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

Guijin Zhai [a], Xiaoyan Wu [b], Qun Luo [a], Kui Wu [a], Yao Zhao [a], Jianan Liu [a], Shaoxiang Xiong [a], Yu-Qi Feng [c], Liping Yang [b,*], Fuyi Wang [a,*,1]

[a] *Beijing National Laboratory for Molecular Sciences; CAS Key Laboratory of Analytical Chemistry for Living Biosystems; Beijing Center for Mass Spectrometry; Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, PR China*

[b] *Cancer Research Center, Tumor Hospital Affiliated to Nantong University, Nantong 226361, Jiangsu, PR China*

[c] *College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, PR China*

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.

© 2014 Elsevier B.V. All rights reserved.

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

* Corresponding authors.
E-mail addresses: liping.yang@ntu.edu.cn (L. Yang), fuyi.wang@iccas.ac.cn (F. Wang).
[1] Fax: (+86) 10-6252-9069.

http://dx.doi.org/10.1016/j.talanta.2014.03.025
0039-9140/© 2014 Elsevier B.V. All rights reserved.

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 coworkers [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

supernatant and washed with 100 μL 50% MeCN-0.1% TFA twice, then the trapped peptides were eluted with 5 μL 2.5% ammonium hydroxide (pH 11.5) and the eluent was collected for MS analysis.

For enrichment of endogenous phosphopeptides in human serum samples, 10 μL of a serum sample was first diluted by addition of 80 μL 50% MeCN-0.1% TFA [13], and incubated with 10 μL of TiO_2/MHMSM (30 mg mL^{-1}) with vibration for 30 min, then the supernatant was removed with using an external magnet and the particles capturing phosphopeptides were washed with 100 μL 50% MeCN-0.1% and 30% MeCN-0.1% TFA in turn. Finally the bound phosphopeptides were eluted by incubation with 30 μL 2.5% ammonium hydroxide (pH 11.5) for 10 min, and the supernatant was collected with using an external magnet and lyophilized to dryness.

2.3. Stable isotopic labeling of phosphopeptides

The lyophilized phosphopeptide mixtures isolated from standard solutions or serum samples were re-dissolved in 4 μL NH_4HCO_3 buffer (50 mM, pH 7.5), then a 20-fold molar excess of H3-NAS was added, reacted at 37 °C for 10 h without stirring [44]. Aliquot of the H3-NAS-labeled sample was mixed with equal volume of the respective D3-labled sample for LC-MS analysis.

2.4. Mass spectrometry

MALDI-TOF mass spectrometry analysis was performed on an Autoflex III mass spectrometer (Bruker, Germany). The instrument was equipped with a delayed ion-extraction device and a pulsed nitrogen laser operating at 337 nm. Typically, 1200 scans were averaged. The MALDI uses a groundsteel sample target with 384 spots. For the analysis of phosphopeptides, 20 mg mL^{-1} 2,5-dihydroxybenzoic acid (DHB) in 50% acetonitrile/1% phosphoric acid was used as the matrix. An aliquot (3 μL) of collected eluents (vide supra) was mixed with the matrix solution in a 1:1 ratio prior to deposition onto the target plate for data collection. The Flexanalysis (ver. 3.0) software was used for analysis and post processing.

Positive-ion electrospray ionization mass spectrometry (ESI–MS) analysis was performed on a Micromass Q-TOF mass spectrometer (Waters) coupled to a Waters CapLC HPLC system. The phosphopeptides captured from human serum samples were separated on a Symmetry-C18 column (1.0 × 50 mm^2, 100 Å, 3.5 μm, Waters). Mobile phases were A: 95% H_2O containing 4.9% acetonitrile and 0.1% formic acid, and B: 95% acetonitrile containing 4.9% H_2O and 0.1% formic acid. The peptides were eluted with a 30 min linear gradient from 1% to 80% of B at a rate of 30 μL min^{-1}. The eluent was directly infused into the mass spectrometer through the ESI probe. The spray voltage of the mass spectrometer was 3.30 kV and the cone voltage 35 V. The desolvation temperature was 413 K and source temperature 353 K. Nitrogen was used as both cone gas and desolvation gas with a flow rate of 40 L h^{-1} and 400 L h^{-1}, respectively. The collision energy was set up to 5 V. All spectra were acquired in the range of 500–2000 *m*/*z*. Mass Lynx (ver. 4.0) software was used for analysis and post processing.

