Chondroitin sulfates play a major role in breast cancer metastasis: a role for CSPG4 and CHST11 gene expression in forming surface P-selectin ligands in aggressive breast cancer cells

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Abstract

Introduction: We have previously demonstrated that chondroitin sulfate glycosaminoglycans (CS-GAGs) on breast cancer cells function as P-selectin ligands. This study was performed to identify the carrier proteoglycan (PG) and the sulfotransferase gene involved in synthesis of the surface P-selectin-reactive CS-GAGs in human breast cancer cells with high metastatic capacity, as well as to determine a direct role for CS-GAGs in metastatic spread.

Methods: Quantitative real-time PCR (qRT-PCR) and flow cytometry assays were used to detect the expression of genes involved in the sulfation and presentation of chondroitin in several human breast cancer cell lines. Transient transfection of the human breast cancer cell line MDA-MB-231 with the siRNAs for carbohydrate (chondroitin 4) sulfotransferase-11 (CHST11) and chondroitin sulfate proteoglycan 4 (CSPG4) was used to investigate the involvement of these genes in expression of surface P-selectin ligands. The expression of CSPG4 and CHST11 in 15 primary invasive breast cancer clinical specimens was assessed by qRT-PCR. The role of CS-GAGs in metastasis was tested using the 4T1 murine mammary cell line (10 mice per group).

Results: The CHST11 gene was highly expressed in aggressive breast cancer cells but significantly less so in less aggressive breast cancer cell lines. A positive correlation was observed between the expression levels of CHST11 and P-selectin binding to cells \((P < 0.0001)\). Blocking the expression of CHST11 with siRNA inhibited CS-A expression and P-selectin binding to MDA-MB-231 cells. The carrier proteoglycan CSPG4 was highly expressed on the aggressive breast cancer cell lines and contributed to the P-selectin binding and CS-A expression. In addition, CSPG4 and CHST11 were over-expressed in tumor-containing clinical tissue specimens compared with normal tissues. Enzymatic removal of tumor-cell surface CS-GAGs significantly inhibited lung colonization of the 4T1 murine mammary cell line \((P = 0.0002)\).

Conclusions: Cell surface P-selectin binding depends on CHST11 gene expression. CSPG4 serves as a P-selectin ligand through its CS chain and participates in P-selectin binding to the highly metastatic breast cancer cells. Removal of CS-GAGs greatly reduces metastatic lung colonization by 4T1 cells. The data strongly indicate that CS-GAGs and their biosynthetic pathways are promising targets for the development of anti-metastatic therapies.
Introduction

Tumor-associated glycans play a significant role in promoting aggressive and metastatic behavior of malignant cells [1-5], participating in cell-cell and cell-extracellular matrix interactions that promote tumor cell adhesion and migration. Among glycans that play a critical role in stromal tumor cell interactions are glycosaminoglycans (GAGs) attached to proteoglycans (PGs). Altered production levels of PGs and structural changes in their GAGs are reported in many neoplastic tissues [6-10]. GAGs are polysaccharide chains covalently attached to protein cores that together comprise PGs [6,11] and based on the prevalence of GAG chains, chondroitin sulfate (CS)/dermatan sulfate (DS) PGs (CS/DS-PGs), heparan sulfate PGs and keratan sulfate PGs have been described [12]. Increased production of CS/DS-GAGs is found in transformed fibroblasts and mammary carcinoma cells [8,13,14] and it has been shown that these polysaccharides contribute to fibrosarcoma cell proliferation, adhesion and migration [15].

Several studies have disclosed the critical involvement of P-selectin in the facilitation of blood borne metastases [16-18]. P-selectin/ligand interaction often requires sialylated and fucosylated carbohydrate such as sialyl Lewis X and sialyl Lewis A [19]; however, P-selectin also binds to heparan sulfate, certain sulfated glycolipids and CS/DS-GAGs [20-23]. In previous studies we found that CS/DS-GAGs are expressed on the cell surface of murine and human breast cancer cell lines with high metastatic capacity and that they play a major role in P-selectin binding and P-selectin-mediated adhesion of cancer cells to platelets and endothelial cells [24]. However, variation in the abundance and function of CS/DS relative to tumor cell phenotypic properties and P-selectin binding are not well defined. It is likely that P-selectin binding to tumor cells and the functional consequences of such binding are dependent on which sulfotransferases define the relevant CS/DS and which core proteins carry the CS polysaccharide.

CS/DS expression is controlled by many enzymes in a complex biosynthetic pathway and this leads to considerable variation in structure and function. The chondroitin backbone of CS/DS-GAGs consists of repetitive disaccharide units containing D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc) residues, or varying proportions of L-iduronic acid (IdoA) in place of GlcA [25,26]. Major structural variability of the CS/DS chains is due to the sulfation positions in repeating disaccharide units by the site-specific activities of sulfotransferases that produce the variants CS-A, CS-B (dermatan sulfate, DS), CS-C, CS-D and CS-E [26,27]. CHST3, CHST7, CHST11, CHST12, CHST13, CHST14 and CHST15 are the enzymes that sulfate the GalNAc residues of chondroitin polymers. The expression levels of these enzymes are hypothesized to affect the production of P-selectin ligands. CHST11 and CHST13 share specificity, as they mediate 4-O sulfation of chondroitin [28-30], CHST12 and CHST14 mediate mostly 4-O sulfation of DS units [30,31], CHST3 and CHST7 share specificity for 6-O sulfation of chondroitin [32,33]. CHST15 transfers sulfate to the carbon-6 of an already 4-O sulfated GalNAc residue producing oversulfated CS-E [34]. The relationship between the relative expression of these sulfotransferases with the expression of P-selectin-reactive CS/DS-GAGs has not been reported and is addressed in this paper.

The variation, abundance and function of CS/DS-GAGs are also affected by the expression of the PG core protein presenting them. Syndecan-1 (SDC-1), syndecan-4 (SDC-4), neuropilin-1 (NRP-1) and CSPG4 are considered major pro-malignancy membrane proteins capable of carrying CS chains [35-39]. Among these PGs, CSPG4 exclusively carries CS chains [40,41]. CSPG4 is a human homolog of Rat NG2, which is also known as High Molecular Weight Melanoma Associated Antigen and Melanoma Chondroitin Sulfate Proteoglycan [40,42,43]. This tumor-associated cell surface PG potentiates cell motility and promotes invasiveness and the metastatic potential of tumor cells in melanoma [44-46]. CSPG4 is also linked to cancer stem cells via signaling mechanisms [39,45]. Therefore, studying whether this PG interacts directly with P-selectin is of particular interest.

