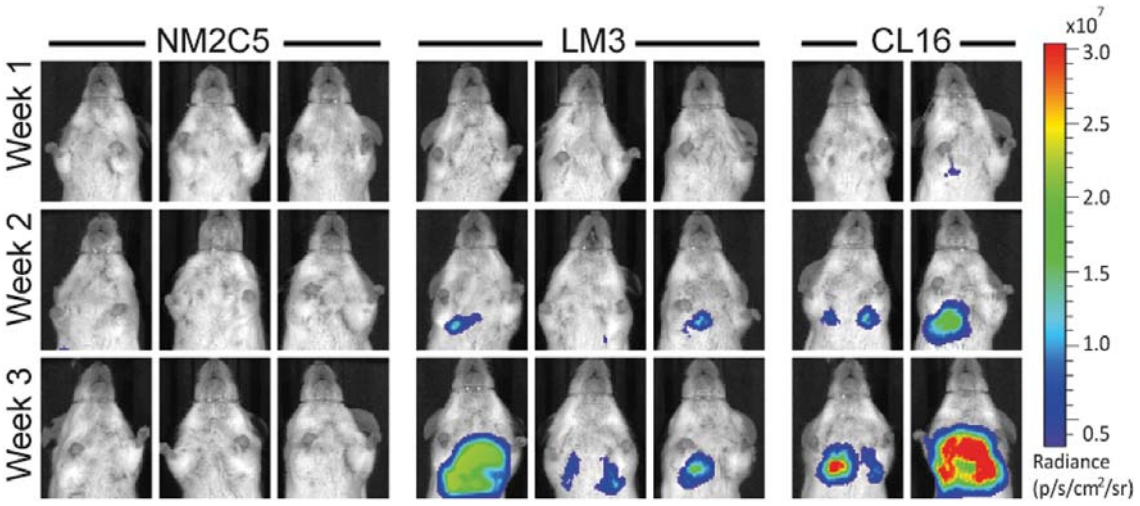

Figure 1. *The three isogenic breast cancer cell lines NM2C5, LM3 and CL16 exhibit different metastatic capabilities. Stably luciferase-expressing NM2C5, LM3 and CL16 cells (10^6) were transplanted into the mammary fat pad of groups of CB17 SCID mice. The primary tumor was surgically removed when at 1.2 cm and the animals were subsequently monitored weekly for lung metastasis development by measuring luciferase activity using an IVIS Spectrum instrument.*

altered expression among the three cell lines (Table I), and thus these proteins may be potential markers of the potential aggressiveness of breast cancer cells to establish distant metastases. Out of these, 15 proteins correlated with the aggressiveness of breast cancer cells in establishing distant metastases, including LRRC59, while 33 proteins correlated inversely with this parameter, including CSPG4 and CD59.

Biochemical validation and characterization of altered protein expression. To validate the quantitative differences in protein expression determined by MS and to further characterize the subcellular localization of the proteins, the staining pattern of the three proteins, LRRC59, CSPG4 and CD59 was examined in the three cell lines using immunocytochemistry. Immunocytochemical analysis showed that CL16 exhibited the highest expression of LRRC59, while LM3 exhibited higher expression than NM2C5, confirming the proteome analysis (Figure 2A). NM2C5 exhibited higher and more distinct membrane expression of CSPG4 compared to LM3, which again exhibited higher CSPG4 expression than CL16. Furthermore, NM2C5 exhibited higher membrane expression of CD59 compared to CL16, while LM3 cells had similar expression to NM2C5 (Figure 2A). To further investigate the association of these proteins with the potential aggressiveness of breast cancer cells in establishing distant metastasis, the expression of the three proteins in the low-metastatic M4A4 cell line was investigated. This analysis showed that M4A4 cells exhibited similar expression of CD59, CSPG4 and LRRC59 to NM2C5 cells (Figure 2A).