3. Results and discussion

3.1. Optimization of phosphopeptide enrichment conditions

The separation and enrichment of phosphopeptides by TiO_2/MHMSM may suffer from nonspecific adsorption of non-phosphorylated peptides, in particular acidic peptides, which increases the chance of false-positive results. Therefore, the tryptic digests of phosphorylated protein α-casein were used to optimize the concentration of trifluoroacetic acid (TFA) and acetonitrile (MeCN), which can reduce the undesirable absorption of acidic non-phosphorylated and hydrophobic peptides, on the surface of

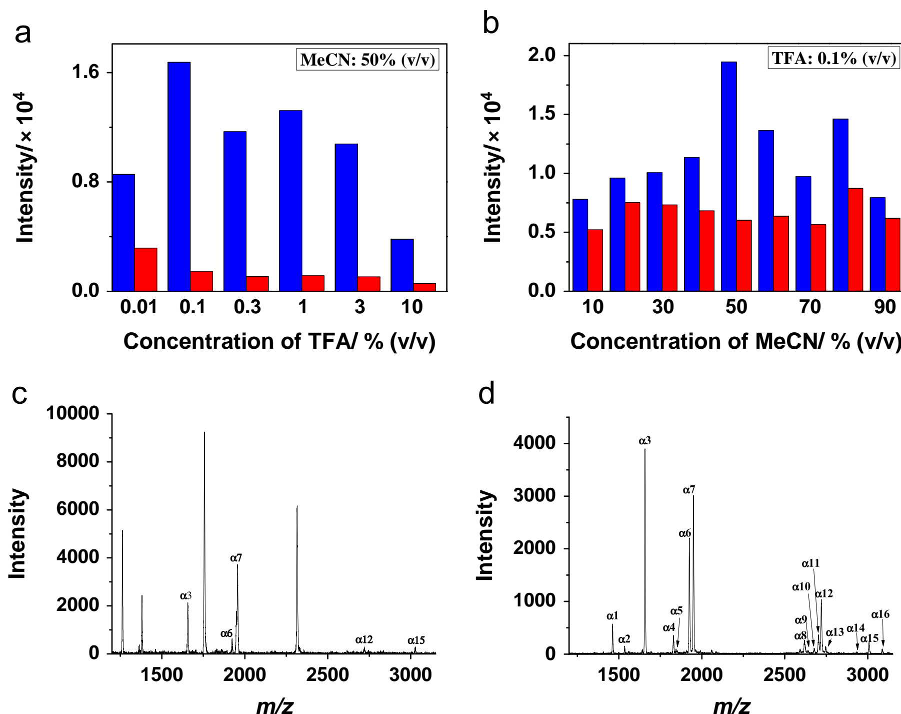

Fig. 1. Effect of the concentration of (a) TFA and (b) MeCN in the loading buffer and washing solvents on the enrichment of phosphopeptides from tryptic digests of α-casein using TiO_2/MHMSM. The bar values represent the total signal intensities detected by MALDI-TOF MS of phosphorylated peptides (blue) and non-phosphorylated peptides (red). Mass spectra of phosphopeptides derived from α-casein obtained by MALDI-TOF MS analysis of the tryptic digest of α-casein (10^{-6} M, 5 μL) (c) before and (d) after enrichment by TiO_2/MHMSM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

TiO_2/MHMSM particles [37,45], in the loading buffer and washing solvent. As shown in Fig. 1a, both low (0.01%) and high (> 0.1) concentration of TFA were ineffective for phosphopeptide enrichment by TiO_2/MHMSM as low concentration of TFA is insufficient for the protonation of carboxyl groups, and a high concentration of TFA resulted in the loss of negative charge on phosphates of phosphopeptides. Thus, 0.1% TFA in the loading buffer and washing solvent was chosen for the rest of this work. When the concentration of TFA was fixed at 0.1%, the change of MeCN concentration in the loading and washing buffers also affected significantly the capturing efficiency of phsophopeptides as shown in Fig. 1b. The concentration of 50% for MeCN in both loading buffer and washing solvent was shown to be suited for selective enrichment of phosphopeptides by TiO_2/MHMSM particles.

