Novel theranostic opportunities offered by characterization of altered membrane lipid metabolism in breast cancer progression

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SUPPLEMENTARY MATERIALS AND METHODS

Breast cancer samples

Hematoxylin & eosin sections were performed for histopathological evaluation. All fresh frozen tumor samples contained ≤20% of adipose tissue upon histopathological quality control. Also the percentage of tumor area in the samples was controlled. The mean tumor area was 85% and 88% in the training and validation sets, respectively. As control samples paired normal breast tissue was used. It was obtained at the time of tumor surgery but did not contain tumor cells. In histopathological quality control the normal samples were selected to contain 80-100% of normal ductal or periductal tissue and only 0-20% of adipose tissue, to ensure comparability with the tumor samples.

Lipidomic analyses of breast cancer tissue

Tissue samples (1-5 mg) were mixed with 100 µl of PBS-buffer and homogenized by using 1-2 grinding balls (Ø 3 and 5 mm) with a Retsch mixer mill MM400 homogenizer at 20 Hz for 2 min. A 10 µl aliquot was separated for protein concentration determination and the rest of the sample was extracted with chloroform:methanol (2:1; 250 µl) after adding an internal standard mixture with PC(17:0/0:0), Cer(d18:1/17:0), PC(17:0/17:0), PE(17:0/17:0) and TG(17:0/17:0/17:0) at concentration level of 0.5-1 µg/sample. The samples were vortexed for 2 min, incubated 30 min at RT and centrifuged at 7800g for 3 min. A labelled lipid standard mixture

containing PC(16:1-D3/0:0), PC(16:1/16:1-D6) and TG(16:0/16:0/16:0-13C3) at concentration level of 0.5 µg/sample was added into the separated lipid extracts (100 µl) before UPLC-MS analysis.

Lipid extracts were analysed on a Waters Q-Tof Premier mass spectrometer combined with an Acquity Ultra Performance LC™ (UPLC™). The column (at 50°C) was an Acquity UPLC™ BEH C18 1 × 50 mm with 1.7 µm particles. The solvent system included A) water (1% 1 M NH_4Ac, 0.1% HCOOH) and B) acetonitrile/isopropanol (5:2, 1% 1M NH_4Ac, 0.1% HCOOH). The gradient started from 65% A / 35% B, reached 100% B in 6 min and remained there for the next 7 min. There was a 5 min re-equilibration step before the following run. The flow rate was 0.200 ml/min and the injected amount 1.0 µl. Leucine enkephaline and reserpine were used as the lock spray reference compounds in ESI- and ESI+ modes, respectively. The data were collected at mass range of m/z 200-1200 with scan duration of 0.2 sec. The data were processed using MZmine 2 software (http://mzmine.sourceforge.net/) and the lipid identification was based on an internal spectral library and tandem mass spectrometry.

Protein content of the samples was determined from the PBS-homogenate of the tissue (5 µl) which was diluted further for Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL). Spectrophotometric determination was performed on a Multiskan EX instrument (Thermo Scientific, Vantaa, Finland). The lipidomics results were normalized according to the protein content of the samples (mg protein / mg tissue) and the lipids were quantified using the internal standards.

Immunohistochemistry

Immunohistochemical staining was performed on tissue microarrays using the following antibodies: ACACA rabbit polyclonal antibody (HPA006554; ATLAS; dilution 1:50), FASN mouse monoclonal antibody (clone 3F2-

1F3, h0002194-M01; Abnova; dilution 1:1000), INSIG1 rabbit anti-human polyclonal antibody (LS-B2420, Lifespan Biosciences; dilution 1:1000), and SREBP1 mouse monoclonal antibody (NB600-582; Novus Biologicals; dilution 1:750). Furthermore, we used peroxidase (FASN, SREBP1) / alkaline phosphatase (INSIG1, ACACA) conjugated secondary antibodies and for color developing (Dako REAL^{TM} Detection System, peroxidase / alkaline phosphatase, rabbit / mouse; Dako, Glostrup, Denmark). Slides were covered manually with Vitro-Clud (Langenbrinck, Emmendingen, Germany) after staining. Stained slides were digitized by a slide scanner (Mirax Scan; Zeiss, Jena, Germany) and evaluated as virtual slides using the VMscope slide explorer including the TMA plugin (VMscope, Berlin, Germany).

Functional experiments in breast cancer cells

The breast carcinoma cells ZR-75-1 and MDA-MB-468 and non-malignant MCF-10A cells were obtained from the American Type Culture Collection (Manassas, VA). The identity of the cells was confirmed by array CGH (Agilent Technologies, Palo Alto, CA) and the cells were passaged no longer than 6 months after receive or resuscitation of frozen aliquots. ZR-75-1 cells were grown in DMEM supplemented with 10 % FBS, 2 mM L-glutamine and 1 % non-essential amino acids (NEAA). Same medium without NEAA was used for MDA-MB-468 cells. The culture conditions for MCF-10A cells were as follows: 50 % DMEM (Sigma): 50% HAM´s F12 (Gibco), supplemented with 5 % horse serum, 1 mM L-glutamate, 10 µg/ml insulin (Sigma), 5 µg/ml hydrocortisone (Sigma), 20 ng/ml EGF (Sigma) and 100 ng/ml cholera toxin. Four siRNAs for each of the selected genes were purchased from Qiagen and plated onto 384-well plates (Greiner), followed by addition of the transfection agent (siLentFect Lipid Reagent, Bio-Rad Laboratories) in Opti-MEM (Invitrogen) and appropriate amount of cells (2000 per well) in media. The final siRNA concentration was 13 nM. For MCF-10A cells the siRNA transfections were done using the same as method as described for cancer cells except that less cells (1600 cells/well for MCF-10A and 2000 for ZR-751 and MDA-MB-468) and less Silentfect (0.05 µl/well compared to 0.07 µl/well used with cancer cells) was required for transfections. After 72 h incubation, cells were assayed