Evaluation of protein expression in human xenograft primary tumor tissues. We next performed immunohistochemical evaluation of the expression of LRRC59, CSPG4 and CD59 in primary tumor tissues, generated by inoculation of the cell lines into immunodeficient mice. CSPG4 exhibited more intense and distinct plasma membrane staining in the primary tumor tissue generated from NM2C5 cells compared to that from CL16 cells, while an intermediate expression level was observed in the LM3 tumor tissue (Figure 2B). Although the expression of CD59 in the primary NM2C5 tumor tissue was heterogeneous, as depicted in Figure 2B, the overall expression of CD59 in NM2C5 tumors was higher compared to that from CL16 cells, while LM3 tumors exhibited intermediate expression. Expression in all tumors was localized to the plasma membrane. The immunohistochemical analysis showed higher cytoplasmic expression of LRRC59 in LM3 and CL16 tumors than in NM2C5 tumors (Figure 2B). The staining pattern for LRRC59 was homogeneous within all tumor tissues.

Discussion

The establishment of a metastasis at distant sites is a complex process that involves interactions between cancer cells and surrounding tissues, as well as cellular signaling within the cancer cells. Since it is difficult to study the individual steps in clinical samples, we used a cell line model system of tumor cell colonization and aggressiveness of distant metastasis formation to identify key proteins involved in the metastatic potential of breast cancer cells. Our proteomic analysis