The performance of TiO_2/MHMSM for capturing phosphopeptides was further investigated under the optimized conditions established above. The direct MALDI-TOF MS analysis of tryptic digest of α-casein resulted in the detection of only 5 phosphopeptides and a few high abundance nonphosphopeptides (Fig. 1c). Moreover, the signal intensities of most of these phosphopeptides were weak due to the ionization suppression arising from highly abundant nonphosphopeptides. With separation and enrichment by TiO_2/MHMSM, the detection of phosphopeptides was significantly improved, and the highly abundant nonphosphopeptides were almost undetected (Fig. 1d). In this case, 16 phosphopeptides derived from α-casein were observed (Table S1 in the Supplementary Information).

In order to evaluate the possible loss of phosphopeptides during the loading and washing steps, the supernatant after phosphopeptide capturing by TiO_2/MHMSM and the washing solution for removing peptides nonspecifically adsorbed on TiO_2/MHMSM were also analyzed by MALDI-TOF MS. As shown in Fig. S1a, only phosphopeptide α3 was detected in the supernatant with low signal intensity. This indicates that the TiO_2/MHMSM materials are of highly capability to capture phosphopeptides. It is essential to remove non-phosphorylated peptides while retaining phosphopeptides captured by TiO_2/MHMSM during the washing steps. As shown in Fig. S1b, a number of nonphosphopeptides were detected together with phosphopeptide α3 in low abundance in the first time washing solution. In the second time of washing eluent, the signal intensities of the nonphosphopeptides and α3 were decreased sharply (Fig. S1c). These results indicate that the washing steps did not result in pronounced loss of phosphopeptides while the nonspecifically bound peptides to TiO_2/MHMSM were washed off.

3.2. Qualification of phosphopeptides in human serum

Human serum contains thousands of peptides that are thought to be fragments generated by proteolytic enzymes. The endogenous phosphopeptides in human serum are present in low abundance [46], thus the directly analysis of phosphopeptides in human serum is an enormous challenge due to the existence of high complexity of the matrix, and wide-dynamic range of endogenous peptides and co-existing proteins. As shown in Fig. 2a, no phosphospeptides were observed by direct MALDI-TOF MS analysis of a human serum sample containing a mixture of sera from healthy adults, whereas, four remarkable ion peaks appeared in the mass spectrum of the serum sample after phosphopeptides capture by TiO_2/MHMSM (Fig. 2b). To confirm the identities of these ions, tandem mass spectrometry (MS/MS) was employed for characterization. The MS^2 spectra obtained by selecting these precursor ions are shown in Fig. S2, indicating that the four species correspond to phosphopeptides, ADpSGEGDFLAEGGGV (F1), DpSGEGDFLAEGGGV (F2), DpSGEGDFLAEGGGVR (F3) and ADpSGEGDFLAEGGGVR (F4) at *m/z* 1389.3, 1460.4, 1545.5 and 1616.6, respectively, which are all derived from fibrinopeptides A.