The expression of CSPG4 in breast cancer has been reported very recently [39]. Depending on its relative expression levels, this PG might be a major core protein presenting CS-GAGs on an aggressive subset of tumor cells, interacting with P-selectin. In the current study we investigated whether CSPG4, via its CS/DS chain can serve as a P-selectin ligand and whether expression of specific chondroitin sulfotransferases contributes to P-selectin interaction with cells. We further examined the involvement of CS-GAG chains in lung colonization by the 4T1 mammary cell line with and without enzymatic removal of CS-GAGs in a murine experimental metastasis model. Our data show for the first time that P-selectin can bind to tumor cells via CSPG4 and that CHST11 expression is linked to P-selectin-reactive cell surface CS/DS-GAGs. The results directly link CS/DS-GAGs to the metastatic spread of breast cancer. These findings have significant implications for understanding mechanisms of breast cancer metastasis, paving the way towards developing alternative strategies to treat and prevent metastasis.

Materials and methods

Reagents

Anti-CS-A mAb 2H6 was from Associates of Cape Cod/Seikagaku America (Falmouth, MA, USA), anti-CSPG4 mAb 225.28 was made as described [47,48], and
recombinant human P-selectin/Fc (human IgG) was from R&D Systems (Minneapolis, MN, USA). Fluorescence-conjugated anti-human IgG, anti-mouse IgM and chondroitinase ABC were from Sigma (St. Louis, MO, USA). R-Phycoerythrin-conjugated polyclonal goat anti-mouse F(ab)_2 fragment was from Dako North America, Inc. (Carpinteria, CA, USA). DNA primers were from Integrated DNA Technologies (IDT, Coralville, IA, USA). Pre-designed siRNA sequences were from Ambion (Austin, TX, USA) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). siPORT™ NeoFX™ Transfection Agent was from Ambion. TRIzol reagent was from Invitrogen (Carlsbad, CA, USA). Real-time PCR reagents were from Applied Biosystems (Foster City, CA, USA). Total RNA isolation and qRT-PCR

For this study, an active human tissue use protocol approved by the UAMS Institutional Review Board was used. In this protocol, informed consent was waived due to the use of de-identified specimens with no link to patient identifiers.

Total RNA isolation and qRT-PCR

Total RNA was isolated from cultured cells and tumor tissues using TRIzol reagent, following the manufacturer’s instructions. The quantity and quality of the isolated RNA was determined by Agilent 2100 Bioanalyzer (Palo Alto, CA, USA). One μg of total RNA was reverse-transcribed using random-hexamer primers with TaqMan Reverse Transcription Reagents (Applied Biosystems). Reverse-transcribed RNA was amplified with SYBR Green PCR Master Mix (Applied Biosystems) plus 0.3 μM of gene-specific upstream and downstream primers during 40 cycles on an Applied Biosystems 7500 Fast Realtime cycler. Data were analyzed by absolute and relative quantification. In absolute quantification, data were expressed in relation to 18S RNA, where the standard curves were generated using pooled RNA from the samples assayed. In relative quantification, the 2^(-ΔΔCT) method was used to assess the target transcript in a treatment group relative to that of an untreated control group using expression of an internal control (reference gene) to normalize data [51]. Expression of GAPDH and β-actin was used as internal control. Each cycle consisted of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 60 s. The primer sequences are shown in Table 1.

Flow cytometry

Binding of the recombinant human P-selectin/Fc molecule, anti-CS-A (2H6) and anti-CSPG4 (225.28) mAb to cells was determined using flow cytometry as previously described [24]. Briefly, cells were incubated with mAb or P-selectin/Fc, washed and stained with FITC-conjugated anti-mouse IgM or anti-human IgG prior to binding detection by flow cytometry. R-phycocerythrin-conjugated polyclonal goat anti-mouse F(ab)_2 was used as secondary for detection of anti-CSPG4 binding.

Switching off gene expression with siRNA

Three pre-designed siRNA sequences for CHST11 (Ambion) were used (Table 1). NG2 siRNA (sc-40771) from Santa Cruz Biotechnology, Inc. was used for inhibition of CSPG4. These sequences were transfected into cells growing in tissue culture using the siPORT™ NeoFX™ Transfection Agent, and mRNA levels were determined 48 hours later by real-time PCR. Expression of GAPDH was used as reference control. Reactivity of anti-CS-A mAb 2H6 and human recombinant P-selectin was assessed 144 hours after siRNA transfection.

Cell lines and tissue specimens

Human breast cancer MCF7, MDA-MB-231, MDA-MB-468 cell lines and the murine 4T1 cell line were from ATCC (Manassas, VA, USA). Human MDA-MET cells were selected in vivo for their bone colonizing phenotype [49]. 4T1 cells were used within 10 passages and less than six months after receipt. We confirmed cell line identities by the Human Cell Line Authentication test (Genetic DNA Laboratories, Inc. Cincinnati, OH, USA). The melanoma cell lines M14 and M14-CSPG4, stably transfected to express CSPG4, were used as homologous CSPG4-non-expressing and expressing cell lines [50] and were characterized by real-time PCR for CSPG4 expression. Cells were cultured in a base medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA, USA), 50 units/mL penicillin, and 50 μg/mL streptomycin. Base medium for MDA-MB-231, MDA-MET, MDA-MB-468, and 4T1 was DMEM (Fisher Scientific, Pittsburgh, PA, USA). For MCF7 base medium was MEM (Fisher Scientific) supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 0.01 mg/ml insulin (Invitrogen). RPMI 1640 medium (Fisher) was used for M14 and M14/CSPG4. For this study, an active human tissue use protocol approved by the UAMS Institutional Review Board was used. In this protocol, informed consent was waived due to the use of de-identified specimens with no link to patient identifiers.

For M14 and M14/CSPG4 RPMI 1640 medium (Fisher Scientific) was supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 0.01 mg/ml insulin (Invitrogen). Human breast cancer MCF7, MDA-MB-231, MDA-MB-468, and 4T1 was fast Realtime cycler. Data were analyzed by absolute and relative quantification. In absolute quantification, data were expressed in relation to 18S RNA, where the standard curves were generated using pooled RNA from the samples assayed. In relative quantification, the 2^(-ΔΔCT) method was used to assess the target transcript in a treatment group relative to that of an untreated control group using expression of an internal control (reference gene) to normalize data [51]. Expression of GAPDH and β-actin was used as internal control. Each cycle consisted of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 60 s. The primer sequences are shown in Table 1.

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De-identified frozen specimens from 15 female breast cancer patients diagnosed with invasive ductal carcinoma were provided by the University of Arkansas for Medical Sciences (UAMS) Tissue Procurement Facility. Specimens were matched for each donor to have both tumor-free and tumor-containing breast tissues. According to the policy of the Tissue Procurement Facility, patients signed consent forms to donate excess specimen tissue and to allow use of related pathological data for research purposes, which were approved by the UAMS Institutional Review Board.
Mice and tumor models

BALB/c female mice (six to eight weeks old) were from Harlan Laboratories (Indianapolis, IN, USA). We used 4T1 cells in an experimental metastasis model [24]. Cells were treated with chondroitinase ABC in HBSS buffer with protease inhibitors [24], or with the buffer and protease inhibitor alone (no chondroitinase ABC treatment) before inoculation through the tail vein. Each mouse (10 mice per group) received 2 × 10^5 4T1 cells. Mice were sacrificed 25 days after cell injection and lungs were harvested to determine clonogenic cells by growing cells in medium containing 6-thioguanine [52]. Animal studies have been reviewed and approved by the Institutional Care and Use Committee of UAMS.