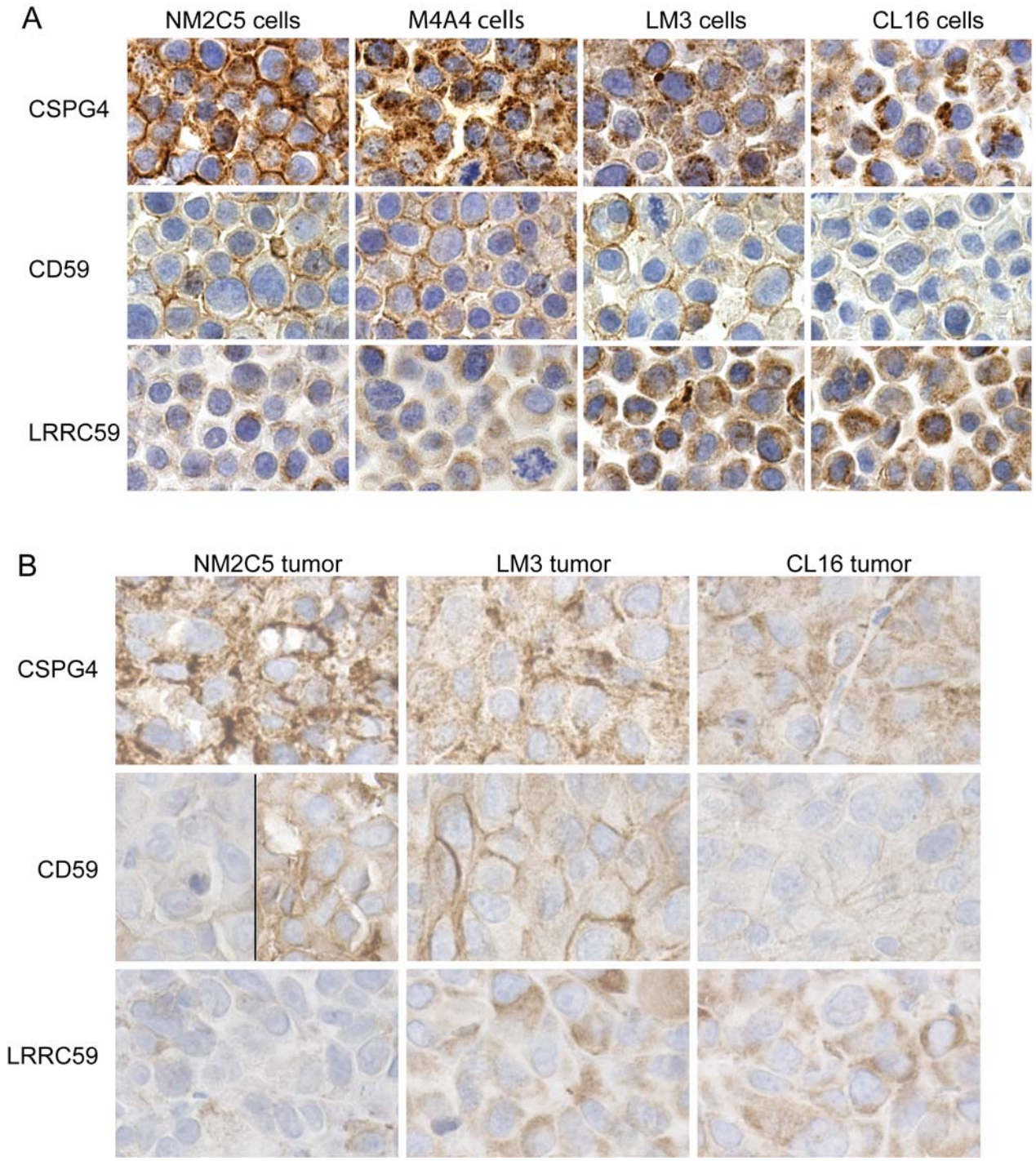

Figure 2. *Biochemical validation and immunohistochemical characterization of expression of selected proteins, leucine-rich repeat containing protein 59 (LRRC59), chondroitin sulfate proteoglycan 4 (CSPG4) and CD59, identified to correlate or inversely correlate with the metastatic capability of cancer cells in the proteomic analysis. A: Cells from the four breast cancer cell lines, NM2C5, M4A4, LM3 and CL16 were analyzed by immunocytochemical staining. A correlation between LRRC59 expression and an inverse correlation between CSPG4 and CD59 expression and metastatic capability was observed. B: Xenografts derived from orthotopic transplantation of the three isogenic breast cancer cell lines NM2C5, LM3 and CL16 were analyzed by immunohistochemical staining. A correlation between LRRC59 expression and an inverse correlation between CSPG4 and CD59 expression and metastatic capability was observed. The expression of CD59 in NM2C5-derived tumors was heterogenic and two representative areas are shown.*

Table I. *Proteins exhibiting altered expression levels in non-metastatic (NM2C5), intermediate-metastatic (LM3), and highly-metastatic (CL16) isogenic breast cancer cells, as determined by comparative quantitative mass spectrometry.*

UniProt	Gene	Protein name	NM2C5 *vs.*		Peptides	
			CL16[a]	LM3[a]	CL16[b]	LM3[b]
O95573	*ACSL3*	Long-chain fatty-acid-CoA ligase 3	**0.65**		3	
P60709	*ACTB*	Actin, cytoplasmic 1	**1.89**	0.93	10	10
O43707	*ACTN4*	Alpha-actinin-4	**2.26**	0.93	3	5
Q09666	*AHNAK*	Neuroblast differentiation-associated protein AHNAK	**1.61**	1.31	18	41
P35613-1	*BSG*	Basigin	**1.70**	1.03	2	2
P62158	*CALM3*	Calmodulin	**1.54**	0.77	2	1
Q6YHK3-1	*CD109*	CD109 antigen		**0.60**		2
P16070-1	*CD44*	CD44 antigen	**2.11**	1.11	5	4
P13987	*CD59*	CD59 glycoprotein	**2.31**	0.96	2	4
P60033	*CD81*	CD81 antigen	**1.57**	1.29	2	2
P21181-4	*CDC42*	Cell division control protein 42 homolog	**1.78**	0.95	3	3
P23528	*CFL1*	Cofilin-1	**1.70**	**1.52**	1	2
Q6UVK1	*CSPG4*	Chondroitin sulfate proteoglycan 4	**3.32**	1.40	22	33
Q96TA1-1	*FAM129B*	Niban-like protein 1		**1.53**		2
P08754	*GNAI3*	GNBP G(k) subunit alpha	**1.52**	0.97	4	4
Q5JWF2-1	*GNAS*	GNBP G(s) subunit alpha isoforms XLas	**2.04**	1.11	2	5
P05534	*HLA-A*	HLA class I histocompatibility antigen, A-24 alpha chain	**1.62**		2	
P18465	*HLA-B*	HLA class I histocompatibility antigen, B-57 alpha chain	**1.69**	0.67	3	2
P01903	*HLA-DRA*	HLA class II histocompatibility antigen, DR alpha chain		**0.40**		3
P07900-1	*HSP90AA1*	Heat shock protein HSP 90-alpha	**1.78**	1.27	4	8
Q9Y4L1	*HYOU1*	Hypoxia up-regulated protein 1	**0.64**	1.00	5	3
P46940	*IQGAP1*	Ras GTPase-activating-like protein IQGAP1	**2.25**	1.36	7	9
P05556-1	*ITGB1*	Integrin beta-1	**1.68**	0.70	7	7
P35527	*KRT9*	Keratin, type I cytoskeletal 9	**0.62**		3	2
Q96AG4	*LRRC59*	Leucine-rich repeat-containing protein 59	**0.56**	**0.66**	5	1
P29966	*MARCKS*	Myristoylated alanine-rich C-kinase substrate	**0.62**	**2.83**	2	1
P43121-1	*MCAM*	Cell surface glycoprotein MUC18	**1.71**	1.22	4	4
P26038	*MSN*	Moesin	**1.70**	1.45	8	13
O43795-1	*MYO1B*	Unconventional myosin-Ib	**2.02**	1.05	2	2
O00159-1	*MYO1C*	Unconventional myosin-Ic	**1.69**	1.10	12	22
O94832	*MYO1D*	Unconventional myosin-Id		**2.38**	1	2
Q6PIU2-1	*NCEH1*	Neutral cholesterol ester hydrolase 1	**0.54**		3	
P07237	*P4HB*	Protein disulfide-isomerase	**0.49**		2	
P61225	*RAP2B*	Ras-related protein Rap-2b	**1.98**	**1.91**	2	2
P17081	*RHOQ*	Rho-related GTP-binding protein RhoQ	**1.82**		2	
P62851	*RPS25*	40S ribosomal protein S25	**1.51**	1.17	3	1
P62753	*RPS6*	40S ribosomal protein S6	1.13	**3.29**	2	2
P61619-1	*SEC61A1*	Protein transport protein Sec61 subunit alpha isoform 1	**0.65**		3	
P43007	*SLC1A4*	Neutral amino acid transporter A	**1.75**	0.89	2	3
Q00325-1	*SLC25A3*	Phosphate carrier protein, mitochondrial	**0.58**		2	
O15260-1	SURF4	Surfeit locus protein 4	**0.44**		2	
P57088	TMEM33	Transmembrane protein 33	**0.61**		2	
P07437	TUBB	Tubulin beta chain	**1.51**	1.18	5	4
P08670	VIM	Vimentin	**0.65**	0.76	17	5