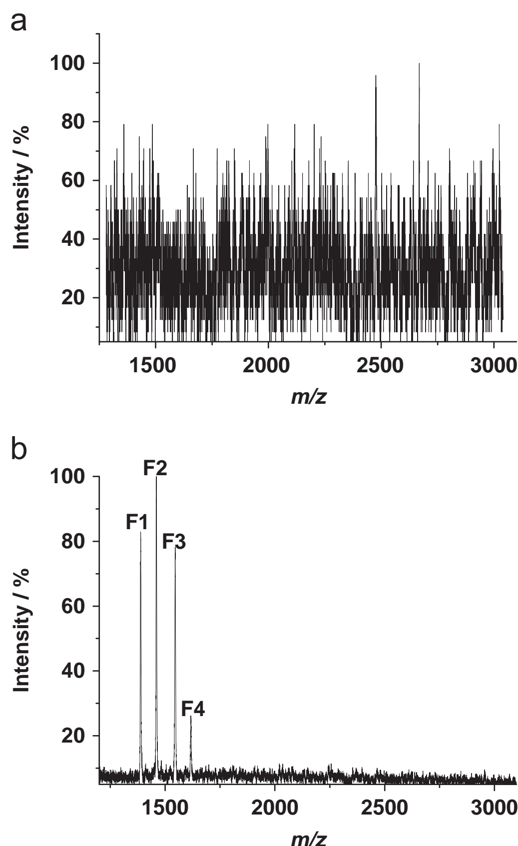

Fig. 2. Mass spectra of phosphopeptides in human serum obtained by (a) direct MALDI-TOF MS analysis and (b) MALDI-TOF MS analysis after enrichment by TiO_2/MHMSM. The phosphopeptides F1 – F4 were identified by ESI–MS/ MS, and the MS/MS data are shown in Fig. S2 in the Supplementary Information.

3.3. MS quantification of phosphopeptides in human serum

Following the effective separation and enrichment of phosphopeptides in human serum samples by TiO_2/MHMSM, the captured phosphopeptides were then labeled light or heavy using N-acetoxy-H3-succinimide (H3-NAS) or N-acetoxy-D3-succinimide (D3-NAS) prior to MS analysis. The isotopic labeling for the quantification of phosphopeptides is generally performed before the phosphopeptide enrichment [40,47]. To avoid large consumption of labeling reagents and to reduce the interference arising from the highly abundant non-phosphorylated peptides on the labeling reactions, we carried out the labeling reactions after the phsophopeptide isolation by TiO_2/ MHMSM. As shown in Fig. S3–S4, the labeling reactions were complete within 10 h with almost no side reaction observed, allowing a high recovery of spiked phosphopeptide standards in serum samples to be obtained (vide infra).

To generate calibration curves for the absolute quantification of the four endogenous phosphopeptides in serum, a series of solutions containing various concentrations of synthetic phosphopeptides F1 – F4 and a solution containing 0.5 μM of F1 – F4 were individually incubated with TiO_2/MHMSM, followed by the labeling of the captured peptides using H3-NAS and D3-NAS, respectively. Then, aliquot of each H3-NAS-labled sample was mixed with an equal-volume of the respective D3-NAS-labled sample, giving a series of standard solutions with a molar ratio of H-pool/D-pool ranging from 0.1 to 4. Fig. 3a shows a typical mass spectrum of H3-NAS-labeled and D3-NAS-labeled F1 in an equimolar mixture of light- and heavy-F1. As the signal overlaps occurred between the last two isotopic peaks of light F1 and the first two isotopic peaks of heavy F1 (Fig. 3b), a correction

