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A mass spectrometry-based plasma protein panel targeting the tumor microenvironment in patients with breast cancer☆

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ABSTRACT

Proteins secreted or shed by cancerous cells are seen as a rich source of biomarkers and novel therapeutic targets. Recently, the importance of the tumor microenvironment, which comprises the surrounding non-tumor cells, has received increased attention for its role in tumor progression. We developed a targeted proteomics assay to monitor a panel of plasma proteins postulated to be present in the tumor microenvironment. The plasma of 76 breast cancer patients was depleted of abundant circulating proteins, enzymatically digested and labeled by reductive methylation. The labeled digests were analyzed by tandem mass spectrometry using a multiple reaction monitoring acquisition method. The protein targets were correlated with the tumor characteristics, the extent of the disease and the clinical staging of the patients. Linear discriminant analysis revealed that infiltrating ductal and invasive mammary breast carcinomas could be grouped based on distinctive peptide levels of fibronectin, clusterin, gelsolin and α-1-microglobulin/Inter-α-trypsin inhibitor light chain precursor (AMBP). These proteins have been previously associated with breast cancer at the tissue level, however, this is the first study to measure plasma levels of these proteins and correlate these levels with clinical features. Significant variability was seen between unique peptides belonging to the same protein. This article is part of a Special Issue entitled: Proteomics from protein structures to clinical applications (CNPN 2012).

1. Introduction

In breast cancer, tissue biopsies play a critical role in determining treatment. These biopsies are typically analyzed by immunohistochemistry to determine the progesterone (PR), estrogen (ER) and HER2/neu receptor status. These markers are both predictive, and prognostic and are crucial to choosing the most appropriate treatment. HER2/neu measurement is a recent addition arising from the development of the targeted therapy Herceptin™, a monoclonal antibody that binds to HER2/neu [1,2]. Other accepted markers include the urokinase-type plasminogen activator (uPA) and the Oncotype DX multiparameter gene expression assay [3]. The use of the biomarkers and their associated treatments has improved outcomes for many breast cancer patients; however, these markers are not expressed in all breast subtypes. For example, the triple negative subtype is an aggressive form characterized by an absence of ER, PR and HER2/neu expression [4]. In addition, these tumor markers do not provide information on the role of the non-tumor components of the microenvironment such as the endothelium, extracellular matrix and cells of the immune system. Therefore, there is a need for the development of novel markers that more completely characterize the tumor and the surrounding microenvironment. In addition, as more therapeutics are being developed

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towards targets in the tumor microenvironment, including a large number of monoclonal antibodies, the need for highly multiplexed assays required to guide treatment decisions will certainly increase.

A significant amount of research has focused on exploring blood as an alternative to tissue biopsies. The rational is that the tumor and the surrounding tissues secrete characteristic proteins into the bloodstream. Furthermore, blood is readily accessible and can be obtained at low cost. These practical considerations make blood-based assays suitable for large-scale, multiplexed assays. Also, unlike tumor biopsies, which are typically collected only once, blood can be sampled frequently in order to monitor response to treatments and, perhaps, modify treatment before disease progression. Different experimental approaches have been tested for blood proteomics in breast cancer. For example, surface enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF) has been used to characterize plasma protein profiles in breast cancer patients at different clinical stages [5,6]. This simple approach aims to identify spectral fingerprints for prognosis and diagnosis but is limited by poor sensitivity and its inability to provide conclusive protein identification. The latter can be achieved using traditional gel-based separation combined with MALDI-TOF [7] or MALDI-TOF/TOF [8] analysis; however, the labor intensive sample preparation steps preclude the use of these techniques for large sample sets. Other research groups have used stable-isotope coded reagents to do comparative studies between healthy control and breast cancer groups [9]. A comprehensive review covering the current applications and challenges of blood proteomics in breast cancer research has been recently published [10]. In spite of the numerous publications in this area, progress is slow, judging by the few protein biomarkers approved or cleared by regulatory agencies in the past decades [11].