### Table 1 Primer and siRNA sequences used in this study

| Primer | Sequence |
|--------|----------|
| 18S forward | 5′ TTCGAAAGCTCTGCCCATCA 3′ |
| 18S reverse | 5′ ATGGTAGCAGGCGGACTA 3′ |
| β-actin forward | 5′ GACTACGAGCAGAGATGGCCAC 3′ |
| β-actin reverse | 5′ CATGAGCCGTTTACGTTTG 3′ |
| CHST3 forward | 5′ GACCTTGTGCAAATGCTTA 3′ |
| CHST3 reverse | 5′ AAGCTTGGGGGAAATTCTGCT 3′ |
| CHST7 forward | 5′ GGGGCCATCCTGCACTACT 3′ |
| CHST7 reverse | 5′ AATTGACGACAGGTTGGG 3′ |
| CHST11 forward | 5′ TCCCCCTGTGCGCGACATC 3′ |
| CHST11 reverse | 5′ CACGTGTCTGTCACTGTTC 3′ |
| CHST12 forward | 5′ GCACACGTCTCCTCTCATGC 3′ |
| CHST12 reverse | 5′ AAATCTGCGCTAGCTGTGC 3′ |
| CHST13 forward | 5′ CCGGCATTTGGAAAACAGG 3′ |
| CHST13 reverse | 5′ GGGGCCGCTCGTGATCTGA 3′ |
| CHST14 forward | 5′ TCTGATCCCCATTATCTCA 3′ |
| CHST14 reverse | 5′ GGAGCAGAAGGGGCGAAAAG 3′ |
| CHST15 forward | 5′ TCACTTCCACAGACACGCC 3′ |
| CHST15 reverse | 5′ GTACAAACAGGGGTCTCTA 3′ |
| CHST16 forward | 5′ CAAACAGGAGAACCTCTGAT 3′ |
| CHST16 reverse | 5′ CTTTACCAGGAAGGACACT 3′ |
| CHST17 forward | 5′ AGGTTGAGTAAAGAGGACC 3′ |
| CHST17 reverse | 5′ AAGGTTGCGACAGTGGACT 3′ |
| GADPH forward | 5′ ACAGTCACCGGCACTCTCT 3′ |
| GADPH reverse | 5′ ACAGCAAAAATCTCGTCTC 3′ |
| NRP-1 forward | 5′ CCAAGGTCTGATGGAAGGCTCA 3′ |
| NRP-1 reverse | 5′ CCAGCAACCTGTTGATCTAG 3′ |
| SDC-1 forward | 5′ GTCCGGGAGTACTGTCGAC 3′ |
| SDC-1 reverse | 5′ GGCTGTGTCTGCAAGGTGA 3′ |
| SDC-4 forward | 5′ GTCTGGTCTGAGCTGCTG 3′ |
| SDC-4 reverse | 5′ CACAAAGGAGTGGACACTT 3′ |

| siRNA | Sequence |
|-------|----------|
| #31 | UCAUGAUGUUCUCGUAGACUtt |
| #32 | UUCUGGUGACUACUCUGUUGCgg |
| #33 | AUUGAGUGUUGUUGACUCUGG |

Transfection with GAPDH siRNA (Ambion) was used as control.

### Results

CHST11 is overexpressed in aggressive human breast cancer cell lines and its expression correlates with P-selectin binding

We have shown that CS/DS-GAGs expressed on the cell surface of MDA-MB-231 and MDA-MET human breast cancer cell lines function as P-selectin ligand and that exogenous CS-E efficiently inhibits P-selectin binding to cells [24]. Among the major sulfotransferases able to sulfate the GlcNAC residue of the chondroitin disaccharide, CHST11, CHST12, and CHST13 are chondroitin 4-sulfotransferases, able to catalyze sulfation of chondroitin on the carbon-4 position of GalNAc sugars in disaccharide units, producing primarily CS-A units (GlcA 1-3GalNAc (4-SO4)) [28-30]. CHST14, dermatan 4-sulfotransferase 1 (D4ST-1), is specific for 4-O sulfation of the GlcNAC residue of the chondroitin disaccharide, inhibiting P-selectin binding [24]. Among the major sulfotransferases able to sulfate the GlcNAC residue of the chondroitin disaccharide, CHST11, CHST12, and CHST13 are chondroitin 4-sulfotransferases, able to catalyze sulfation of chondroitin on the carbon-4 position of GalNAc sugars in disaccharide GAG units, producing primarily CS-A units (GlcAβ1-3GalNAc (4-SO4)) [28-30]. CHST14, dermatan 4-sulfotransferase 1 (D4ST-1), is specific for 4-O sulfation of the GalNAc in CS-B (DS) units after dermatan sulfate epimerase activity and its specificity can be shared by CHST12 [30,31]. CHST3 and CHST7 are chondroitin 6-sulfotransferases that transfer sulfate to carbon-6 position of GalNAc, competing with 4-sulfotransferases for the same substrate, producing primarily CS-C units (GlcAβ1-3GalNAc (6-SO4)) [32,33]. CHST15, GalNAc 4-sulfotransferase (GalNAc4S-6ST), transfers sulfate to the carbon-6 of a 4-sulfated GalNAc, producing CS-E (GlcAβ1-3GalNAc (4-, 6-SO4)) [34].
Quantitative real-time PCR was used to monitor the expression levels of these genes in several human breast cancer cell lines differing in their cancer phenotype. The human breast cancer cell lines MCF7, MDA-MB-468, MDA-MB-231, and MDA-MET represent increasing aggressiveness and metastatic capacity (MCF7 < MDA-MB-468 < MDA-MB-231 = MDA-MET). While expression of all genes was detected in these breast cancer cells, CHST13 expression was observed to be very low with no significant differences in expression between cell lines (Figure 1). Among the seven sulfotransferases tested, only CHST11 expression levels trended well with the metastatic potential of cells, ranging from a low in MCF7, intermediate to high in MDA-MB-468 and high in MDA-MB-231 and MDA-MET cells (Figure 1). The expression level of CHST11 was elevated with the increase in metastatic potential of cells. Such a trend was not observed with the expression levels of other genes examined. Therefore, the data may implicate the immediate product of CHST11 expression, CS-A, in the metastatic behavior of these cells.

