Sialylation and Metastasis: from tumor-associated antigens discovery to therapeutic development

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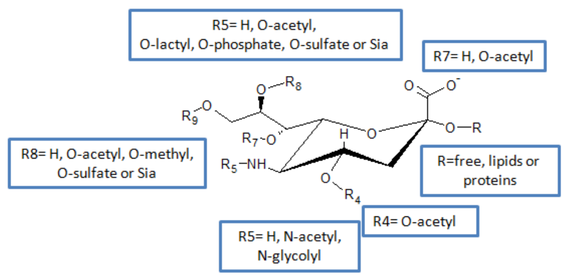
Overview and Conclusions

- A fast, efficient and selective method for purification of **N-linked sialylated peptides** from membrane protein fractions employing titanium dioxide (TiO_2) in combination with Hydrophilic Interaction Chromatography (HILIC) fractionation is presented. Using this method we have detected 651 glycoproteins with more than 1200 sialylated glycosites.
- A quantitative strategy based on selective enrichment of sialylated glycopeptides from SILAC-labeled isogenic breast cancer cells is presented as a tool to understand the sialylation changes during **metastatic progression**.
- The identification of tumor-specific sialylated glycoprotein alterations add important knowledge to **breast tumor therapy**.

INTRODUCTION

Breast cancer is the leading female cancer in Europe and in the USA with metastasis recognized as the main reason that leads to the mortality in the patients. The glycocalyx of cancer cells changes during metastatic progression and **sialic acids** (SA), shown below, are regulated during cancer progression. Indeed aberrant sialylation on the cell surface affects the intercellular and cell-matrix recognition processes, influencing the metastatic behaviour of cancer cells. Altered sialylated glycostructures have been associated to the cancer invasive process so the discovery of tumor-specific sialylated antigens represents an important step to develop efficient therapeutics. Because the levels of many membrane proteins may have an effect on cell motility, and therefore metastasis, identifying these changes in cell lines of differing metastatic potential could aid in our understanding of the underlying mechanisms of this process. Due to that the sialoglycobiology of tumor cells, identification of tumor-specific glycoprotein antigens and of the enzymatic steps required for their in vivo synthesis represent important tools to breast tumor therapy.



We have compared two isogenic breast cancer cells with different metastatic potential, NM2C5 and M4A4, using a newly method for enrichment of SA-containing glycopeptides using TiO_2 chromatography and capillary HILIC fractionation (1). The deglycosylated sialoglycopeptides we analyzed by liquid chromatography Electrospray Ionization tandem Mass Spectrometry (LC-ESI-MS/MS).

MATERIAL AND METHODS

Cell culture:

Human breast tumour cells from a patient were orthotopically transplanted into mice and metastatic cell line was selected for enhanced growth rate and faster metastasis. After selection, the non-metastatic (NM2C5) and metastatic (M4A4) Her2-negative cell lines were grown in SILAC medium respectively in $^{13}C_6$-Lys,Arg and $^{12}C_6$-Lys,Arg. Cells lysis and preparation of membrane fraction were carried out according to the workflow shown**.**

Purification of membrane proteins from human breast cancer cells:

Cell pellets were resuspended in ice cold sodium carbonate, pH 11 and after probe-tip sonication, unbroken cells were removed by centrifugation at 2,500 g for 10 min. The carbonate treated membranes were collected by ultracentrifugation at 100,000 g for 1 hr.

In-solution digestion:

