Talanta 125 (2014) 411–417

Contents lists available at ScienceDirect

Talanta

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

Evaluation of serum phosphopeptides as potential cancer biomarkers by mass spectrometric absolute quantification

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ARTICLE INFO

Article history:
Received 24 January 2014
Received in revised form
10 March 2014
Accepted 12 March 2014
Available online 20 March 2014

Keywords:
Absolute quantification
Cancer biomarker
Mass spectrometry
Serum phosphopeptides
Enrichment

ABSTRACT

Mass spectrometric quantification of phosphopeptides is a challenge due to the ion suppression effect of highly abundant non-phosphorylated peptides in complex samples such as serum. Several strategies for relative quantification of serum phosphopeptides based on MS have been developed, but the power of relative quantities was limited when making direct comparisons between two groups of samples or acting as a clinical examination index. Herein, we describe an MS absolute quantification strategy combined with Titania Coated Magnetic Hollow Mesoporous Silica Microspheres (TiO_2/MHMSM) enrichment and stable isotopic acetyl labeling for phosphopeptides in human serum. Four endogenous serum phosphopeptides generated by degradation of fibrinogen were identified by LC–ESI–MS/MS following TiO_2/MHMSM enrichment. The ESI–MS signal intensity ratios of the four phosphopeptide standards labeled with N-acetoxy-H_3-succinimide (H3-NAS) and N-acetoxy-D_3-succinimide (D3-NAS), following TiO_2/MHMSM capture are linearly correlated with the molar ratios of the "light" to "heavy" phosphopeptides over the range of 0.1–4 with an r^2 of up to 0.998 and a slope of close to 1. The recovery of the four phosphopeptides spiked at low, medium and high levels in human sera were 98.4–111.9% with RSDs ranging 2.0–10.1%. The absolute quantification of the phosphopeptides in serum samples of 20 healthy persons and 20 gastric cancer patients by the developed method demonstrated that 3 out of the 4 phosphopeptides showed remarkable variation in serum level between healthy and cancer groups, and the phosphopeptide DpSGEGDFLAEGGGVR is significantly down-regulated in the serum of patients, being a potential biomarker for gastric cancer diagnosis.

1. Introduction

Cancer is the second leading cause of death in developing countries and even the leading cause of death in developed countries [1]. Early detection of cancer is the most promising way to improve long-term survival of patients. Cancer-specific biomarkers play important roles in cancer diagnosis and prognosis as most cancer-related deaths can be prevented through early diagnosis and surgical removal of early stage cancer and precancerous lesions [2]. Serum as one of biological fluids has become one of the best resources for biomarker discovery [3]. Several biomarkers in serum, including prostate-specific antigen (PSA) for prostate cancer [4], alpha fetoprotein (AFP) for hepatocellular carcinoma (HCC) [5], CA125 for ovarian cancer [6], carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) for gastric cancer (GC), have been used for cancer diagnosis in clinic. However, none of these biomarkers have been produced as well-accepted screening tools due to their low sensitivity or specificity for diagnosing cancer [7,8]. Thus, it is very essential to identify and characterize novel biomarkers for the detection of cancers.

The reversible phosphorylation of proteins is the most common post-translational modification in mammals, and plays an important role in protein function regulation and signaling transmission [9]. The abnormal protein phosphorylation has been thought to be a cause or consequence of diseases such as cancers [10]. Therefore, the analysis of phosphorylated proteins or endogenous phosphopeptides derived from tissues or biological fluids is critical with respect to clinical diagnostic and prognostic information for cancer and other diseases [11]. Serum with endogenous phosphopeptides either degraded from larger proteins or secreted from cells and tissues provides a direct link between peptide profiles of disease

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http://dx.doi.org/10.1016/j.talanta.2014.03.025
0039-9140/

and differential protease activity [3]. Human serum is a very complex matrix in which protein concentrations can range over more than 10 orders of magnitude, and 90% of the total protein composition is constituted by about 20 abundant proteins [12]. The complexity of serum proteins and peptides as well as the high background level of the highly abundant proteins make the mass spectrometry (MS)-based phosphopeptide profiling a great challenge. To overcome serum complexity and reduce the ion suppression effect arising from non-phosphorylated proteins/peptides, robust and highly selective enrichment of phosphorylated proteins or peptides for subsequent MS analysis is in great demand [13,14].

A number of strategies have been applied to isolate phosphorylated proteins and peptides from complicated mixtures. Immobilized metal ion affinity chromatography (IMAC), which relies on the special affinity of the phosphate group to metal ions such as Fe^{3+}, Ga^{3+}, Ti^{4+}, and Zr^{4+}, is one of the widely used methods. Amoresano and co-workers successfully identified four free phosphopeptides in human serum using an improved IMAC strategy in conjugation with iterative mass spectrometry based scanning techniques with selective "precursor ion", constant "neutral loss" triple quadrupole scan modes and multiple reaction monitoring [11]. However, the specificity of these IMAC methods is not high enough as some non-phosphopeptides also bind to the adsorbents, which results in serious interference for the analysis of target phosphopeptides [15–19]. To reduce the unexpected absorption of non-phosphorylated peptides, metal oxide affinity chromatography (MOAC) has attracted more and more attention [20,21]. Titanium dioxide (TiO_2) and zirconium oxide (ZrO_2) have been alternatively used as chromatographic materials because they can reduce nonspecific binding of non-phosphopeptides and show higher selectivity for phosphopeptides compared to IMAC [21–30]. Recently, functionalized mesoporous TiO_2 [31,32] and ZrO_2 [33] have been applied as affinity materials for the separation and enrichment of phosphopeptides in the enzymatic digests of multiple proteins. The metal oxides are coated to magnetic core (Fe_3O_4) *via* a thin layer of carbon [34], silicon thin film [35] or graphene platform [36], conferring the fabricated mesoporous spheres or networks with the virtue of magnetic separability and enhanced surface area for efficient and rapid capture of phosphopeptides. More recently, we have developed an MS method for rapid screening of epidermal growth factor receptor (EGFR) inhibitors by using the TiO_2 coated magnetic hollow mesoporous silica microspheres (TiO_2/MHMSM) [31] as a solid phase microextraction (SPME) device to concentrate phosphorylated substrates of EGFR [37].