UniProt: Accession numbers. [a]Fold difference between the indicated cell lines. Proteins exhibiting altered expression (≥1.5 fold) are indicated in bold. [b]The maximum number of peptides used for quantification. HSP: Heat-shock protein; GNBP: guanine nucleotide-binding protein.

identified a total of 414 proteins, out of which 44 were differentially expressed between the cell lines. Fifteen proteins had expression levels that correlated with the metastatic potential of the cell lines, while the expression levels of 33 proteins correlated inversely with this parameter.

LRRC59 was one of the proteins identified as being associated with metastatic potential, and its altered expression was biochemically validated. LRRC59 expression in primary tumor tissues generated from the metastasizing LM3 and CL16 cell lines was increased compared to tumors

from NM2C5 cells, suggesting that this protein is involved in the metastatic process. LRRC59 is predicted to be a tail-anchored ER membrane protein containing a leucine-rich repeat domain and a putative coiled-coil domain, which has been shown to bind fibroblast growth factor 1 (FGF1). It has recently been suggested that LRRC59 is involved in nuclear import of FGF1 (25). Moreover, FGF1 has been shown to inhibit p53-dependent apoptosis and cell growth arrest *via* an intracrine pathway (26, 27). Increased levels of LRRC59 might increase the nuclear import of FGF1 and thus induce a more proliferative phenotype. The increased expression of LRRC59 in the CL16 and LM3 xenograft tumors *vs*. the NM2C5 primary tumors might facilitate a growth advantage of metastatic cells.