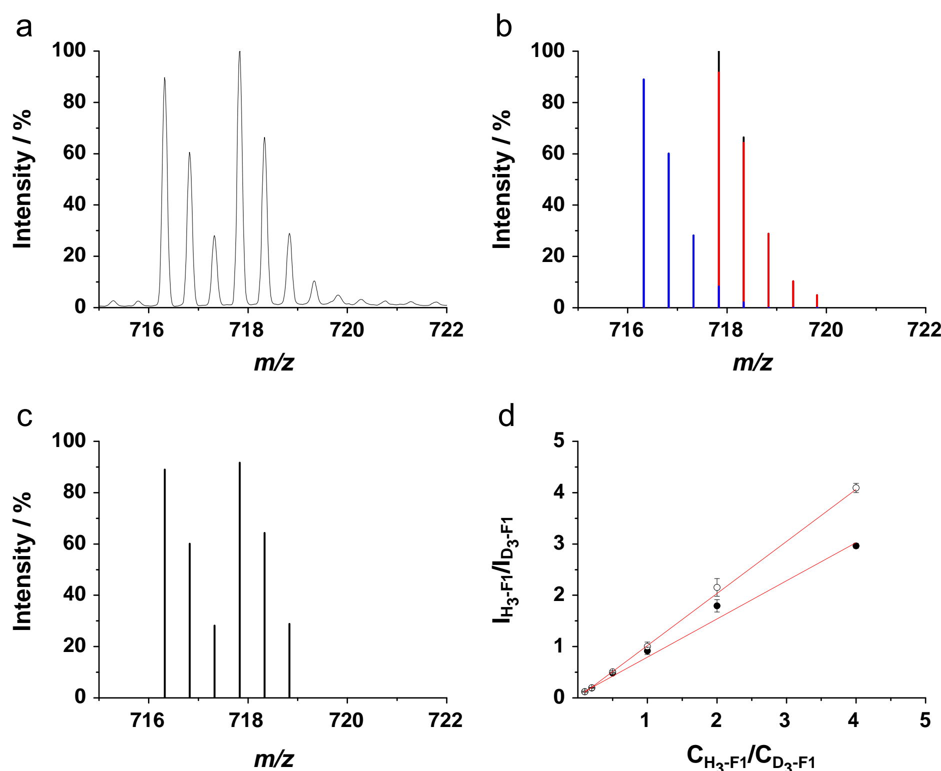

Fig. 3. The mass spectrum (a) before and (b) after centering of the doubly-charged ions of H3-NAS-labeled and D3-NAS-labeled F1 in an equimolar mixture of the light and heavy phosphopeptides, showing the signal overlap between the light and heavy F1; (c) the mass spectrum showing the first three isotopic peaks of the equimolar light- and heavy-labeled F1 after correcting overlap between the last two isotopic peaks of light F1 and the first two isotopic peaks of heavy F1; and (d) calibration curves plotted by the intensity ratios of the first isotopic peaks of light and heavy F1 versus their molar ratios without (●) and with (○) overlap correction.

Table 1
Recovery of phosphopeptides F1 – F4 spiked into human serum at different levels.

	Original conc./μM	Recovery (%, $n=3$) of Spiked F_i		
		0.1 μM	0.5 μM	1.5 μM
F1	0.36 ± 0.02	107.4 ± 10.1	105.5 ± 2.8	111.8 ± 2.3
F2	0.20 ± 0.02	99.4 ± 8.3	102.6 ± 6.0	103.6 ± 9.2
F3	0.10 ± 0.01	100.7 ± 9.4	104.1 ± 7.3	99.1 ± 2.0
F4	0.09 ± 0.01	98.4 ± 3.1	111.9 ± 4.7	98.6 ± 4.9

procedure was applied on the basis of the theoretical isotopic ratio of F1. It can be seen that with this correction, the signal intensity of the first three isotopic peaks of light-labeled F1 was almost equal to that of equimolar heavy-labeled F1 (Fig. 3c). As a consequence, the calibration curve plotted by the corrected intensity ratio of the first isotopic peak of H-pool/H-pool F1 versus their molar ratio is better linear than that without correction (Fig. 3d, Table S2). Analogically, the calibration curves of F2 to F4 were obtained and are shown in Fig. S5–S7. The results indicate that the calibration equations with signal correction yield a slope close to 1 and an R^2 of up to 0.998 (Table S2), suggesting that the signal ratios of light/heavy pair measured by ESI-MS allow accurate quantitative analysis of the phosphopeptides captured by TiO_2/MHMSM. To verify this absolute quantification approach, the recovery of spiked synthetic phosphopeptides in a serum mixture of healthy adults was determined. The results (Table 1) indicate that at the low (0.1 μM), mediate (0.5 μM) and high (1.5 μM) spiking levels, the recovery of the four phosphopeptides ranges from 98.4% to 111.9%, implying that the developed approach is reliable for absolute quantification of endogenous phosphopeptides in human serum.

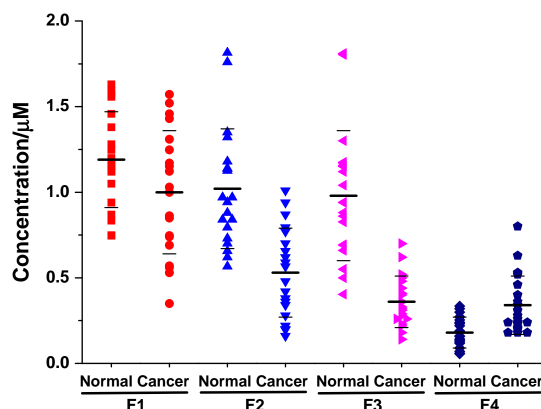

Fig. 4. Scatter plots demonstrating the concentration distribution of the four endogenous phosphopeptides F1 – F4 in serum samples of healthy persons and gastric cancer patients. The short solid lines represent the average value (thick) and standard derivation (thin), respectively, of each phsophopeptide.