with CellTitre-Glo (Promega Inc., Madison, USA) for viability and with homogenous Apo-ONE assay (Promega, Madison, WI) for apoptosis according to the manufacturer's instructions. The Envision Multilabel Plate Reader (Perkin-Elmer, Massachusetts, MA) was used for fluorometric signal quantification. The raw results were normalized using loess log. An average of three replicate transfections per siRNA was calculated and siRNAs reducing cell viability by at least 1.5 SD from the median of the controls were considered as putative anti-proliferative or pro-apoptotic siRNAs. AllStars Negative Control (scrambled siRNA) and lipid only were used as negative controls, siRNAs against KIF11 (kinesin family member 11) and PLK1 (polo-like kinase 1) as positive controls.

Based on the cell viability results seven siRNAs (and two positive controls) were selected for lipidomics analysis. ZR-75-1 cells were transfected in two replicates for each timepoint. After 48 and 72 hours, approximately 5 million cells were detached, washed two times with cold PBS and the pellets were freezed to -80°C. Lipidomic analyses for the cells were performed as for the tissue samples with the following modifications: The cell pellets were homogenized with 50 µl PBS-buffer. From the homogenate, 5 µl was taken for the protein assay and 15 µl was used for lipid extraction and spiked with an internal standard mixture of PC(17:0/0:0), MG(17:0/0:0/0:0)[rac], PG(17:0/17:0)[rac], Cer(d18:1/17:0), PS(17:0/17:0), PC(17:0/17:0), PA(17:0/17:0), PE(17:0/17:0), DG(17:0/17:0/0:0)[rac] and TG(17:0/17:0/17:0) at concentration levels of 0.1-0.2 µg/sample. After extraction, the lower phase (60 µl) was separated and spiked with the labelled internal standard mixture at concentration level of 0.1 µg/sample. The column used for cell extracts was an Acquity UPLC^{TM} BEH C18 2.1 × 100 mm with 1.7 µm particles. The solvent system was A) ultrapure water (1% 1 M NH_4Ac, 0.1% HCOOH) and B) LC/MS grade acetonitrile/isopropanol (1:1, 1% 1M NH_4Ac, 0.1% HCOOH) and the gradient started from 65% A / 35% B, reached 80% B in 2 min, 100% B in 7 min and remained there for 7 min. The flow rate was 0.400 ml/min and the injected aliquot 2.0 µl (Acquity Sample Organizer, at 10 °C). The protein concentration was measured

as for the tissue samples, and the total protein content of the sample was used in the normalization of the data.

To validate the silencing of the target genes, aliquots of siRNA transfected cells were collected for RNA extraction from each sample prepared for lipidome analysis. Total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's protocol and processed to cDNA with Applied Biosystem´s cDNA synthesis kit. TaqMan gene expression probes and primers from the Universal Probe Library (Roche Diagnostics, Espoo, Finland) were used to study gene expression (Supplementary Table S1). Real-time quantitative PCR was done using ABI Prism 7900 (Applied Biosystems, Foster City, CA). Quantitation was carried out using the ΔΔCT method with RQ manager 1.2 software (Applied Biosystems). Average expression of the untreated control samples was considered for the calculation of the fold changes. Three replicate samples were studied for detection of mRNA expression. Three replicate samples were also used to investigate the expression levels of investigated genes in the three cell lines (ZR-75-1, MDA-MB-468 and MCF-10-A).

Statistical analyses of the data

Fold changes between the tumor types were calculated by comparing the median value of the groups (Table 1 and Supplementary Tables S4-S7), and if the fold change was <1, then the inverse negative of the value was taken (*e.g.* fold change 0.5 was turned to -2.0). The heatmap (Fig. 1B) showing the fold changes for tumors of different grade and ER status was constructed by taking the median value of each group and comparing it to the median value of normal tissue samples. For survival analyses, the median value of all samples was calculated for each lipid, and then the samples were divided into two groups, those having concentration above and below of the median of all samples. The survival analyses were performed using the R package survival with the Kaplan-Meier method (Fig. 1D). For the heatmap showing the results of immunohistochemistry (Fig. 3B), the median of all samples was calculated for each lipid, and then median of

samples within certain group was compared to all samples by log2 fold change. The heatmap for the lipidomics after gene silencing (Fig. 4D) was constructed by taking the log2 fold change of the mean of the two replicates relative to the scrambled siRNA samples (48h and 72h separately). The p-values in Supplementary Table S8 were calculated using χ^2 test.