CD59 and CSPG4 were among the group of proteins with expression levels that correlated inversely with metastatic potential in our proteomic analysis, and the altered expression was further confirmed by immunocytochemical analysis. In the xenografts derived from the three cell lines, the expression of CD59 was found to be more heterogeneous in primary NM2C5 tumors, compared to those from LM3 and CL16. However, CD59 expression was higher overall in tumors from NM2C5 compared to CL16 tumors, while LM3 exhibited intermediate expression compared to the other two tumor types, suggesting that a decrease in CD59 expression is a selective advantage for very aggressive metastasizing cells. CD59 is an 18-20-kDa glycosylphosphatidylinositol-anchored protein that inhibits the complement cascade (28). It has been shown in a clinical study by Madjd *et al*. including 520 breast carcinomas with varying histopathological origins, tumor grades and estrogen receptor status, that high CD59 expression correlated with low histological grade and good prognosis (29). Furthermore, the expression of CD59 was found to correlate inversely with development of distant metastases (29). Our results support this finding and furthermore suggest that decreased expression of CD59 favors metastatic colonization. Heterogeneity within the staining pattern of CD59 was observed in many of the breast carcinomas assessed by Madjd *et al*., which we also observed, especially in the NM2C5 derived-tumors. Thus, our model demonstrates a clinically relevant expression pattern for CD59.

CSPG4 expression in the primary tumor specimens generated in mice, was shown to be increased in tumors from NM2C5 cells compared to that from CL16 cells, while LM3 tumors exhibited intermediate expression compared to the other two tumor types. CSPG4, also known as high-molecular weight melanoma-associated antigen or melanoma chondroitin sulfate proteoglycan, is a membrane-bound proteoglycan composed of an N-linked 280-kDa glycoprotein and a 450-kDa chondroitin sulfate proteoglycan. Interestingly, increased CSPG4 was recently associated with a triple-negative subtype of breast cancer, the same subtype our cell line model is derived from (30). Furthermore, targeting CSPG4 with monoclonal antibodies reduced growth, adhesion and migration *in vitro* and tumor growth and metastasis *in vivo* (30). These findings seemingly contradict our findings wherein the non-metastatic and the low-metastatic cell lines (NM2C5 and M4A4) exhibited higher CSPG4 expression than the intermediate- and highly-metastatic cell lines (LM3 and CL16). Further studies should address whether the expression level of CSPG4 in primary triple-negative breast cancer can predict the risk of metastasis and outcome.

Interestingly, although the three proteins LRRC59, CD59 and CSPG4 were previously identified in earlier proteomic studies of ours, where we compared the expression levels in the non-metastatic cell line NM2C5 *vs*. the low-metastatic cell line M4A4, none of them was found to have altered expression when comparing these two cell lines (15). In agreement with this, our immunocytochemical analysis showed that LRRC59, CD59 and CSPG4 were similarly expressed in NM2C5 and M4A4 cells. Together, this indicates that the altered expression levels of the three identified proteins are unique to cancer cells exhibiting the aggressive metastatic phenotype and not simply associated with any metastatic cancer cell line.

Although the MDA-MB-435S cell line that our isogenic cell model system has derived from was originally derived from a highly aggressive human invasive ductal carcinoma, there has been a debate as to whether the cell line can be unambiguously defined as breast cancer since it, along with breast- and epithelial-specific markers, also expresses melanoma-specific genes (31). It has been suggested that MDA-MB-435S was derived from M14 melanoma cells, although this cannot be the case since this cell line was isolated from a male patient and the current stocks of the MDA-MB-435S cell line are of female origin (32). Furthermore, MDA-MB-435S cells can be induced to express breast differentiation-specific proteins and secrete milk lipids, as observed in other well-established breast cancer cell lines (33). Furthermore, in a recent study by Montel and colleagues, it was shown that primary breast tumors often exhibit expression of melanocyte-related proteins and that the histopathology of the tumors and metastases generated by the MDA-MB-435S cell line are similar to those seen in breast carcinoma, supporting the breast origin of this cell line (34). As a result, it is commonly recognized that the MDA-MB-435S cell line is a breast cancer cell line.

In conclusion, our proteomic analysis identified a number proteins in which expression levels correlated positively or inversely with metastatic capability. We focused on three proteins in particular, LRRC59, CD59 and CSPG4, which exhibited altered expression in the aggressive metastatic cancer cell lines LM3 and CL16 compared to low-metastatic M4A4 and non-metastatic NM2C5 cell lines. This finding supports the

contention that these three proteins are associated with a more aggressive phenotype of metastasizing cancer cells. Some of the proteins have already been related to the metastatic process, and our work reported herein provides further insights in to the contribution of these proteins, particularly with regard to colonization and the aggressive phenotype.

Conflicts of Interest

The Authors have declared no conflicts of interest.

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