Cancer Letters 343 (2014) 33–41

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

A serum nuclear magnetic resonance-based metabolomic signature of advanced metastatic human breast cancer

Elodie Jobard [a,b], Clément Pontoizeau [a], Benjamin J. Blaise [a], Thomas Bachelot [b], Bénédicte Elena-Herrmann [a,*], Olivier Trédan [b,*]

[a] Université de Lyon, CNRS/ENS Lyon/UCB-Lyon-1, Institut des Sciences Analytiques, Centre de RMN à Très Hauts Champs, 5 rue de la Doua, 69100 Villeurbanne, France
[b] Université de Lyon, Centre Léon Bérard, Département d'Oncologie Médicale, 28 rue Laennec, 69008 Lyon, France

ARTICLE INFO

Article history:
Received 3 July 2012
Received in revised form 3 September 2013
Accepted 9 September 2013

Keywords:
Breast cancer
Metabonomics
Metabolomics
NMR
Serum
Metastatic

ABSTRACT

Breast cancer (BC) displays a high heterogeneity from histology to prognosis, metastatic evolution and treatment responses. We report here a ^{1}H NMR-based metabolic phenotyping study aiming at identifying coordinated metabolic serum changes associated with advanced metastatic breast cancer (MBC) in comparison to the localized early disease (EBC). A model discriminating EBC and MBC patients is obtained (n = 85: 46 EBC and 39 MBC), and validated with an independent cohort (n = 112: 61 EBC and 51 MBC; 89.8% sensitivity, 79.3% specificity). We identify 9 statistically significant metabolites involved in this discrimination: histidine, acetoacetate, glycerol, pyruvate, glycoproteins (N-acetyl), mannose, glutamate and phenylalanine. This work illustrates the strong potential of NMR metabolic phenotyping for the diagnosis, prognosis, and management of cancer patients.

1. Introduction

Breast cancer (BC) incidence has slightly but steadily increased over the past three decades while its mortality rate has declined over the same period, thanks to advances in screening methods, early diagnosis and treatments. BC is the most common cancer and the leading cause of cancer death in women worldwide [1]. Breast cancer displays a high heterogeneity in terms of etiology and histology as well as prognosis, metastatic evolution and response to treatments. Deciphering the molecular basis of such heterogeneity is a major challenge, now achievable through new biomolecular and analytical techniques, aiming at a comprehensive cancer characterization for risk stratification, therapeutic target identification and appropriate treatment selection [2].

DNA microarray-based technology has provided researchers with a tool to perform comprehensive genetic profiling of breast cancer, and therefore to identify gene expression signatures that can help to predict breast cancer recurrences. For instance, the 21-gene Recurrence Score assay (Oncotype DX) predicts the 10-year risk of distant recurrence and survival for patients treated in the adjuvant setting [3,4]. Amsterdam signature (Mammaprint) [5] and EndoPredict signature [6] also offer independent prognostic information beyond what can be achieved with all common clinical parameters. However, the challenge remains to evaluate the relative contributions of multiple levels of data (clinical, pathological, genomics and proteomics) in predicting breast cancer outcome and response to anticancer treatments.

Specific changes in metabolism during tumor progression have also early been reported by Warburg [7] and appear as keystone for understanding cancer evolution or response to drug treatment [8,9]. Yet, detailed characterization of BC tumor metabolism as well as broader investigations of the global alterations to individual metabolism still represent a tremendous challenge.

Metabolic phenotyping studies that provide untargeted identification of all detectable low molecular-weight molecules by profiling without any *a priori* the metabolic signatures of biological samples in connection to patho/physiological events [10,11], are prone to play a key role towards this objective. Versatile analytical techniques mainly based on Nuclear Magnetic Resonance spectroscopy (NMR) and Mass Spectrometry (MS) allow the analysis of various samples from bio-fluids such as serum or urine, and exhaled breath condensate, to intact cells or tissues [12–15]. Meanwhile, metabolomic approaches have already found promising applications in different fields from toxicology [16], functional genomics [11,17], to oncology [18,19].

* Corresponding authors. Addresses: Centre de RMN à Très Hauts Champs, 5 rue de la Doua, 69100 Villeurbanne, France. Tel.: +33 (0)426233880; fax: +33 (0)478896761 (B. Elena-Herrmann). Centre Léon Bérard, 28 rue Laennec, 69008 Lyon, France. Tel.: +33 (0)478782644; fax: +33 (0)478782727 (O. Trédan).
E-mail addresses: benedicte.elena@ens-lyon.fr (B. Elena-Herrmann), olivier.tredan@lyon.unicancer.fr (O. Trédan).

0304-3835/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canlet.2013.09.011

In BC research, metabolomics has been so far generally used for the direct characterization of tumor metabolism alterations, mainly through analysis of intact biopsies by high-resolution magic angle spinning (HR-MAS) NMR spectroscopy. Specific tumor metabolic profiles have been identified by comparison of control and tumor tissues [20–23], and correlation between metabolic tumor profiles and histological grade, hormone grade and axillary lymphatic spread were also observed [23,24], highlighting the potential of tumor metabolic signatures as prognosis factors. Recently work also showed the possibility to refine tumor gene-expression-based classification from tumor metabolic profiles, emphasizing the synergic use of transcriptomic and metabolomic approaches [25,26].

