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Targeted proteomics driven verification of biomarker candidates associated with breast cancer aggressiveness

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ABSTRACT

Breast cancer is the most common and molecularly relatively well characterized malignant disease in women, however, its progression to metastatic cancer remains lethal for 78% of patients 5 years after diagnosis. Novel markers could identify the high risk patients and their verification using quantitative methods is essential to overcome genetic, inter-tumor and intra-tumor variability and translate novel findings into cancer diagnosis and treatment. We recently identified 13 proteins associated with estrogen receptor, tumor grade and lymph node status, the key factors of breast cancer aggressiveness, using untargeted proteomics. Here we verified these findings in the same set of 96 tumors using targeted proteomics based on selected reaction monitoring with mTRAQ labeling (mTRAQ-SRM), transcriptomics and immunohistochemistry and validated in 5 independent sets of 715 patients using transcriptomics. We confirmed: (i) positive association of anterior gradient protein 2 homolog (AGR2) and periostin (POSTN) and negative association of annexin A1 (ANXA1) with estrogen receptor status; (ii) positive association of stathmin (STMN1), cofilin-1 (COF1), plasminogen activator inhibitor 1 RNA-binding protein (PAIRBP1) and negative associations of thrombospondin-2 (TSP2) and POSTN levels with tumor grade; and (iii) positive association of POSTN, alpha-actinin-4 (ACTN4) and STMN1 with lymph node status. This study highlights a panel of gene products that can contribute to breast cancer aggressiveness and metastasis, the understanding of which is important for development of more precise breast cancer treatment.

1. Introduction

Breast cancer is the most common malignant disease in women, representing the most serious oncological cause of death among women worldwide [1]. Currently, several clinicopathological parameters are being used in clinical practice as prognostic and predictive factors of breast cancer: age at diagnosis, tumor size, tumor grade, presence of lymph node metastases, distant metastases, status of estrogen (ER), progesterone (PR) and Her2/Neu (HER2) receptors [2]. Some of these factors are strongly associated with tumor aggressiveness: The most important factor is tumor grade which positively correlates with high cell proliferation and low cell differentiation. ER and HER2 are potent predictive factors for relatively effective treatment of ER and HER2 positive tumors using hormonal and biological therapy, respectively. A combination of the key factors currently determines cancer phenotypes with extremely poor prognosis, such as triple negative breast cancer subtype (ER-, PR-, HER2-, high grade), while another phenotypes (e.g., luminal A subtype, ER +, PR +, HER2-, low grade) have relatively good prognosis. Remaining breast cancer subtypes called luminal B (ER +, PR ±, HER2 ±, high grade) and HER2 enriched (ER-, PR-, HER2 +, high grade) have medium prognosis in general. Presence of lymph node metastases is an indicator of higher risk of cancer spreading [3]. Currently used classification is effective in treatment prediction, however, it fails in some clinical scenarios: For instance, it does not prevent

Abbreviations: ACTN4, alpha-actinin-4; AGR2, anterior gradient protein 2 homolog; ANXA1, annexin A1; BGH3, transforming growth factor-beta-induced protein ig-h3; COF1, cofilin-1; DDA, data-dependent acquisition; ER, estrogen receptor; G1, tumor grade 1; G3, tumor grade 3; GSN, gelsolin; HER2, Her2/Neu receptor; IHC, immunohistochemistry; iTRAQ, isobaric tags for relative and absolute quantitation; MIDAS™, MRM Initiated Detection And Sequencing; mTRAQ, mass differential tags for relative and absolute quantification; mTRAQ-SRM, selected reaction monitoring with mTRAQ labeling; N0, lymph node negative; N1, lymph node positive; PAI-1, plasminogen activator-inhibitor 1; PAIRBP1, plasminogen activator inhibitor 1 RNA-binding protein; TSP2, thrombospondin-2; POSTN, periostin; PR, progesterone receptor; RFS, relapse free survival; RNF25, E3 ubiquitin-protein ligase RNF25; SRM, selected reaction monitoring; STMN1, stathmin; TEAB, triethylammonium bicarbonate; TMAs, tissue microarrays; u-PA, urokinase-type plasminogen activator.

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development of metastatic disease that remains lethal to 78% of patients 5 years after diagnosis [4]. It is thus essential to recognize additional markers and molecular targets associated with various aspects of breast cancer aggressiveness to improve clinical decisions. Although we have to realistically expect that any novel markers will somehow correlate with traditional prognostic factors, they have an indisputable potential to improve diagnosis via more precise classification, and/or into treatment as alternative therapeutic targets applicable in more stratified therapy.

Involvement of shotgun proteomics with "isobaric tags for relative and absolute quantitation" (iTRAQ) labeling to discover such novel targets has led to high number of proteins presented as potential biomarkers in last decade [5]. Due to limited reproducibility of data-dependent acquisition (DDA) [6,7] as well as co-fragmentation of co-eluting peptides that may affect protein quantification, it is appropriate to verify quantitative findings of DDA-based proteomics with an independent method in the same sample set to highlight the most suitable candidates for validation in independent sample cohorts. Targeted mass spectrometry based on selected reaction monitoring (SRM), a method of the year 2012 by Nature Methods [8], is a sensitive, quantitative and high throughput method for both verification and validation steps. The "mass differential tags for relative and absolute quantification" (mTRAQ) labels have been designed for relative quantification in SRM and are commercially available in triplex format (mTRAQ-Δ0, mTRAQ-Δ4 and mTRAQ-Δ8). In a typical biomarker verification experiment, global internal standard (pooled from aliquots of all samples) is prepared by labeling with mTRAQ-Δ8 label whereas individual samples are labeled with any of two remaining variants (mTRAQ-Δ0, mTRAQ-Δ4). The relative quantification is then based on the ratio of signal intensities of product ions originating from differently tagged peptides.