The purpose of this study is to determine the correlation between the levels of blood proteins and traditional tumor characteristics. The major difference in our study is the inclusion of protein targets from the tumor microenvironment in addition to tumor-specific, surface and secreted cell proteins. Specifically, we selected our targets from studies performed by laser capture microdisection of breast cancer vasculature tissue [12] and bioinformatics studies of cell surface and secreted proteins identified from public transcriptomics data and metastasis network modules [13]. These proteins, derived from fibroblasts, immune cells, blood vessel or the extracellular matrix immediately surrounding tumor cells, have been shown to play a key role in regulating tumor growth, development and metastasis [14–16]. For our study, blood samples from 76 patients with breast cancer were depleted of abundant circulating proteins, enzymatically digested and labeled by reductive methylation. The labeled digests were analyzed by LC–MS/MS using a multiple reaction monitoring (MRM) acquisition method. The data obtained from the samples was compared to the histological and clinical data using linear discriminant analysis (LDA). The results of the statistical analysis revealed that certain protein targets had significant predictive trends, especially for grouping patients with infiltrating ductal and invasive mammary types of breast cancer. Interestingly, there were significant differences in behavior of unique peptides from the same protein. This intraprotein variability could be due to differential post translational processing of the proteins.

2. Materials and methods

2.1. Cell culture

MDA-MB-231 cells (ATCC HTB-26, Manassas, VA) were grown in DMEM (Gibco 11995) supplemented with 10% fetal bovine serum (Gibco 12484). Cells were cultured at 37 °C and 5% CO_2 until reaching 80% confluence. The cell monolayer was rinsed twice with PBS (Gibco 14190) and harvested by scraping into PBS. Cells were pelleted at 380 ×*g*, washed twice with PBS and stored at −20 °C as a dry pellet.

Cells were resuspended in 50 mM triethylammonium bicarbonate (TEAB, Sigma T7408) pH 8.0 with 100 mM NaCl and protease inhibitor (Set III, Calbiochem 539134) and lysed by probe sonication, 3×10 second cycles at power setting 2 (Fisher Sonic Dismembrator, model 100). The lysate was centrifuged at 8000 ×*g* to remove debris, then centrifuged at 110,000 ×*g* for 45 min to pellet total membranes. Cytosolic fraction was transferred to a separate tube and the total membrane pellet was rinsed with TEAB. Membrane pellet was resuspended in 50 mM TEAB pH 8.0 with 0.1% Rapigest (Waters 186001861) using probe sonication, 3×10 second cycles.

Protein concentrations of the cytosolic and membrane fractions were determined using the Bradford assay (Bio-Rad 500-0006). 1 mg of each fraction was reduced with 5 mM DTT (Sigma D9163) at 60 °C for 30 min and alkylated with 15 mM iodoacetamide (Sigma I6125) for 30 min at room temperature. Samples were then digested overnight, 37 °C, with trypsin (Promega V5113) at a 50:1 protein to trypsin ratio. The resulting peptides were acidified to pH<3 with TFA (Sigma 299537), filtered through 0.45 μm filter to remove hydrolyzed Rapigest detergent and finally desalted using SPE cartridges (Waters HLB 186000383).

2.2. Protein standards

AXL long isoform of tyrosine-protein kinase receptor (UFO), Isoform 1 of Disintegrin and metalloproteinase domain-containing protein 9 (ADAM9), Carboxypeptidase A4 (CPA4), Isoform 1 of ICOS ligand (ICOSLG), Microfibril-associated glycoprotein 4 (MFAP4), Isoform 1 of Periostin (POSTN) and Semaphorin-7 (SEMA7A) were expressed in HEK293 in serum-free medium with a 6xHis tag and purified by IMAC as previously published [17,18]. Purified proteins were resuspended in 50 mM TEAB pH 8.0, reduced in 5 mM DTT for 30 min at 60 °C, alkylated in 15 mM iodoacetamide for 30 min at room temperature and digested overnight with trypsin (50:1 protein to trypsin ratio).

The resulting peptides were labeled by reductive methylation of primary amino groups (n-term and K). An excess of formaldehyde, 10 μL of 7 M formaldehyde, was added to each sample and incubated for 5 min at room temperature. Then, 16.7 μL of 6 M $NaCNBH_3$ was added to each digest and incubated for 2 h at room temperature. Samples were desalted using SPE cartridges (Waters HLB 1860003831) and reconstituted in 5% acetonitrile 0.1% formic acid.

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