Flow cytometry analysis of the binding of the anti-CS-A mAb 2H6, consistent with qRT-PCR data, indicates that the expression of CS-A was lower in the least aggressive MCF7 cell line and high in the most aggressive MDA-MB-231 and MDA-MET cell lines (Figure 2A). While MDA-MB-468 showed higher expression of CS-A than MCF7 cells, the CS-A expression in MDA-MB-468 was not different than its expression in MDA-MB-231 and MDA-MET. The lack of a difference in CS-A between these three cell lines could be due to the expression level of CHST15 that can turn the 4-sulfated product of CHST11 to CS-E. The expression of CHST15 was significantly lower in MDA-MB-468 than in MDA-MB-231 or MDA-MET (Figure 1) making the conversion of some CS-A to CS-E much more likely in MDA-MB-231 and MDA-MET than in MDA-MB-468. Among the sulfotransferase genes examined, P-selectin binding to these cells correlated the best with CHST11 expression. We performed a correlation analysis of the gene expression levels and the mean fluorescence intensities of P-selectin binding among all cell lines studied and observed that only CHST11 (r = 0.85, P < 0.0001) showed a statistically significant correlation with P-selectin binding (Table 2). P-selectin reactivity with cell lines was comparable to anti-CSA 2H6 binding (Figure 2A, B). These data suggest that CHST11 (a chondroitin-4-sulfotransferase) expression in these cells is associated with the production of P-selectin ligands.

Inhibition of CHST11 expression results in inhibition of CS-A production and P-selectin binding

Our data indicate that CHST11 is required for P-selectin binding. To confirm a role of 4-O sulfated structures in P-selectin binding, the expression of CHST11 in MDA-MB-231 cells was inhibited by siRNA. We observed that CHST11 mRNA levels, anti-CS-A binding and P-selectin binding were all significantly reduced upon treatment with the three siRNAs tested (Figures 3A, B). Transfection with siRNA #31 (Table 1) showed the highest inhibitory effect on binding of anti-CS-A and recombinant P-selectin (Figure 3B). In three independently conducted experiments, transfection with siRNA #31 significantly reduced the mean fluorescence intensity for anti-CS-A (P ≤ 0.015) and P-selectin (P ≤ 0.001) binding, as compared with vehicle-treated cells.

We repeated the CHST11 siRNA assay and did not observe any effect on the CSPG4 transcript or its surface expression as assayed by anti-CSPG4 mAb (Additional file 1). GAPDH siRNA was used as control in siRNA assays and reduced GAPDH mRNA by 75% in multiple assays (data not shown). Treatment of MDA-MB-231 cells with GAPDH siRNA did not inhibit CHST11 expression (data not shown). CHST11 siRNA sequences did not affect expression of GAPDH (data not shown). Therefore the expression of CS-A and binding of P-selectin to this cell line depends on the expression of the CHST11 gene.

CSPG4 expressed on aggressive breast cancer cells functions as a P-selectin ligand through its CS-GAGs

Because CS/DS-GAG expression and function depends on the composition of PGs expressed, studying the nature of the PG(s) involved in presentation of P-selectin-reactive CS/DS is important. Such studies should help us understand the functional consequences of CS/DS-GAG, PG and P-selectin interactions and provide data that may, in future studies, be used to manipulate the expression of the polysaccharide by targeting the core protein(s). Several membrane PGs, including SDC-1, SDC-4, NRP-1, and CSPG4 can potentially present GAG chains on the surface of tumor cells [53-55]. CSPG4 is the only cell surface PG that is exclusively decorated with CS-GAGs [41] and therefore, it may play a major role in forming cell surface CS-GAGs. We compared the expression of CSPG4 in the above described human breast cancer cell lines. The results of qRT-PCR indicate that the less aggressive epithelial-like cell lines MCF7 and MDA-MB-468 did not express CSPG4, while the gene was highly expressed in the highly aggressive mesenchymal-like cell lines MDA-MB-231 and MDA-MET (Figure 4A). Flow cytometry analysis further confirmed that the expression of CSPG4 was high in aggressive cell lines MDA-MB-231 and MDA-MET with almost no expression detected in the less aggressive cell lines MCF7 and MDA-MB-468 (Figure 4B).

Because CSPG4 is abundant on aggressive cells, we hypothesized that it may function as a major core protein presenting CS-A and CS-related P-selectin ligands. We
used CSPG4 siRNA to inhibit CSPG4 expression (Additional file 2). Inhibiting CSPG4 transcript in turn inhibited cell surface expression of the PG (Figure 4C), and that led to a drop in P-selectin (Figure 4D) and anti-CS-A binding (Figure 4E). We examined the transcript levels for CHST11 after transfection with CSPG4 siRNA (Additional file 2). Inhibition of expression of CSPG4 did not affect the expression of CHST11, indicating independence of expression of these two genes. This suggests that a decrease in CS-A after CSPG4 siRNA treatment (Figure 4E) is due to reduced expression of CSPG4 and not of CHST11. These data suggest that CSPG4 participates in forming P-selectin ligands on the surface of highly aggressive human breast cancer cells, but obviously it is not the only PG involved in CS-GAG presentation. Others have reported the expression of syndecans and NRP-1 in these breast cancer cell lines and it is known that these PGs can present CS-GAGs [36-38,54-56]. To confirm, we further examined the expression of SDC-1, SDC-4, NRP-1, and CSPG4 by qRT-PCR (Table 3). The expression of SDC-1 was lower in cells with highest metastatic potential and the expression of NRP-1, SDC-4 and CSPG4 was significantly higher in the most aggressive cells. The abundance of CS-A expression in MDA-MB-468 (a cell line with intermediate aggressiveness (Figure 2A), combined with the lack of CSPG4 expression in this cell line (Figure 4A, B), suggest that CS chains on this cell line are probably presented by other PGs (Table 3).

In order to confirm that P-selectin binds to CSPG4 we used CSPG4-transfected M14 melanoma cell line

Figure 1 The expression of the chondroitin sulfotransferase genes, in the breast cancer cells used. The expression of CHST3, CHST7, CHST11, CHST12, CHST13, CHST14, CHST15 was measured by qRT-PCR and normalized using 18S. Data were analyzed by one-way ANOVA and post hoc analysis using data from three (CHST7, CHST12, CHST13, CHST14) and four (CHST3, CHST11, CHST15) independent experiments. Means and standard deviations are shown. Statistically significant differences and P values are shown.
available in the lab. In prescreening of the M14 cell line, it appeared that this cell line expresses *CHST11* with low binding of anti-CS-A 2H6 mAb and P-selectin, indicating that this cell line and its transfected version are excellent candidates for studying participation of CSPG4 and its CS GAGs in P-selectin binding. We observed that anti-CSPG4 225.28 mAb reacted with the transfected cells (M14-CSPG4) but not with mock-transfected M14 cells (Figure 5A, B, C). Anti-CS-A 2H6 mAb reacted with M14-CSPG4 but not with mock-transfected

![Figure 2](http://breast-cancer-research.com/content/13/3/R58)
Table 2 Correlation coefficients between P-selectin binding to cells and the expression of the sulfotransferases studied

| Sulfotransferase gene | Pearson r | number | P value (two-tailed) |
|----------------------|-----------|--------|---------------------|
| CHST3                | 0.25      | 16     | 0.36                |
| CHST7                | 0.45      | 12     | 0.14                |
| **CHST11**           | **0.85**  | **16** | **<0.0001**         |
| CHST12               | 0.32      | 12     | 0.30                |
| CHST13               | -0.37     | 12     | 0.24                |
| CHST14               | 0.20      | 12     | 0.52                |
| CHST15               | 0.29      | 16     | 0.31                |