In our recent breast cancer discovery study [9] we employed iTRAQ-based shotgun proteomics to identify proteins connected with lymph node status specifically of luminal A tumors. In the current study, we use the same sample set of 96 breast tumor lysates with equal composition of grade 1 and grade 3 tumors, lymph node positive and lymph node negative tumors and containing both ER positive and ER negative tumors within grade 3 group (see Table 1 and Supplementary File 1 for more details of the sample set) to further verify our previous screening findings of proteins related to ER status, tumor grade and lymph node status regardless the current breast cancer subtypes. For this purpose we used mTRAQ-SRM driven targeted proteomics approach and transcriptomics; the strongest candidates were subjected to additional verification using immunohistochemistry. Validation was performed via meta-analysis of the mRNA levels from publicly accessible datasets to confirm a connection of gene expression with the clinicopathological parameters. The validated gene products may be connected to mechanisms of tumor aggressiveness and metastasis and as such can be considered as molecular targets of more personalized treatment.

2. Materials and methods

2.1. Tissue processing and patient characteristics

Patient informed consent forms along with tissue processing procedures were approved by the ethics committee of the Masaryk Memorial Cancer Institute (MMCI) (Supplementary File 1). Tissues were frozen in liquid nitrogen within 20 min after surgical removal and stored at −180 °C in tissue bank at MMCI. A complementary formalin fixed, paraffin embedded tissue block was available for each sample for histological evaluation and immunohistochemical (IHC) analysis. A set of 96 preoperatively untreated breast carcinomas of 11–20 mm maximum diameter (pT1c) was selected for the study. The sample set included 48 grade 1 tumors both ER and PR positive, 24 of them having lymph node metastases at the time of surgery. Another 48 cases were grade 3 carcinomas, 24 of them lymph node positive and 24 lymph node negative. The sample set characteristics are shown in Table 1 and individual details including involved immunophenotypes are available in Supplementary File 1. Finally, our set of 58 paired breast cancer and adjacent normal tissues as well as 10 FFPE normal breast tissues were also involved to compare the gene expression/protein levels in cancer vs. normal tissues as detailed in Supplementary File 5.

Table 1
Number of breast cancer specimens involved in the study including their ER status, tumor grade and lymph node status.

	Grade 1		Grade 3	
	Lymph node positive	Lymph node negative	Lymph node positive	Lymph node negative
Total	24	24	24	24
ER positive	24	24	12	12
ER negative	0	0	12	12

2.2. Sample preparation

Frozen breast cancer tissue (approx. 20 mm^3) was homogenized in 150 μl of the lysis buffer (6 M guanidine hydrochloride; 0.1 M Na-phosphate buffer, pH 6.6; 1% Triton X-100) in MM301 mechanic homogenizer (Retsch, Germany) using a metal ball for 2 × 2 min at 20 s^{-1}, needle-sonicated (Bandelin 2200 Ultrasonic homogenizer, Bandelin, Germany; 30 × 0.1 s pulses at 50 W) and kept on ice for 1 h. After centrifugation, protein concentration was measured in the supernatant using RC-DC protein assay (Bio-Rad, USA), a modified Lowry method [10] that includes protein precipitation to avoid interferences of reducing agents and detergents. The lysate (volume corresponding to 60 μg of total protein) was digested using modified filter aided sample preparation protocol. Briefly, aliquots of the lysate were mixed with 200 μl of 8 M urea in 0.5 M triethylammonium bicarbonate (TEAB), pH 8.5 on Vivacon 500 filter device, cut-off 10 K (Sartorius Stedim Biotech GmbH, Germany). The device was centrifuged at 14,000 × *g* at 20 °C for 20 min (all following centrifugation steps were performed applying the same conditions). Subsequently, 100 μl of 5 mM tris(2-carboxyethyl)phosphine in 8 M urea in 0.5 M TEAB, pH 8.5 was added to the filter, incubated at 37 °C for 60 min at 600 rpm and centrifuged. Next, 100 μl of 10 mM S-methyl methanethiosulfonate in 8 M urea in 0.5 M TEAB, pH 8.5 was added to the filter, incubated at 20 °C for 10 min and centrifuged. The resulting concentrate was diluted with 100 μl of 8 M urea in 0.5 M TEAB, pH 8.5 and centrifuged again; this washing step was repeated twice. The concentrate was subjected to proteolytic digestion by adding 100 μl of 0.5 M TEAB, pH 8.5 containing L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin (AB Sciex, USA) reconstituted in water (trypsin to protein weight ratio 1:30) and incubation at 37 °C for 16 h. The digests were collected by centrifugation into clean tubes and dried in a vacuum concentrator.

2.3. Labeling

For mTRAQ labeling, the evaporated digests were reconstituted in 24 μl of 0.5 M TEAB, pH 8.5 and two aliquots from each sample corresponding to 10 μg of digested protein were processed. One sample group was labeled with mTRAQ-Δ0 whereas the other sample group was labeled with mTRAQ-Δ8 label. Stock solution of each label was prepared by adding 50 μl of 2-propanol per one unit of the label as recommended by the manufacturer. For each labeling reaction, 0.15 unit was added to 10 μg of digested protein (the label-to-protein ratio was optimized and found similar to the ratio published by Holzmann et al. [11]), pH of the mixture was ~8.3 (no pH adjustment). After 1 h incubation at RT, the labeling was stopped by adding 15 μl of water. Samples labeled with mTRAQ-Δ8 were pooled to create global internal standard, which