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Transgelin is upregulated in stromal cells of lymph node positive breast cancer

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ABSTRACT

Transgelin and transgelin-2 have been discussed as potential markers of various cancers. Here we identified increased transgelin level in lymph node positive *vs.* negative, low grade primary breast cancer tissues using 2-DE in the cohort of 12 patients. We further clinically validated 2-DE results in an independent cohort of 48 low grade breast cancer patients through untargeted and targeted proteomics analysis (iTRAQ-2D-LC–MS/MS, mTRAQ-SRM), at transcript level and using immunohistochemistry. Another group of 48 high grade tumors of different breast cancer subtypes was analyzed together with the low grade samples to test transgelin specificity for low grade tumors and to study transgelin relation to known molecular markers and histological features. The results confirmed transgelin connection with the lymph node metastasis. As a marker of a reactive tumor stroma, transgelin can be connected with the higher risk of metastasis development. Moreover, we observed significant down-regulation of transgelin in high *vs.* low grade tumors caused by decreased content of stromal cells (mainly expressing transgelin) in high grade tumor tissue. We also analyzed expression of transgelin-2 in the second cohort using proteomics and immunohistochemistry. Transgelin-2 was mainly expressed by epithelial cancer cells and its levels were increased in metastatic and poorly differentiated tumors.

Biological significance: Both transgelin and transgelin-2 have been previously described as potential markers of many types of cancer. We are specifying this connection to metastatic affection of lymph nodes and cell differentiation in breast cancer. In the wider context, the results of our study highlight tumor stroma as a source of cancer biomarkers and point out how measured levels of tissue markers can actually reflect cellular feature of cancer mass.

1. Introduction

Metastasis is the most life-threatening event in cancer development. Lymph node status is an important prognostic factor of metastasis formation and lymph node negative breast cancer patients have generally favorable prognosis. However, approximately 30% of lymph node negative patients of grade 1 suffer from metastatic disease in disagreement with the theoretical prognosis [1]. This is because reliable markers for precise assessment of metastatic disease are still missing. So far, only two markers of the urokinase-plasminogen activator system, urokinase plasminogen activator and plasminogen activator inhibitor 1 were recommended by the American Society for Clinical Oncology for the determination of prognosis in lymph node negative breast cancer [2]. The prognostic value of multiparameter gene expression analysis (Oncotype DX and MammaPrint) is still under evaluation for the same purpose [3].

Here, we aim to identify new targets related to lymph node metastasis in low grade breast cancer. We postulate that analysis of lymph node positive *vs*. negative low-grade tumors could reveal key changes involved specifically in lymph node metastasis, avoiding general characteristics of tumor aggressiveness present in more proliferative high grade cancers. Having analyzed 12 patients using 2-DE, we identified transgelin as a protein up-regulated in lymph node positive *vs.* lymph node negative samples. To clinically validate our 2-DE results and to further study transgelin specificity for low grade tumors and its relation to known breast cancer molecular markers, we first analyzed an independent proteomics dataset of 48 low grade and 48 high grade primary breast cancer samples differing in lymph node status obtained by iTRAQ-2DLC–MS/MS method (Supplemental file 4 in the paper by Bouchal et al. [4]). Since the data were supportive to 2-DE results, transgelin protein level was verified also by targeted mTRAQ-SRM proteomics. Moreover, transgelin gene expression was assessed at transcript level using quantitative real-time PCR (qPCR) and its cell-

Abbreviations: qPCR, quantitative Real-time PCR; IHC, immunohistochemistry; TMA, tissue microarray.

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type specific expression was studied using immunohistochemistry. Transgelin is highly homologous to transgelin-2. Both proteins are potential cancer markers and, interestingly, Rho et al. described their cell type specific expression in tumor stroma *vs.* neoplastic epithelial cells in lung cancer [5]. We thus included analysis of transgelin-2 expression into validation part of the study and compared the data with transgelin results.

2. Materials and methods

2.1. Study design

The study was designed to find markers of lymph node metastasis in low grade breast cancer. The pilot 2-DE study compared 6 lymph node positive with 6 lymph node negative low grade breast carcinomas (cohort I). The study identified transgelin as a potential marker of lymph node metastasis in low grade breast cancer. Clinical validity of transgelin was verified on the additional group of 48 low grade breast carcinomas (cohort II) with same characteristics as in the cohort I. The cohort II contained another group of 48 high grade tumors differing in lymph node status and belonging to different breast cancer subtypes. The reason to use the group of high grade samples was to describe whether transgelin up-regulation in lymph node positive samples is specific for low grade tumors and to suggest any other correlation of transgelin with known molecular markers and subtypes in breast cancer.

2.2. Tissue specimens

Breast cancer tissue specimens were obtained from the Masaryk Memorial Cancer Institute (MMCI), Brno, Czech Republic. Ethics committee of MMCI approved the study and all patients gave written informed consent. Tissues were frozen in liquid nitrogen within 20 min after surgical removal and stored at − 196 °C in a tissue bank of MMCI until sample preparation. Two different cohorts of patients were included as follows: Cohort I used in the 2-DE experiment consisted of 12 primary breast carcinomas, estrogen receptor positive, progesteron receptor positive, Her2/Neu negative, grade 1 (G1), luminal A subtype, including 6 lymph node negative (N0) and 6 lymph node positive (N1–2) cases.

