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Putative circulating markers of the early and advanced stages of breast cancer identified by high-resolution label-free proteomics

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ABSTRACT

This study evaluated the plasmatic proteomic profile of breast cancer patients in the early (ED) and advanced (AD) stages, employing high-throughput proteomics. We identified 92 differentially expressed proteins in ED and 73 proteins in AD patients. Gelsolin, lumican, clusterin, SALL4 and PMS2, as well hTERT, TNF-α and GRHL3 were chosen for further investigation. ED presented augmented expression of GRHL3 and reduced circulating TNF-α with high expression of GRHL3 in tumors. AD displayed high TNF-α and a significant expression of PMS2 in tumors. These findings suggest processes enrolling stem cell division in ED, with TNF-α signaling and DNA mismatch repair in the advanced stage.

1. Introduction

Ductal carcinoma is a major histological breast cancer subtype representing approximately 70% of all diagnosed cases [1]. Since most breast cancer deaths are caused by disease evolution to distant metastases [2], breast tumors must have specific biological capabilities to ensure successful development from early to metastatic stages [3]. These capabilities include differential oxidative metabolic status [4], escape from immune surveillance [5], dissemination capability [6], and preparation of the pre-metastatic niche by exosome delivery [7]. In this context, a systemic cancer progression overview has been recently proposed based on independent progression of metastasis arising from early-disseminated tumor cells [2]. However, a major challenge that remains in translational research is to understand how breast cancer metastasizes by identification of circulating tumor-derived proteins. To this end, a proteomics strategy provides a powerful tool to investigate potential biomarkers in several types of cancers due to its high sensitivity, precise characterization of protein interactions, and detection of post-translational modifications [8].

Several studies have employed proteomic strategies in cancer to identify serological indicators of disease [9–11] based on a shotgun proteomics approach. Recently, the proteomic characterization of breast cancer progression by Geiger et al. [12] has provided a system-wide analysis of breast cell lineages that resulted in a stage-specific signature associated with estrogen negative cells. Although high throughput technologies have improved our understanding of breast cancer, little progress has been made regarding circulating indicators of disease, particularly in human disease. Thus, reliable circulating markers of different human breast cancer stages are still missing, and according to ASCO guidelines, present data regarding tumor circulating markers are insufficient to define the progress of breast cancer stages by blood analysis [13].

Here, we sought to identify circulating proteins from different disease stages in breast cancer patients with an innovative strategy employing high sensitivity label-free proteomics. The MS-based method for the quantification of proteins termed data-independent analysis (or MS^E) has been chosen based on its applicability to global proteomic profiling in several studies. This method includes both analysis of preclinical models and clinical studies [14,15], producing reliable and accurate quantification of proteins in various background matrices with high peptide and sequence coverage [16]. To achieve this goal, plasma from breast cancer patients was analyzed employing qualitative and quantitative bidimensional

Abbreviations: ED, early disease; AD, advanced disease; TERT, telomerase reverse transcriptase; TNF, α-tumor necrosis factor alpha; GRHL3, grainy head-like 3; ASCO, American Society of Clinical Oncology; MS, mass spectrometry; REMARK, Reporting Recommendations for Tumor Marker Prognostic Studies; TNM, tumor node metastasis classification; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TFA, trifluoracetic acid; ADH, alcohol dehydrogenase; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins.

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nanoUPLC tandem nanoESI–MS^E mass spectrometry to identify differentially expressed proteins between early and advanced breast cancer stages. Further investigations were also conducted identifying the expression levels of these circulating markers in tumor samples and evaluating plasmatic levels of several proteins involved in a common network identified by in silico interaction analysis.

2. Methods

2.1. Study patients

A total of 200 women were enrolled in this prospective study based on a series of 65 healthy controls and 135 patients diagnosed with unilateral primary ductal infiltrative carcinoma of the breast from March 2009 to December 2010 at Londrina Cancer Institute, Londrina, Paraná, Brazil. The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria [17] were followed regarding patient selection, assay performance, and data analysis throughout this study. The institutional board previously approved this study, and all participants signed informed consent forms. This study was designed and conducted in accordance with the ethical principles for medical research involving human subjects from the Declaration of Helsinki.

A schematic representation of patient selection and study design is shown in Fig. 1. Breast cancer patients diagnosed with primary unilateral infiltrative carcinoma were included in this study ($n = 62$) and stratified into two cohorts according to disease staging: early disease patients (ED group, TNM staging IIa/IIb, $n = 28$) and advanced disease patients (AD group, TNM staging IIIc/IV, $n = 34$). To further validate the findings, an additional cohort of 73 patients was also studied (33 ED patients and 40 AD patients). ED patients were characterized by a slower tumor progression and locoregional disease, while AD patients presented with an aggressive disease profile and distant metastases. Clinicopathological data from patients were collected from medical records and included age at diagnosis, weight, height, clinical and family history, menopausal status, comorbidities, TNM staging, tumor histological type, histological tumor grade, lymph nodal status, tumor size, presence of distant metastases, systemic treatment, death occurrence, and tumor molecular status to estrogen receptor (ER), progesterone receptor (PR), and human epidermal receptor 2 (HER-2/neu).

All included cancer patients received standard surgical treatment based on radical mastectomy or breast-conserving surgery, and sample collecting was performed before the first chemotherapeutic regimen began (paclitaxel 175 mg/m^2, 1-h infusion or doxorubicin 60 mg/m^2, 1-h infusion, according to standard institutional protocols). The control group ($n = 65$) consisted of healthy women, age-matched with cancer patients (ranging from 32 to 79 years) without previous history of any type of cancer, chemotherapy, hormonal therapy, and chronic diseases. Current smoking, previous or current hepatic, cardiac, or renal dysfunction, obesity, use of drugs, hypertension, diabetes, or other chronic conditions were considered exclusion criteria for all groups.