Gene expression was measured by qRT-PCR and normalized using 18S transcript. Mean Fluorescence Intensities of P-selectin binding to cells was assayed by flow cytometry. Cell lines were MCF7, MDA-MB-468, MDA-MB-231, and MDA-MB-157. Each assay was repeated independently 3 times per cell line for CHST7, CHST12, CHST13 and CHST14 and 4 times per cell line for CHST3, CHST11, CHST15. Then the data were log transformed and subjected to correlation analysis using both Pearson (parametric) and Spearman (nonparametric) correlation tests in order to check validity of assumptions. Correlation coefficients were similar, assumptions were considered valid, and the Pearson coefficient was reported. CHST3, Carbohydrate (chondroitin 6) sulfotransferase 3; CHST7, Carbohydrate (N-acetylgalactosamine 6-O) sulfotransferase 7; CHST11, Carbohydrate (chondroitin 4) sulfotransferase 11; CHST12, Carbohydrate (chondroitin 4) sulfotransferase 12; CHST13, Carbohydrate (chondroitin 4) sulfotransferase 13; CHST14, Carbohydrate (N-acetylgalactosamine 4-O) sulfotransferase 14; CHST15, Carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15.

M14 cells (Figure 5D). P-selectin also reacted with M14-CSPG4 cells but not mock-transfected M14 cells, and treatment of M14-CSPG4 cells with chondroitinase ABC reduced the reactivity (Figure 5E). Pre-incubation of cells with 225.28 did not inhibit P-selectin binding (data not shown). These data suggest that CSPG4, via its CS chain, serves as a P-selectin ligand on the cell surface and that anti-CSPG4 225.28 mAb does not react with the GAG part of CSPG4. The data further suggest that overexpression of both CSPG4 and CHST11 genes in tumor cells may contribute to a superior metastatic potential.

**CHST11 and CSPG4 are overexpressed in malignant tissues of breast cancer patients**

In order to establish a translational relevance, we examined the expression of CSPG4 and CHST11 in specimens from breast cancer patients to compare the level of expression of these genes between normal and malignant tissues. Frozen sample-pair specimens from 15 breast cancer patients diagnosed with invasive ductal carcinoma were obtained from the UAMS tissue bank. In each sample pair, a tumor-containing sample was matched with tumor-free tissue from the same donor. We observed that these genes were overexpressed in tumor-containing tissues versus normal tissues (Figure 6). CSPG4 and CHST11 expression showed an increase in tumor tissue over normal tissue in 10 out of 14 sample pairs and 8 out of 15 sample pairs, respectively. CSPG4 was elevated 3.2-fold ($P < 0.02$) in tumor tissue over normal tissue among 14 subjects, while CHST11 was elevated 1.8-fold ($P = 0.034$) in tumor over normal among 15 subjects (Figure 6). Gene overexpression was detected in both ER-positive and ER-negative samples, but the majority of specimens with increased expression of CHST11 (seven out of eight), were HER2-neu-negative. These data suggest that despite the stromal expression of both CHST11 and CSPG4 genes the expression can be targeted specifically in aggressive tumors for therapeutic purposes. In this regard P-selectin binding to a relevant receptor on tumor cells may have profound impact on tumor cell dissemination and understanding the detail of such interaction may lead to development of novel anti-metastatic approaches.

**CS-GAG removal inhibits tumor metastasis**

We have previously suggested a role for CS/DS-GAGs in metastasis of the murine mammary cell line 4T1 [24]. To directly link CS/DS-GAGs to tumor metastasis, we examined whether removal of the cell surface CS/DS-GAGs affects metastasis of 4T1 cells in vivo. Here we demonstrate that removing CS/DS-GAGs by treating cells with chondroitinase ABC attenuated lung metastases in the 4T1 murine tumor model (Figure 7). Chondroitinase treatment of cells prior to tail-vein injection significantly reduced lung metastases ($P = 0.0002$). The data indicate a significant role for tumor surface CS/DS-GAGs in establishing lung metastases in this breast cancer model and further support a likely role for P-selectin interaction with CS/DS-GAG in breast cancer metastasis.

**Discussion**

Tumor cell dissemination by platelets leads to colonization of cancer cells to secondary organs resulting in poor prognosis and high mortality of cancer patients. P-selectin is present on activated platelets and endothelial cells while CS/DS-GAGs on the surface of breast cancer cells with high metastatic potential serve as P-selectin ligands [24]. The role of P-selectin in heterotypic adhesion is a critical component determining the efficiency of tumor cell dissemination [16,17]. The study of P-selectin-reactive molecules on tumor cells is crucial for the assessment of metastatic risk and the development of possible ways of dealing with metastatic disease. Such studies are needed to ultimately reveal the functional consequences of P-selectin/ligand interaction in tumor progression, making specific links between platelets and tumor metastasis.

Our results suggest the CHST11 gene as a major player in production of such P-selectin ligands. The results using CHST11 siRNA further suggest that among the chondroitin sulfotransferases tested, CHST11 expression has a rate limiting role in constructing both CS-A chains and P-selectin ligands on MDA-MB-231 cells. This data directly links expression of the CHST11 gene...
to P-selectin-reactive GAGs on this cell line and has significant implications for further functional studies. CS-A is an immediate product of \textit{CHST11} expression and is considered a precursor in forming CS-E units [57]. P-selectin has been shown to bind to CS-E [23]. We have also shown that exogenous CS-E inhibits P-selectin binding to cancer cells [24]. While we do not rule out participation of CS-E in P-selectin binding, our data do not support CS-E as the P-selectin reactive GAG unit on these cells. The combined high expression of these

\begin{figure}
\centering
\includegraphics[width=\textwidth]{inhibition.png}
\caption{Inhibition of CHST11 expression and P-selectin binding to MDA-MB-231 cells by CHST11 siRNA. MDA-MB-231 cells were treated with three different siRNAs for the CHST11 gene. RNA was harvested after 48 hours and gene expression was assayed at the mRNA level (A). GAPDH was used as the housekeeping gene to normalize mRNA-based expression data using the delta-delta CT method. CHST11 mRNA levels are shown relative to mRNA level in cells treated with transfection agent only (vehicle). Data were log transformed and subjected to one-way ANOVA with post-hoc Tukey’s analysis. B) Binding of anti-CS-A (2H6 mAb) (top) and P-selectin (bottom) was tested at Day 6 post siRNA transfection. Binding of secondary antibodies only serves as control. Binding of anti-CS-A 2H6 mAb and recombinant human P-selectin to vehicle-treated and siRNA-treated MDA-MB-231 cells with the three siRNAs is shown. Mean fluorescent intensities of three independent experiments were log transformed and analyzed by ANOVA and post-hoc comparison. Treatment with CHST11 siRNA #31 significantly reduced mean fluorescent intensities for anti-CS-A 2H6 mAb ($P \leq 0.015$) and P-selectin ($P \leq 0.001$) binding, as compared with vehicle-treated cells.}
\end{figure}
two genes may associate with P-selectin-reactive glycans and aggressiveness. This possibility should be investigated in future studies. However, the data indicate that the expression of the CHST11 gene in tumor cells is associated with synthesis of P-selectin ligands and a metastatic phenotype. Others have suggested a role for CS-A in tumor progression and metastasis in a melanoma model [46]. However, our data suggest that surface presentation of CS-A may be required but is not sufficient for a metastatic phenotype to occur. Besides its role in constructing CS-A, CHST11 also plays a role in chain elongation and production of more CS [57]. Chain elongation activity of CHST11 with a fine balance in the expression of other enzymes might be needed for constructing conformational epitopes. Thus, CHST11 activity may lead to larger CS polymers with multiple.