Cohort II consisted of additional 96 primary breast carcinomas of 11–20 mm maximum diameter (pT1c). 48 low grade (G1) tumors with the same characteristics as in cohort I, 24 lymph node positive, 24 lymph node negative and 48 high grade (G3) tumors representing different molecular subtypes: luminal B HER2 negative (LB, N = 16), luminal B HER2 positive (LBH, N = 8), HER2 positive (HER, N = 8) and triple negative (TN, N = 16), always half of them with and half without metastasis (see Supplementary File 1 for details of cohort I and cohort II).

2.3. 2-DE

Fresh frozen tissues were lysed in buffer containing 7 M urea, 2 M thiourea, 1% (*w*/*v*) 3-(4-heptyl)phenyl-3-hydroxypropyldimethylammoniopropanesulfonate (C7BzO), 70 mM DTT, 5 mM NaF, 0.2 mM $NaVO_3$, Complete Mini (Roche) protease inhibitor cocktail (1 tablet/10 ml) and 1 mM PMSF. The samples were homogenized in a mechanical homogenizer (Retsch, Haan, Germany) using a metal ball for 2×2 min at 25 s^{-1} and then using needle sonication (Bandelin 2200 Ultrasonic homogenizer, Bandelin, Germany, 30×0.1 s pulses at 50 W). The lysates were incubated for 1.5 h, the last 15 min with addition of 58 U benzonase at room temperature. After adding of 4 μl of Pharmalyte 3–10, homogenates were centrifuged at 16 000 g at 4 °C for 20 min. Protein concentration in supernatants was measured by the RC-DC Protein Assay (Bio-Rad, USA).

250 μg/350 μg (analytical/micropreparative 2-DE) of protein in 2% Pharmalyte 3–10 was supplemented by rehydration solution (7 M urea, 2 M thiourea, 1% C7BzO, 70 mM DTT, bromophenol blue) to the final volume of 350 μl. The whole volume was applied to IPG Ready Strips (pH 3–10 NL, 18 cm, Bio-Rad, USA). After rehydration for 14 h, proteins were separated by isoelectric focusing at a maximum voltage 8000 V and a maximum current 50 μA/strip for 100 kVh. Equilibration of the IPGs, SDS-PAGE in the 2nd dimension and protein staining was performed as described previously [6]. The stained gels were scanned by GS-800 densitometer or Pharos FX Plus fluoroimager and analyzed by PDQUEST software version 8.0 (all Bio-Rad, USA). Top four protein spots with the highest fold change of spot integral optical density between the lymph node positive (N = 6) and lymph node negative (N = 6) tissues were selected for MS identification, statistical significance was determined by Mann–Whitney U-test. The MS was done as described previously [7] with the search against NCBInr database (release 2008_03).

2.4. Reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was isolated using the RNeasy mini kit (Qiagen, Germany) following the manufacturer's protocol. Isolated RNA was quantified at 260 nm using NanoDrop ND-1000 (Thermo Fisher Scientific, USA) and its quality was checked by RNA integrity number (RIN) measurement on Agilent 2010 Bioanalyzer (Agilent, USA). cDNA was synthesized using H Minus M-MuLV Reverse Transcriptase and random hexamer primers (both Fermentas Life Sciences, Canada) according to the manufacturer's protocol.

The mRNA levels were measured by qPCR using 7900HT Fast system (Applied Biosystems, USA). Each sample was assayed in triplicates using SYBR Green for transgelin and TaqMan for 18S rRNA as endogenous control (both Applied Biosystems, USA). Sequences of the primer pairs for transgelin were as follows: Forward: 5′-CAAGAATGATGGGCACTACCGT-3′ and reverse: 5′-TCTGCCGAGGTCGTCCGTA-3′. PCR reaction was performed in a total volume of 20 μl including 1 μl of cDNA and 0.5 μM primers. Absolute mRNA quantity was determined using the standard curve method. The calibration curve was made of 10-fold serial dilutions of standard for each analyzed gene and was constructed for each plate separately. The standard for transgelin was cDNA-carrying plasmid (calibration samples contained 10^6 to 10^3 cDNA copies). The standard for 18S rRNA was a mixture of the same volume of all 96 analyzed samples (calibration samples were 1–10^3 times diluted standard). Statistical analysis was performed using STATISTICA 7.1 software (StatSoft, USA) and Mann–Whitney U-test was used to determine statistical significance of expression changes.

2.5. ITRAQ-2DLC–MS/MS

The relative protein levels of transgelin and transgelin-2 in groups within cohort II were obtained by data analysis of previously published dataset acquired by iTRAQ-2DLC–MS/MS technique (Supplementary File 4 in [4]).

2.6. Targeted proteomics-selected reaction monitoring

The samples were homogenized similarly as described above for the 2-DE experiment with the following modifications: (i) lysis buffer composition was 6 M guanidium chloride, 0.1 M Naphospate buffer pH 6.6, 1% Triton X-100, (ii) incubation time was 1 h. Protein concentration was measured by RC-DC protein assay (Bio-Rad, USA). The S–S bridges in 60 μg of total protein lysate were reduced by 5 mM tris(2-carboxyethyl)phosphine and −SH groups were blocked by 10 mM S-methylmethanethiosulfonate, both in 8 M urea and 0.5 M triethylammonium bicarbonate pH 8.5 prior to protein digestion with trypsin (37 °C for 16 h, trypsin-to-protein ratio 1:30) using filter aided sample preparation [8]. Peptides were then labeled with mTRAQ labels as follows: Half