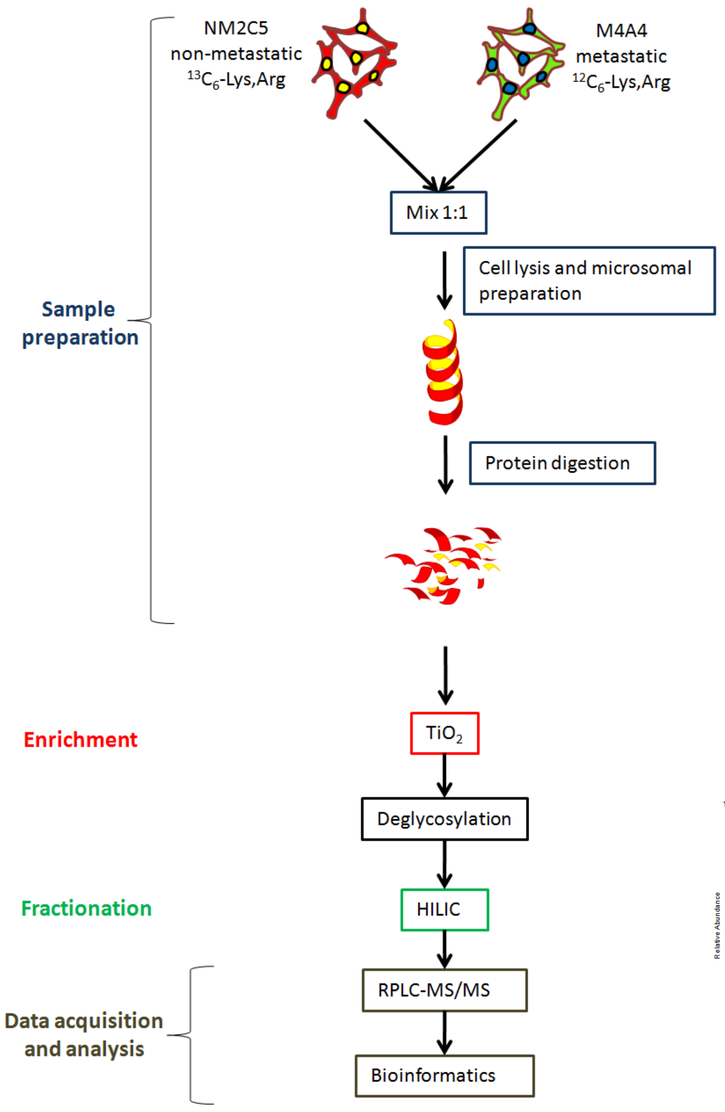
The membrane pellet was resuspended in 8M Urea, reduced with 20mM dithiotreitol (DTT) and alkylated for 40min. with 40mM iodoacetamide at room temperature in the dark. The reduced and alkylated sample was incubated for 3 hrs with lysyl endopeptidase. The Lys-C digested sample was digested with trypsin at room temperature overnight in 1:50 protein/enzyme ratio. Alkaline phosphatase and lamba phosphatase treatment was performed to remove the phosphate group.

Batch-mode Titanium Dioxide (TiO_2) Purification:

SA-containing glycopeptides were enriched using TiO_2 as previously described (2) using low pH buffers to bind and remove non-sialylated glycopeptides and high pH solutions to elute the SA-containing glycopeptides. The enriched peptides were treated with N-glycosidase F and fractionated with capillary flow HILIC chromatography.

Nano-HPLC-MS/MS experiments were performed using the Easy-nLC (Proxeon Biosystems) connected to a linear ion trap-orbitrap hybrid mass spectrometer (Thermo). The peptides were separated using C18 reversed phase column and analyzed in data-dependent mode. The MS raw data were analyzed using the software MaxQuant (3).