Table 3 Ratio of mRNA for some PG genes to 18S RNA

| Antigen     | MCF7       | MDA-MB-468 | MDA-MB-231 | MDA-MET   |
|-------------|------------|------------|------------|-----------|
| CSPG4       | 0.02 (± 0.006) | 0.03 (± 0.02) | 2.43 (± 0.56) | 2.15 (± 0.8) |
| NRP-1       | 0.96 (± 0.10)  | 1.00 (± 0.08) | 1.55 (± 0.20) | 1.96 (± 0.07) |
| SDC-1*      | 0.45 (± 0.07)  | 2.09 (± 0.18) | 0.22 (± 0.03) | 0.13 (± 0.04) |
| SDC-4**     | 0.58 (± 0.08)  | 0.86 (± 0.07) | 1.50 (± 0.30) | 1.90 (± 0.11) |
| ER1         | 4.84 (± 0.59)  | 0.12 (± 0.03) | 0.14 (± 0.03) | 0.11 (± 0.02) |

Gene expression was measured by qRT-PCR assays, repeated 3 times for each gene and each cell line and averages (± standard deviations) are shown. One-way ANOVA with Tukey’s post-hoc procedure on log-transformed data was used for statistical analysis as described in Materials and Methods. Bold entries are statistically different (at P < 0.05) than non-bold entries in each row. *The expression of SDC-1 in MDA-MB-468 cells is significantly higher than that in MDA-MB-231 and MDA-MET cells. **The expression of SDC-4 in MDA-MB-468 cells is significantly less than that in MDA-MB-231 and MDA-MET cells. The expression of ER1 was used as a control knowing that MCF7 is ER-positive and the other three cell lines are ER-negative. CSPG4, Chondroitin Sulfate Proteoglycan 4; ER1, Estrogen Receptor 1; NRP-1: Neuropilin-1; SDC-1, Syndecan-1; SDC-4, Syndecan-4.

Figure 4 The expression of CSPG4 in aggressive breast cancer cell lines contributes to P-selectin binding. A) CSPG4 mRNA was measured by qRT-PCR and normalized to 18S values and log transformed. Means and standard deviations are shown. Comparisons were made by ANOVA and post-hoc analysis. B) Cell surface expression of CSPG4 was examined in the indicated breast cancer cell lines by flow cytometry using anti-CSPG4 225.28 mAb (10 μg/ml). Open histogram shows binding of the secondary antibody only, while filled histogram shows anti-CSPG4 225.28 mAb binding. One representative experiment out of three is shown. C) Expression of CSPG4 was inhibited by transient transfection of MDA-MB-231 cells with CSPG4 siRNA that led to a decrease in the binding of P-selectin (D) and anti-CS-A (E) to transfected cells. In C, D and E the filled histograms show the binding of secondary antibodies only (control), the open histograms with solid lines show binding to vehicle-treated cells while the open histograms with dotted lines show binding to siRNA-transfected cells.
sequences embedded with distinct sulfation patterns resulted from activity of multiple sulfotransferases, leading to the production of conformational epitopes or highly concentrated sequences with specific reactivities. Thereby, the expression of CHST11 may correlate better with the tumor cells’ aggressive phenotype than does the prevalence of any particular CS isomers.

Interestingly, our current results implicate CSPG4 in the presentation of CS moieties as P-selectin ligands. CSPG4 exclusively presents CS-GAGs, and the data described here suggest that these structures interact with P-selectin and, therefore, may contribute to distant metastasis of tumor cells. Our data indicate that P-selectin binds to CSPG4 through CS-GAGs and that CSPG4
is involved in P-selectin binding to CSPG4-expressing breast cancer cells. However, other PGs may also participate in P-selectin binding as expression of SDC-4 and NRP-1 is also higher in MDA-MB-231 and MDA-MET. Lack of expression of CSPG4 in MDA-MB-468 further suggests that anti-CS-A and P-selectin binding to these cells is probably due to expression of other PGs and not CSPG4. However, because of the role of CSPG4 in signaling and tumor phenotype, we speculate that its interaction with P-selectin may lead to an exclusive tumor cell activation and consequently survival in circulation. Therefore, concerted upregulation of CSPG4 and CHST11 may induce expression of a unique molecular entity that may increase the metastatic capabilities of tumor cells. More studies are needed to understand the consequences of P-selectin binding to CS-GAGs of multiple PGs and to reveal how and at what stage of the metastatic cascade the CS-GAGs and their carrier proteins contribute to metastasis.

We have shown glycan interactions with P-selectin and the significance of P-selectin binding in metastasis of a murine mammary cell line [24,52]. Our previous findings support the concept that CS chains promote survival in the circulation and tumor cell extravasation via P-selectin-mediated binding to platelets and endothelial cells. In the current study, the significance of the cell surface expression of CS-GAGs in a breast cancer model is established. The data demonstrate that enzymatic removal of the CS chains significantly attenuated formation of lung metastases in a highly metastatic mammary cell line. Others have shown that P-selectin ligands are critical components of heterotypic adhesion, determining the efficiency of tumor cell dissemination [16,17]. Sugahara’s group demonstrated that highly sulfated CS-GAGs, in particular CS-E, are involved in metastasis of murine lung carcinoma and osteosarcoma cells [58,59]. However, our data suggest a role for 4-O sulfation of chondroitin in the metastatic phenotype. Moreover, the data suggest that the presence of CS/DS-GAGs may not be sufficient for a phenotype with high metastatic capacity to occur. The data emphasize a combination of the polysaccharide and a core protein as a pro-metastatic entity. Future studies are needed to understand the contribution of each PG in P-selectin mediated tumor cell behavior.

We further showed that the expression of CHST11 and CSPG4 is elevated in tumor tissues from breast cancer patients.
Conclusions

This study demonstrates the significance of CS-GAGs in the lung colonization of an aggressive murine mammary cell line. The study reveals that CSPG4 can serve as a P-selectin ligand through its CS chain and that the expression of the CHST11 gene controls P-selectin reactive CS-GAGs formation. The data suggest that CS-GAGs, their biosynthetic pathway, or the core protein carrying them can be potential-targets for the development of therapeutic strategies for treatment of aggressive breast tumors. The knowledge and perspective gained from this line of research together with further mechanistic studies may pave the road to target CS-GAGs, their carrier PGs and their interaction with P-selectin as novel antitumor strategies.