2.2. Sample collection and processing

Plasma samples were obtained from a 20 mL whole blood sample collected in sodium EDTA tubes that had been centrifuged for 5 min at 1400g at 4 °C. Proteomics analysis was performed on each group using the pooled plasma samples (500 μL sample from each sample). Protein concentrations of cleared supernatants were determined using the Bradford method. Samples were concentrated 16× and exchanged with 50 mM ammonium bicarbonate using a 3-kD ultra-filtration device (Millipore). Proteins were then denatured (0.1% RapiGEST at 60 °C for 15 min) (Waters, Milford, USA), reduced (10 mM dithiothreitol at 60 °C for 30 min), alkylated (10 mM iodoacetamide for 30 min at room temperature in the dark), and enzymatically digested with trypsin at a 1:50 (w/w) enzyme to protein ratio. Digestion was terminated by the addition of 10 L of 5% trifluoroacetic acid (TFA), which also destroyed RapiGest in the incubation. Yeast alcohol dehydrogenase (ADH; P00330) was added to the digests (200 fmol μL^{-1}) as an internal standard for absolute quantitation.

Tumor samples of the same patients evaluated in plasmatic analysis were evaluated by a pathologist to diagnostic stratification and histopathological processing to posterior immunohistochemical analysis.

2.3. 2D-nanoUPLC tandem nanoESI–MS^E (MudPIT) conditions

Qualitative and quantitative bidimensional nanoUPLC tandem nanoESI–MS^E experiments were conducted using a 1.5-h reversed phase gradient from 5% to 40% (v/v) acetonitrile (0.1% v/v formic acid) at 600 nL min^{-1} on a nanoACQUITY UPLC core system. A nanoACQUITY UPLC C18 BEH 1.7 μm, 100 μm × 10 cm column was used in conjunction with an SCX (Strong Cation Exchange) 5 μm, 180 μm × 23 mm column. Typical on-column sample loads were 2 μg of total protein digests, as previously described [16]. All analyses were performed using electrospray ionization in the positive ion mode ESI(+) and a NanoLockSpray source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with a GFP solution (200 fmol μL^{-1}) delivered through the reference sprayer

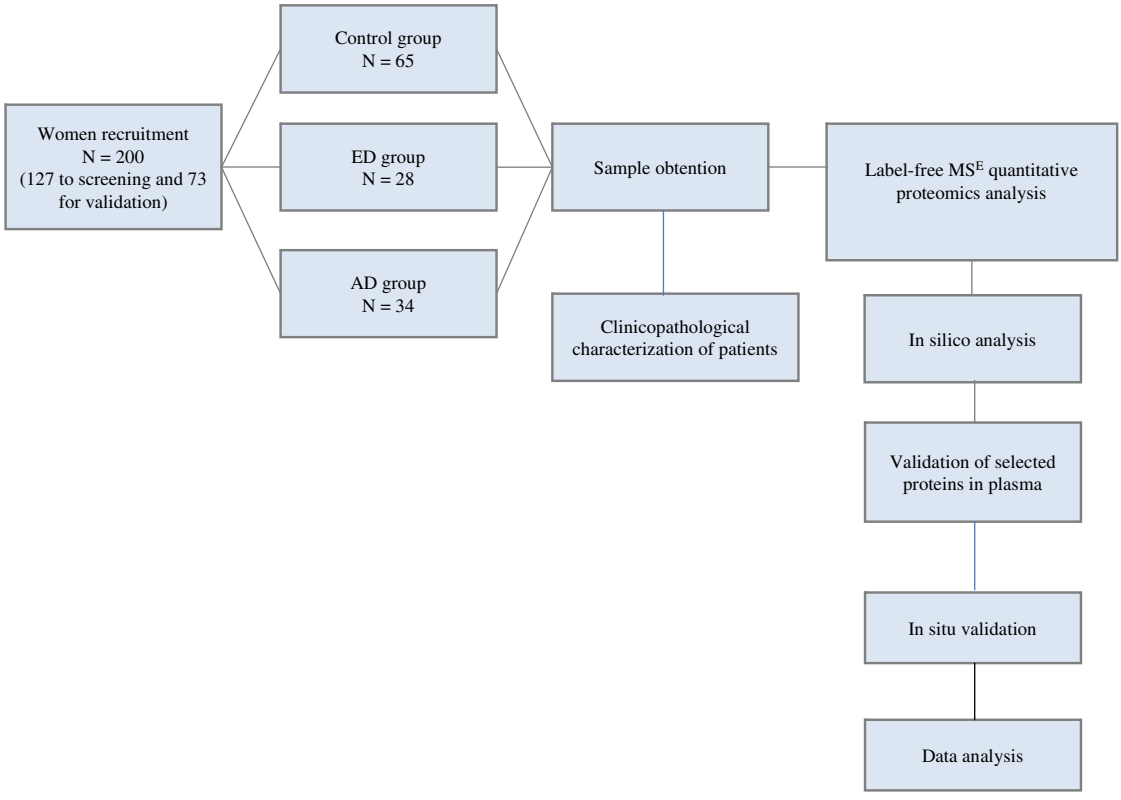

Fig. 1. Design of the study. ED = patients diagnosed with infiltrative ductal carcinoma of the breast in stages I–IIb, AD = patients diagnosed with infiltrative ductal carcinoma of the breast in stages IIIc–IV, MudPIT = qualitative and quantitative bidimensional nanoUPLC tandem nanoESI–MS^E mass spectrometry. ED = patients diagnosed with infiltrative ductal carcinoma of the breast in stage II, AD = patients diagnosed with infiltrative ductal carcinoma of the breast in stage IV.

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