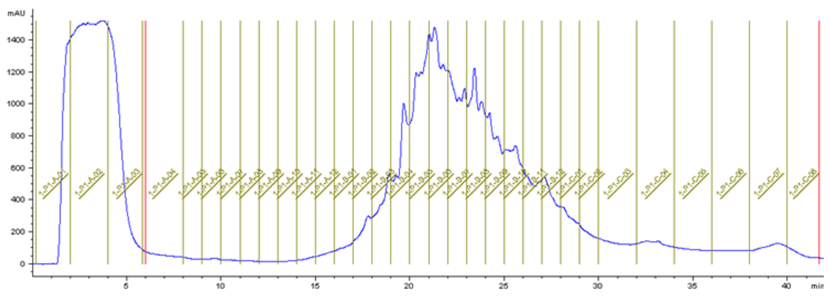
STRATEGY



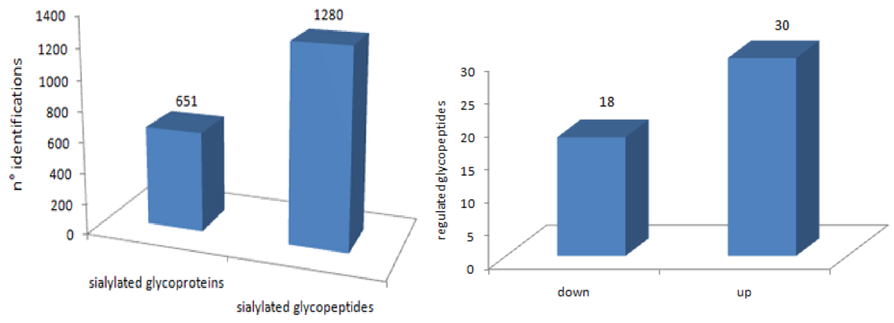
Workflow showing sialic acid containing membrane proteins quantitative profile in Stable Isotope Labeling with Amino Acids (SILAC) cell culture method. NM2C5 (non-metastatic) and M4A4 (high metastatic) cells were cultured in SILAC media. After isolation of membrane proteins and tryptic digestion, glycopeptides were enriched with TiO_2 and deglycosilated with PNGaseF. After capillary HILIC fractionation, the enriched peptides were analyzed by mass spectrometry to quantitatively determine the difference between two conditions. Quantitative data were determined by comparing the extracted ion chromatograms during elution of light and heavy peptides using MaxQuant software.

RESULTS

Chromatogram of the HILIC separation of the deglycosylated SA-containing glycopeptides indicating the fractions collected throughout the gradient and analyzed by RPLC-MS/MS.

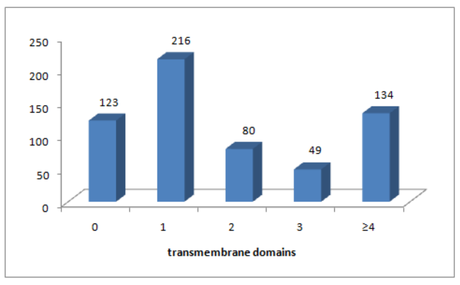


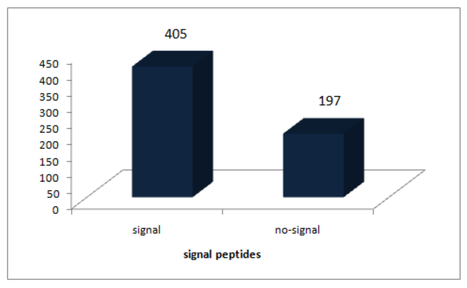
A total of 1280 sialylated glycopeptides within 651 membrane glycoproteins were identified using this strategy representing the highest number of glycosylation sites identified and quantified in human breast cancer. Regulated glycopeptides: 18 and 30 glycopeptides were identified respectively to be statistically significant down and up-regulated with a significance B <0.001 (3)



Example of differentially regulated glycopeptides between metastatic and non-metastatic condition

Glycoproteins	Fold change (Metastatic/Non-Metastatic)
SLIT	4.2
Integrin beta 1	3
Receptor tyrosine-protein kinase erbB-2	1
CD44	0.3