Additional material

Additional file 1: Supplemental Figure S1. Temporal transfection of MDA-MB-231 cells with CHST11 siRNA inhibits CHST11 expression (A), anti-CS-A (C) and P-selectin (D) binding with no effect on CSPG4 mRNA (B) or the surface expression of the PG (E).

Additional file 2: Supplemental Figure S2. Fold change in the expression of CHST11 and CSPG4 mRNA after transient transfection with CSPG4 siRNA. The expression of genes was measured 48 hours post transfection by qRT-PCR. Fold change is calculated based on the expression of genes in vehicle-treated cells. GAPDH message was used to normalize the data. Transfection with a scrambled siRNA was used as additional control. **, significantly different than expression in either cells only or cells transfected with scrambled siRNA at P < 0.001.

Abbreviations

18S: 18S ribosomal RNA; ANOVA: Analysis of Variance; CS: Chondroitin Sulfate; CS-GAGs: Chondroitin Sulfate Glycosaminoglycans; CS/DS: Chondroitin Sulfate/Derermatan Sulfate; CS/DS GAGs: Chondroitin Sulfate/Derermatan Sulfate Glycosaminoglycans; CHST3: Carbohydrate (chondroitin 6) sulfotransferase 3; CHST7: Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7; CHST11: Carbohydrate (Chondroitin 4) Sulfotransferase 11; CHST12: Carbohydrate (Chondroitin 4) Sulfotransferase 12; CHST13: Carbohydrate (Chondroitin 4) Sulfotransferase 13; CHST14: Carbohydrate (N-acetylgalactosaminiose 4-O) Sulfotransferase 14; CHST15: Carbohydrate (N-acetylgalactosaminiose 4-sulfate 6-O) Sulfotransferase 15; CS-A: Chondroitin Sulfate A unit; CS-E: Chondroitin Sulfate E unit; CSPG4: Chondroitin Sulfate Proteoglycan 4; DS: Dermatan Sulfate; DS4S-1: Dermatan Sulfate 4-sulfotransferase 1; ER1: Estrogen Receptor 1; GAGs: Glycosaminoglycans; GaNAC: N-acetyl-D-galactosamine; GaNAC5-6ST: N-acetylgalactosaminiose 4-sulfato 6-O-sulfotransferase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GlcNAc: N-acety-D-glucosamine; GlcA: Glucuronic acid; IdoA: Iduronic acid; mAb: monoclonal Antibody; NPR-1: Neuruplin-1; PG: Proteoglycan; qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction; SDC-1: Syndecan-1; SDC-4: Syndecan-4; siRNA: short interfering RNA; UAMS: University of Arkansas for Medical Sciences.

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cancer patients. Consistent with our data, CHST11 expression has been shown to be greater in human breast carcinoma compared to normal breast tissue [60] and in malignant plasma cells from myeloma patients compared to normal bone-marrow plasma cells [61]. The current research should lead to future studies of functional relationships between CS and tumor progression. Existing knowledge and further mechanistic studies might suggest CS-GAGs and their presenting PGs as targets for antitumor strategies. In support of work done in melanoma [62] and recent studies in breast cancer [39], the studies outlined here strongly suggest that CSPG4 can be an available target for immunotherapy of breast cancer. However, in order to efficiently block tumor cell dissemination by interrupting P-selectin/CS interaction, targeting any single PG does not seem enough as other PGs can probably compensate and support metastatic processes. In this regard, global targeting of specific CS isoforms may be a particularly effective approach.

Breast cancer cell surface is decorated with CS-GAGs and due to tumor-specific expression patterns of chondroitin sulfotransferases and PGs, the composition and binding specificity of these polysaccharides differ from those of normal tissues. Therefore, these molecules and their interaction with P-selectin should be considered as viable targets for the development of novel therapeutic strategies.
4. Kawaguchi T: Cancer metastasis: characterization and identification of proteoglycans: structures and interactions.

11. Kjellen L, Lindahl U:

5. Korourian S, Siegel E, Kieber-Emmons T, Monzavi-Karbassi B: On the role of cell surface carbohydrates and the behavior of metastatic tumor cells and the cell adhesion molecules, including carbohydrates.

7. Iozzo RV:

6. Poole AR:

2. Couldrey C, Green JE: Analysis of carbohydrate antigens in ductal carcinoma in situ of the breast by lectin histochemistry.

9. Vynios DH, Theocharis DA, Papageorgakopoulou N, Papadas TA, Mastronikolis NS, Goumas PD, Stylianou M, Stylianou M: Expression in human laryngeal cancer. Anticancer Res 2008, 28:1245-1251.

12. Esko JD, Kimata K, Lindahl U: Proteoglycans and sulfated glycosaminoglycans. In Essentials of Glycobiology. Edited by: Varik A, Cummings RD, Esko JD, Freeze H, Hart G, Marsh JD. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1999:653.

28. Hiraoka N, Nakagawa H, Ong E, Akama TO, Fukuda MN, Fukuda M: Glycosaminoglycans in human breast cancer. Acta Obstet Gynecol Scand 1988, 67:539-542.

17. Borsig L, Wong R, Feramisco J, Nadeau DR, Varik NM, Varik A: Heparin and cancer reviewed: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci USA 2001, 98:3352-3357.

 Garcia J, Callewaert N, Borsig L: P-selectin mediates metastatic progression through binding to sulfatides on tumor cells. Glycobiology 2007, 17:185-196.

19. McEver RP: Selectin-carbohydrate interactions during inflammation and metastasis. Glycobiology 1997, 14:585-591.

20. Aruffo A, Kolanus W, Walz G, Fredman P, Seed B: CD62/P-selectin recognition of myeloid and tumor cell sulfatides. Cell 1991, 67:35-44.

24. Koenig A, Norgard-Sumnicht K, Linhardt R, Varik A: Differential interactions of heparin and heparan sulfates with glycosaminoglycans with the selectins. Implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. J Clin Invest 1998, 101:877-889.

25. Silbert JE, Sugumaran G: Molecular cloning and expression of human chondroitin 6-sulfotransferase. J Biol Chem 2002, 277:12921-12929.

26. Sugahara K, Mikami T, Endo M, Itoh T, Takase K, Takeuchi K: Expression analysis of carbohydrate antigens in ductal carcinoma in situ of the breast by lectin histochemistry. BMC Cancer 2008, 8:136.

27. Iozzo RV: Proteoglycans in health and disease: structures and functions. Biochem J 1996, 326:1-14.

28. Hiraoka N, Nakagawa H, Ong E, Akama TO, Fukuda MN, Fukuda M: Glycosaminoglycans in human breast cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci USA 2001, 98:3352-3357.