Stable isotope labeling in combination with mass spectrometry techniques has been widely used in quantitative proteomics. These methods involve metabolic, e.g., stable isotope labeling by amino acids in cell culture (SLIAC) [38], or chemical, e.g., isobaric tag for relative and absolute quantitation (iTRAQ) labeling [39,40]. Metabolic labeling is probably the most accurate quantitative MS method in terms of overall experimental process. This makes it particularly suitable for assessing relatively small changes in protein levels. However, metabolic labeling is typically expensive, and does not applicable to clinical samples, and the complete incorporation of labeled amino acids needs to be optimized for individual cell types [41]. Chemical labeling, on the other hand, provides researchers with many choices with the manner of selectively introducing an isotope tag by chemical reactions onto a desired site on a protein or peptide. Recently, Zou and co-workers [42] developed a type of modified phosphoric acid functionalized mesoporous organo-silica (EPO) nanomaterials as the adsorbent for *in situ* enrichment and isotope labeling of endogenous phosphopeptides in serum. The subsequently relative MS quantification revealed differences in the abundance of the phosphopeptides between the cancer patients and healthy controls. However, to the best of our knowledge, the absolute quantitative analysis of phosphopeptides by isotope differential mass spectrometry has remained less explored.

In this work, we developed an MS-based strategy in combination with phosphopeptide enrichment by the TiO_2 coated magnetic hollow mesoporous silica microspheres (TiO_2/MHMSM) and stable isotope labeling by acetylation of peptides for the absolute quantification of endogenous phosphopeptides in human serum. This method relies on isotopic labeling for isolated phosphopeptides from standards and samples, differing from isotope dilution strategies and making the method a high-throughput and economical approach for measuring phosphopeptides in biological samples. The isotopic labeling was generated by treatment of phosphopeptides with N-acetoxysuccinimide and its deuterated analog, which results in acetylation and deuterioacetylation of the primary amines of endogenous peptides and synthetic peptide standards, respectively. Under the optimized conditions, over a range of 0.1–4, the molar ratios of four endogenous phosphopeptides to the respective standards in sera maintained a good linear relationship with their MS signal ratios, allowing accurate quantification of phophospeptides in serum samples of healthy persons and gastric cancer patients. The subsequently receiver operating characteristic (ROC) analysis indicated that a phosphopeptide in serum derived from fibrinogen is significantly down-regulated in sera of patients, perhaps being a promising biomarker for detection of GC with high specificity and sensitivity.

2. Experimental

2.1. Reagents and materials

α-casein, trifluoroacetic acid (TFA) and N-acetoxy-D_3-succinimide (D3-NAS) were purchased from Sigma-Aldrich (USA). N-acetoxy-H_3-succinimide (H3-NAS) and a mixture of sera from healthy adults were purchased from Heowns Biochem Technologies (Tianjin, China). Sequencing grade trypsin was obtained from Promega (USA). The phosphopeptides ADpSGEGDFLAEGGGV (F1), DpSGEGDFLAEGGGV (F2), DpSGEGDFLAEGGGVR (F3) and ADpSGEGDFLAEGGGVR (F4) were purchased from HysBio Ltd. (Beijing, China). Acetonitrile (MeCN) was purchased from Tedia, and the chemicals for fabrication of Titania Coated Magnetic Hollow Mesoporous Silica Microspheres (TiO_2/MHMSM) from Shanghai General Chemical Reagent Factory (Shanghai, China). The deionized water used in the experiments was prepared by a Milli-Q system (Millipore, Milford, MA).

Serum samples of healthy persons and gastric cancer patients were collected at the Tumor Hospital Affiliated to Nantong University following the standard clinical protocol described in the literature [3,43]. Briefly, all blood samples were collected in 7.0 ml glass red-top tubes (BD; 366431), allowed to clot at room temperature for 1 h, and centrifuged at 2000 g for 10 min at room temperature. Sera (upper phase) were then transferred to 1.5 ml cryovials with about 1 mL serum in each and stored frozen at −80 °C until further use. The utilization of human sera complied with guidelines of Ethics Committee of the Hospital and the Institute.

2.2. Enrichment of phosphopeptides by TiO_2/MHMSM

α-Casein (1 mg) was dissolved in 1 mL Tris–HCl (50 mM, pH 8.2) and treated with trypsin using an enzyme to substrate ratio of 1:50 (w/w), and incubated for 16 h at 37 °C. Aliquot (5 μL, 10^{-6} M) of tryptic digest of α-casien was diluted with 30 μL 50% MeCN-0.1% TFA, then a suspension of 5 μL TiO_2/MHMSM (30 mg mL^{-1}) was added. The resulting mixture was vibrated for 30 min at room temperature. After that, with the help of magnet, the peptides-loaded TiO_2/MHMSM were collected by removal of the