Changes in global metabolism of individuals have also been identified in BC from biofluids such as urine and serum, most efforts being dedicated to the research of early non-invasive diagnosis biomarkers [27–29]. One recent attempt was made so far to detect micrometastatic disease in early breast cancer patients from metabolomic analysis of peripheral blood serum [30]. We report here a ^{1}H-NMR-based metabolomic study aiming at deciphering metabolic serum changes associated with advanced metastatic breast cancer by comparison to the localized disease.

2. Materials and methods

2.1. Populations

This observational study was conducted in the Centre Léon Bérard, (Lyon, France). A cohort of female patients constituted of two subgroups was recruited from May 2005 to January 2012, including patients suffering from early breast cancer (EBC; Group A) or metastatic breast cancer (MBC; Group B). Inclusion criteria for group A were: patients older than 18, with histologically proven EBC, who underwent a negative CT scan of the chest, abdomen, and pelvis, a negative whole body bone scan and were scheduled for a surgical procedure at our institution. Inclusion criteria for the group B were: patients older than 18 with histologically proven MBC, eligible for a first line of chemotherapy; previous hormonal treatment was allowed. Diabetic patients were excluded due to the high concentration of glucose in their blood. For each patient, recorded clinical data included age, menopausal status, hormonal and HER2 receptor status, medical history and treatments, molecular subtype, SBR (Scarff Bloom and Richardson) grade and tumor histological type. A first cohort of 85 patients, named the *training cohort*, was recruited to derive the statistical model, and a second independent cohort of 112 individuals, named the *validation cohort*, was used for model validation. Written informed consent was obtained from each patient. The institutional ethics committee approved the study protocol before implementation.

2.2. Data collection and storage

Blood collection was performed in fasting conditions (12 h without food intake) for each patient, in the morning before breast surgery for the group A, or in the morning before first chemotherapy cure for the group B. Blood samples were recovered from dry tubes and centrifuged 30 min after collection at 800 g for 10 min. Sera were stored in cryo-plastic straws at −80 °C before acquisition of the NMR data.

2.3. Sample preparation

Samples were prepared as previously described by Beckonert et al. [5,31]. Serum samples were thawed at room temperature before use. 200 μL of each was diluted with 400 μL of a 0.9% saline solution (NaCl 0.9% wt./vol, D_2O 10% vol/vol) in a microtube, then centrifuged for 5 min at 4 °C at 12,000 g. Finally, 550 μL of supernatant was transferred into 5 mm NMR tubes. Samples were kept at 4 °C until analysis.

2.4. ^{1}H-NMR spectroscopy of serum samples

All NMR experiments were carried out on a Bruker Avance III spectrometer operating at 800.14 MHz (proton resonance frequency) equipped with a 5 mm TXI probe, and high-throughput sample changer that maintained the samples temperature at 4 °C until actual NMR acquisition. The temperature was then regulated at 300 K throughout the NMR experiments. Standard ^{1}H 1D NMR pulse sequences, NOESY and CPMG with water presaturation, were applied on each sample to obtain corresponding metabolic profiles. 128 transient free induction decays (FID) were collected for each experiment with a spectral width of 20 ppm. For both sequences, the relaxation delay was set to 2 s. The NOESY mixing time was set to 100 ms and the CPMG spin-echo delay to 300 μs allowing an efficient attenuation of the lipid NMR signals. The 90° pulse length was automatically calibrated for each sample at around 9.25 μs. In addition, 2D NMR experiments (^{1}H—^{13}C HSQC, ^{1}H—^{1}H TOCSY and J-Resolved) were recorded on a subset of samples to achieve structural assignment of the metabolic signals.

2.5. Data processing

All FIDs were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening factor, prior Fourier transformation. ^{1}H-NMR spectra were automatically phased and referenced to the α-glucose anomeric proton signal (δ = 5.23 ppm) using Topspin 2.1 (Bruker GmbH, Rheinstetten, Germany). Residual water signal (4.66–5.11 ppm), and polyethylene Glycol signal at 3.70 ppm, a contamination from sample storage containers, was excluded. Spectra were divided into 0.001 ppm-wide buckets over the chemical shift range [0.5; 9 ppm] using the AMIX software (Bruker GmbH). Spectra were normalized to their total intensity and mean-centered prior to analysis. Spectra were aligned using the module Icoshift [6,32] in Matlab (The Mathworks Inc., Natick, MA). Data were then exported to SIMCA-P 13 (Umetrics, Umea, Sweden) for statistical analysis.

2.6. Population characterization

Descriptive statistical analysis was performed to characterize the two populations, using the Student *t*-test and Chi^2 test for quantitative and qualitative data respectively. The significance threshold was set to 0.05 for both tests.

2.7. Multivariate analysis of serum metabolic profiles

Unsupervised and supervised statistical multivariate methods were used to build models for sample classification and extract group-specific metabolic signatures. Principal component analysis (PCA) was performed to derive the main sources of variance within the dataset, check population homogeneity and eventually identify technical or biological outliers. Data were visualized as score plots, where each point stands for the projection of a single sample on the main principal components and as loading plots, which represent the contribution of the metabolic variables to principal components. Supervised regression methods such as Orthogonal Partial Least-Squares (O-PLS) [33] were performed to build a robust sample classification model and derive group-specific metabolic phenotypes. The O-PLS analysis was run to discriminate populations by regressing a Supplementary data matrix **Y**, containing information about the disease severity (EBC or MBC status) on