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Quantitative assessment of human serum transferrin receptor in breast cancer patients pre- and post-chemotherapy using peptide immunoaffinity enrichment coupled with targeted proteomics

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ABSTRACT

Background: sTfR, a soluble form of transferrin receptor in serum, has been suggested as an indicator of bone marrow failure in breast cancer patients receiving chemotherapy. However, intensive chemotherapy could also cause a reduction of sTfR to a level below the LOQ of most assays.
Methods: An advanced liquid chromatography-tandem mass spectrometry (LC-MS/MS)–based targeted proteomics assay coupled with peptide immunoaffinity enrichment (SISCAPA) was developed and validated for monitoring sTfR.
Results: Tryptic peptide 681VEYHFLSPYVSPK693 was selected as a surrogate analyte for quantification. High-abundant proteins were first removed from serum, followed by SISCAPA that was effective in surrogate peptide enrichment and sensitivity enhancement. The resulting LOQ can achieve 100 ng/ml (~10-fold increase). Then, sTfR levels in breast cancer patients pre- and post-chemotherapy, and healthy volunteers were accurately quantified as 1.77 ± 0.53 μg/ml, 0.98 ± 0.26 μg/ml and 1.66 ± 0.50 μg/ml, respectively, using a standard addition method. While there was no evidence for a difference between patients and healthy volunteers, differential levels of sTfR pre- and post-chemotherapy were obtained. Comparison between SISCAPA-targeted proteomics and ELISA indicated that the former approach provided a lower value of sTfR.
Conclusions: SISCAPA-targeted proteomics may allow the quantification of low-abundant proteins in a more accurate manner.

1. Introduction

Human transferrin receptor (TfR) is a transmembrane dimeric glycoprotein composed of 2 identical 95 kDa subunits, linked by disulphide bonds. As a result of externalization of TfR during the endocytic cycle, a soluble form of TfR (sTfR) consisting of 101–760 amino acid residues of TfR can be detected in serum. Although sTfR is frequently used to identify iron deficiency anaemia, especially in the context of inflammation [1], a few studies have reported its elevation in a variety of cancers, including breast cancer [2,3]. Moreover, a reduction of sTfR at a value much greater than the previous increase has been observed in patients receiving chemotherapy [4]. Specifically, chemotherapy can induce a state of temporary bone marrow aplasia and erythropoiesis suppression, during which the patient must be supported with blood product transfusions [5]. Therefore, quantitative assessment of sTfR in clinical practice is important to indicate the degree of bone marrow failure and transfusion requirements.

In recent years, several commercial tests have become available for the determination of sTfR. Most of them are immunoassays based on antibodies against sTfR. While these tests provide valuable information on protein levels and can be extremely sensitive, they often lack specificity and reproducibility [6]. In addition, antibody-based methods are not easy to scale up and are labor-intensive [7]. Furthermore, due to a lack of standardization, results obtained from different tests cannot be compared to each other with respect to both reference and pathological ranges. Because of these issues, liquid chromatography coupled online to tandem mass spectrometry (LC-MS/MS)–based targeted proteomics has been developed and applied as an alternative technique.

There has been a growing interest in targeted proteomics where a limited number of proteins are pre-selected and quantified [8,9]. In this approach, proteins are digested to component peptides using an enzyme such as trypsin. One or more selected proteolytic peptides whose sequence is unique to the target protein in that species are then measured as a quantitative stoichiometric surrogate for the protein in samples [10]. To detect the surrogate peptide, selected reaction

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monitoring (SRM or MRM) on a triple quadrupole instrument is generally employed. Quantification is then accomplished by relating the intensity of surrogate peptide to the corresponding signal of stable isotope–labeled peptides with an identical sequence. Our previous work has successfully determined TfR in cultured breast cells and breast tissue samples using targeted proteomics [11].

In this study, we further applied the LC-MS/MS–based targeted proteomics assay into clinical practice. Compared to the amounts of TfR in cells and tissue samples, sTfR in human serum is much less abundant (males, 2.2 μg/ml; females, 1.9 μg/ml) [12]. Additionally, its value in post-chemotherapy patients dropped far below the LOQ of most assays. Thus, stable isotope standards with capture by antipeptide antibodies (SISCAPA), in which proteolytic peptide and its corresponding spiked stable isotope–labeled internal standard are captured by an antibody raised against the peptide, was employed here [13]. As reported previously, volume reduction on the order of 10^2–10^3 and quantification in the ng/ml range can be achieved using SISCAPA [14].

2. Materials and methods

2.1. Chemicals and reagents

The synthetic proteolytic peptides and the stable isotope–labeled internal standard were developed by ChinaPeptides Co. Ltd. The purity of these peptides was also provided by the manufacturer. sTfR was from RayBiotech. Anti-peptide antibody was produced by Genscript Co. Ltd., and the preparation procedure was described in the supplementary material. Ammonium bicarbonate was from Qiangshun Chemical Reagent Co. Ltd. DL-Dithiothreitol (DTT) and iodoacetamide (IAA) were from Sigma-Aldrich. Sequencing-grade modified trypsin was from Promega. Phosphate-buffered saline (PBS) was from Beyotime Institute of Biotechnology. Acetonitrile (ACN) and methanol were from Tedia Company, Inc. Trifluoroacetic acid (TFA), acetic acid and formic acid (FA) were from Sigma-Aldrich and Xilong Chemical Industrial Factory Co. Ltd., respectively. Sodium dodecyl sulfate (SDS) was obtained from Generay Biotech Co. Ltd. Water was purified and deionized with a Milli-Q system from Millipore).