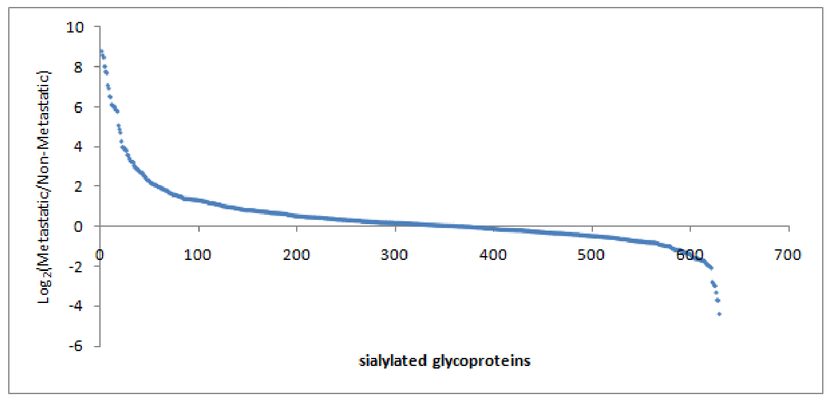




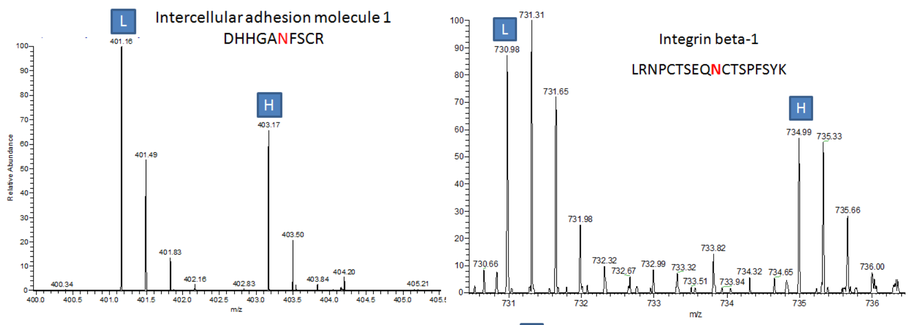
Transmembrane domains and signal peptides for all glycoproteins. The high number of transmembrane domain indicate a high enrichment of membrane proteins, moreover several identified glycoproteins contain a signal peptide for targeting them to specific compartment. The identified glycoproteins were analyzed using Protein Center (Proxeon) software. The program was used to study the gene ontology (GO) cellular location and molecular function of the glycoproteins.

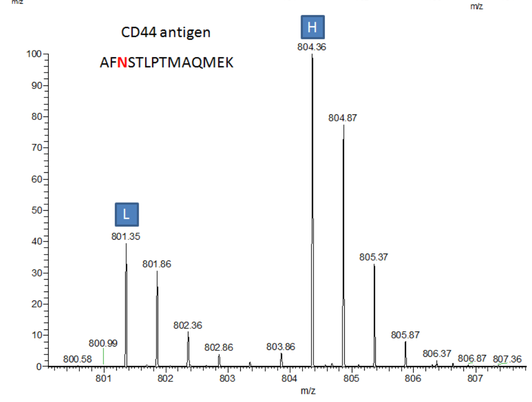
RESULTS

Distribution of glycoproteins expression ratios as determined by SILAC. The SILAC ratio for each protein represents the relative expression difference between the high-(M4A4) non-(NM2C5) metastatic breast cancer cells. Proteins were sorted and plotted by SILAC ratio expressed as Log_2 function. The distribution of glycoproteins ratio shows mostly the upregulation of sialylated proteins during metastatic progression even though there is a portion of them downregulated. The regulation could be due to the oversialylation during metastatic progression

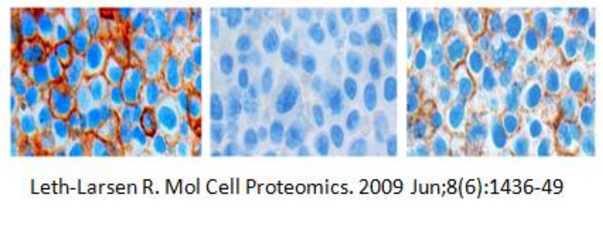


MS signals of ICAM-1, Integrin beta-1 and CD44 antigen showing the differential regulation between non metastatic (H) and metastatic (L). Intercellular ashesion molecule 1 has been previously shown to be upregulated in gastric, pancreatic, and breast cancer tissues, with highest levels in samples from patients with metastases.





Immunohistochemistry validation. CD44 is reported as prognostic marker in breast cancer. Increased B1 integrin signaling is involved in malignant progression and inhibitory antibody to B1 integrin leads to selective apoptosis and decreased proliferation in three-dimensional cultures and in xenograft models of breast cancer in vivo.



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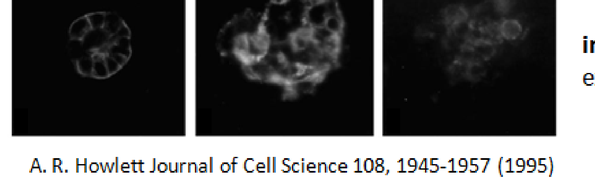

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CORRESPONDANCE

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