29. Takasaki H, Fujita M, Ito N, Sugahara K: Molecular cloning and expression of a novel breast carcinoma cell specific chondroitin 6-sulfotransferase. J Biol Chem 2000, 275:21075-21080.
34. Habuchi O, Moro R, Ohtake S. Enzymatic synthesis of chondroitin sulfate E by N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase purified from squid cartilage. *Anal Biochem* 2002, 310:129-136.

35. Barbarese M, Masionneuve P, Aldrovini D, Cang MG, Peccarini L, Angela Maur F, Veronese S, Gaffo O, Lucenti A, Palma PO, Galigioli E, Doglioni C. High syndecan-1 expression in breast carcinoma is related to an aggressive phenotype and to poorer prognosis. *Cancer* 2003, 98:474-483.

36. Burbach BJ, Friedl A, Mundhenke C, Rapparee AG. Syndecan-1 accumulates in lysosomes of poorly differentiated breast cancer cells. *Matrix Biol* 2003, 22:163-177.

37. Baba F, Swartz K, van Buren R, Eckhoff J, Zhang Y, Wolberg W, Friedl A. Syndecan-1 and syndecan-4 are overexpressed in an estrogen receptor-negative, highly proliferative breast carcinoma subtype. *Breast Cancer Res Treat* 2006, 98:91-98.

38. Gotte M, Kersting C, Radke I, Kiesel L, Wullf P. An expression signature of syndecan-1 (CD138), E-cadherin and c-met is associated with factors of angiogenesis and lymphangiogenesis in ductal breast carcinoma in situ. *Breast Cancer Res* 2007, 9:R6.

39. Wang X, Wang Y, Yu L, Sakakura K, Visu C, Schwab JH, Ferrone CR, Favone I, Koya Y, Campoli MR, McCarthy JB, DeLeo AB, Ferrone S. CSPG4 in cancer: multiple roles. *Curr Mol Med* 2010, 10:419-429.

40. Bumol TF, Reisfeld RA. Unique glycoprotein-proteoglycan complex defined by monoclonal antibody on human melanoma cells. *Proc Natl Acad Sci USA* 1982, 79:1245-1249.

41. Nishiyama A, Dahlin KJ, Prince JT, Johnstone SR, Stallcup WB. Chondroitin sulfates play a major role in cancer: multiple roles. *Curr Mol Med* 2003, 3:249-257.

42. Burg MA, Grako KA. Characterization of a cell-surface and a cytoplasmic antigen detectable in human melanoma cells. *Proc Natl Acad Sci USA* 1996, 93:9710-9715.

43. Pluschke G, Vanek M, Evans A, Dittmar T, Schmid P, Itin P, Filardo EJ, et al. The NG2 antigen, a putative lineage marker: immunofluorescent localization in primary cultures of rat brain. *Dev Biol* 1981, 83:154-165.

44. Burg MA, Grako KA. Stallcup WB. The primary structure of NG2, a novel membrane-spanning proteoglycan. *J Cell Biol* 1991, 114:359-371.

45. Stallcup WB. The NG2 antigen, a putative lineage marker: immunofluorescent localization in primary cultures of rat brain. *Dev Biol* 1981, 83:154-165.

46. Iida J, Wilhelmson KL, Ng J, Lee P, Morrison C, Tam E, Overall CM, et al. Differentiation immunogenicity of two peptides isolated by high molecular weight melanoma-associated antigen-specific monoclonal antibodies with different affinities. *J Immunol* 2005, 174:7104-7110.

47. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001, 25:402-408.

48. Monzavi-Karbassi B, Whitehead TL, Jousseghayn F, Artaud C, Hennings L, Shaif S, Slaughter A, Koriyan S, Kelly T, Blaszczyk-Thurn M, Kieber-Emmons T. Deficiency in surface expression of E-selectin ligand promotes lung colonization in a mouse model of breast cancer. *Int J Cancer* 2005, 117:398-408.

49. Faasen AE, Schrager JA, Klein DJ, Oegema TR, Couchman JR, McCarthy JB. A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. *J Cell Biol* 1992, 116:521-531.

50. Deepa SS, Yamada S, Zako M, Goldberger O, Sugahara K. Chondroitin sulfate chains on syndecan-1 and syndecan-4 from normal murine mammary gland epithelial cells are structurally and functionally distinct and cooperate with heparan sulfate chains to bind growth factors. A novel function to control binding of midkine, pleiotrophin, and basic fibroblast growth factor. *J Biol Chem* 2004, 279:37368-37376.

51. Shintani Y, Takahisha S, Asano Y, Kato H, Liao Y, Yamazaki S, Tsukamoto O, Seguchi O, Yamamoto H, Fukushima T, Sugahara K, Kitakaze M, Hori M. Glycosaminoglycan modification of neuropilin-1 modulates VEGFR2 signaling. *Embo J* 2006, 25:3045-3055.

52. Bielenberg DR, Pettaway CA, Takahisha S, Klagsbrun M. Neuropilins in neoplasms: expression, regulation, and function. *Exp Cell Res* 2006, 312:584-593.

53. Uyama T, Ishida M, Ikumikawa T, Trylba E, Tufaro F, Bergstrom T, Sugahara K, Katagawa H. Chondroitin 4-O-sulfotransferase-1 regulates E disaccharide expression of chondroitin sulfate required for herpes simplex virus infection. *J Biol Chem* 2006, 281:38668-38674.

54. Li F, ten Dam GB, Murugan S, Yamada S, Hashiguchi T, Mizumoto S, Ofrit K, Okayama M, van Kuppevelt TH. Sugahara K. Involvement of highly sulfated chondroitin sulfate in the metastasis of the Lewis lung carcinoma cells. *J Biol Chem* 2008, 283:34294-34304.

55. Baissapa , Murugan S, Sugahara KN, Lee CM, ten Dam GB, van Kuppevelt TH, Miyasaka M, Sugahara K. Involvement of chondroitin sulfate E in the liver tumor focal formation of murine osteosarcoma cells. *Glycobiology* 2009, 19:735-742.

56. Potapenko IO, Haakensen VD, Luders T, Helland A, Bukholm I, Sorlie T, Kristensen VN, Lingjaerde OC, Borresen-Dale AL. Glycan expression signatures in normal and malignant breast tissue: possible role in diagnosis and progression. *Mol Oncol* 2010, 4:98-118.

57. Breit C, Hose D, Remé T, Sprynski AC, Mahlouk K, Schved JF, Quitter P, Rossi JF, Goldschmidt H, Klein B. Expression of genes encoding for proteins involved in heparan sulfate and chondroitin sulfate chain synthesis and modification in normal and malignant plasma cells. *Br J Haematol* 2009, 145:350-368.

58. Campoli MR, Chang CC, Kagihara T, Wang X, McCarthy JB, Ferrone S. Human high molecular weight-melanoma-associated antigen (HMMW-MAA): a melanoma cell surface chondroitin sulfate proteoglycan (MSCP) with biological and clinical significance. *Cytomembr Biol* 2004, 24:267-296.

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