2.2. Preparation of stock solutions, calibration standards and quality controls (QCs)

A 1 mg/ml stock solution of sTfR protein (purity > 90%) in deionized water was prepared. Its concentration was determined as 0.876 mg/ml using a BCA protein assay kit (Pierce Biotechnology, Inc.). The solution was stored at −20 °C in an amber glass tube to protect it from light. In this report, an isotope-labeled synthetic peptide was used as an internal standard and 5 μg/ml stock solution was prepared in deionized water. A 20 ng/ml internal standard solution was prepared by diluting the stock solution into a mixture of ACN:water (50:50, v/v) containing 0.1% FA.

sTfR calibration standards were prepared by serial dilution of the stock solution using pooled serum from healthy volunteers as the matrix. Calibration standards were prepared at 100, 200, 500, 1000, 2500, 5000 and 10,000 ng/ml. QC standards, i.e., lower limit of quantification (LLOQ), low QC, mid QC and high QC were prepared at 100, 300, 1000 and 8000 ng/ml, respectively, in the same matrix and frozen prior to use.

2.3. Sample collection

Human serum sample collection in this study was approved by the institutional review board of Nanjing Medical University. Sixty pairs of serum samples from breast cancer patients pre- and post-chemotherapy (the fifth day after the onset of chemotherapy) were collected consecutively between January 2014 and September 2014 at the Jiangsu Cancer Hospital and First Affiliated Hospital of Nanjing Medical University, Nanjing, China (mean patient age, 51.3 ± 8.4 years; age range, 38–65 years). The patients were biologically unrelated, but all belonged to the Han Chinese ethnic group from Jiangsu Province in China. Informed consent was obtained from the subjects. In addition, 60 healthy volunteers were recruited and serum samples were collected. To their knowledge, they were healthy and had no reason to consult their local doctors during the preceding 12 months. This study was approved by the institutional review board of Nanjing Medical University, Nanjing, China.

2.4. Serum depletion and in-solution tryptic digestion

Primary proteins were depleted from the samples using the Seppro IgY 14 Spin Columns (Sigma Aldrich) according to the manufacturer's protocol. After depletion, 500 μl of each sample was mixed with 250 μl of 50 mmol/l NH_4HCO_3. Denaturation was performed at 95 °C for 8 min. Subsequently, the protein was reduced with the addition of 50 mmol/l DTT to a final concentration of 10 mmol/l and incubated at 60 °C for 20 min. The sample was then alkylated by adding 400 mmol/l IAA to a final concentration of 50 mmol/l and incubated at room temperature for 6 h in the dark. Finally, 175 μg of sequencing grade trypsin was added, and the sample was incubated at 37 °C for 24 h. The reaction was stopped by adding 50 μl of 0.1% TFA. Then, 100 μl of the internal standard solution was added to the tryptic peptide mixture before transferring it into an Oasis HLB cartridge (60 mg/3 ml; Waters) that was preconditioned with 3 ml ACN and 3 ml deionized water. After the sample was loaded, the cartridge was washed with 2 ml of water and 2 ml of ACN:water (50:50, v/v) and eluted with 1 ml of 100% ACN. Finally, the eluent was evaporated to dryness and then resuspended in 0.5 ml PBS.

2.5. Peptide/protein immunoaffinity enrichment

Immunoaffinity enrichment of target analytes can further improve the sensitivity of the targeted proteomics approach. Within this technology, either intact protein is captured using a protein antibody or a surrogate peptide is captured employing SISCAPA. Both of these approaches were evaluated in this study. Since protein enrichment has been widely used in proteome studies and our previous work has described the related details (please see Supplementary Material) [15], only SISCAPA is illustrated here. Early developed rabbit polyclonal anti-peptide antibody bound to Protein A/G-agarose beads (Abmart, Shanghai, China) was added to the peptide resuspension obtained above. The sample was incubated with gentle rotation overnight at 4 °C. After centrifugation, the beads were washed 3 times with ice-cold PBS buffer and finally resuspended in 100 μl 5% acetic acid for 6 h with gentle rotation at room temperature. The supernatant was then injected on the LC-MS/MS system.

2.6. LC-MS/MS

An Agilent Series 1200 HPLC system (Agilent Technologies) and a 6410 Triple Quad LC/MS mass spectrometer (Agilent) were used. The liquid chromatography separations were performed on a hypersil gold column (3 μm, 20 mm × 2.1 mm; Thermo Fisher Scientific) at room temperature. The mobile phase consisted of solvent A (0.1% FA in water) and solvent B (0.1% FA in methanol). A linear gradient with a flow rate of 0.3 ml/min was applied in the following manner: B 10% (0 min) → 10% (1 min) → 90% (4 min) → 90% (8 min) → 10% (9 min). The injection volume was 10 μl.

The mass spectrometer was interfaced with an electrospray ion source and operated in the positive MRM mode. Q1 and Q3 were both set at unit resolution. The flow of the drying gas was 10 l/min, while the drying gas temperature was held at 350 °C. The electrospray capillary voltage was optimized to 4000 V. The nebulizer pressure was set