Table 2
Statistical analysis of levels of phosphopeptides F1 – F4 in the serum samples of gastric cancer patients and healthy persons.

	Healthy (μM)		Cancer (μM)		*F*	*P*
	Mean	SD	Mean	SD		
F1	1.19	0.28	1.00	0.36	3.67	0.06
F2	1.02	0.35	0.53	0.26	25.3	1.2E-5
F3	0.98	0.38	0.36	0.15	47.2	3.6E-8
F4	0.18	0.09	0.34	0.17	13.9	6.3E-4

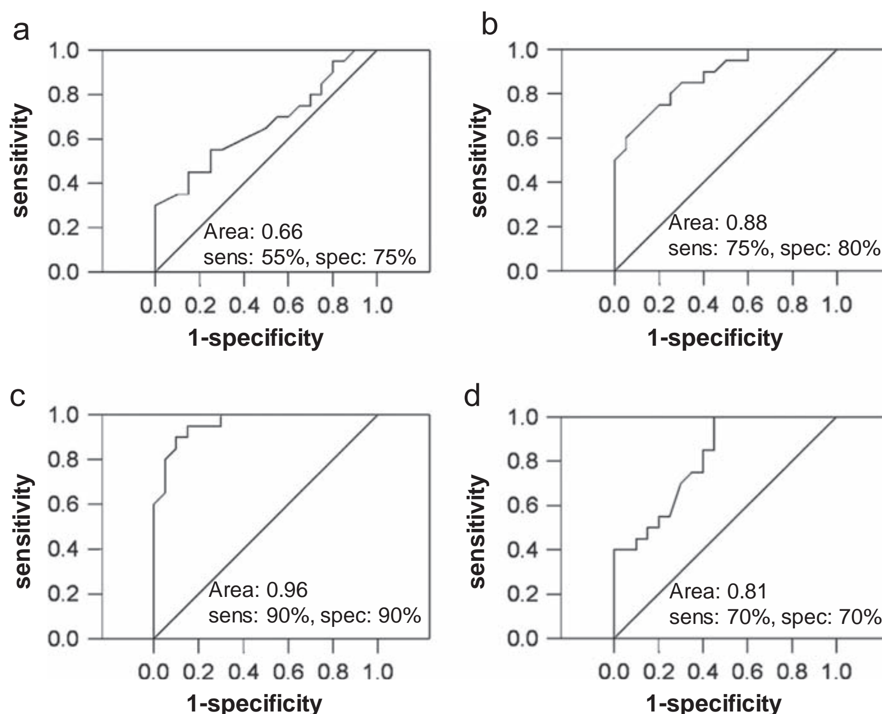

Fig. 5. ROC curves for phosphopeptides (a) F1, (b) F2, (c) F3 and (d) F4 in human serum. The cut-off value was set at the point whose distance from the (sensitivity, specificity)=(1,1) reached the minimum. The sensitivity (sens), specificity (spec) and area under the curve (AUC) were shown in each graph.

The established method was then applied to quantitatively characterize phosphopeptide levels in clinically collected serum samples of 20 healthy persons and 20 gastric cancer patients. The results are listed in Table S3 in the Supplementary Information, and diagrammatically shown in Fig. 4. It can be seen that phosphopeptide F4 was up-regulated, while F2 and F3 were both down-regulated in the serum samples of cancer patients in comparison with the healthy controls. Furthermore, the one-way ANOVA was applied to evaluate the differences in the serum level of the four phosphopeptides between the healthy group and the cancer group. The statistical analysis (Table 2) indicates that except for F1, the P values are <0.01 with $F>10$. This means that the serum level of phosphopeptides F2, F3 and F4 of the patient group is significantly different from that of the healthy group. We next assessed the sensitivity and specificity of the phosphopeptides as a potential biomarker for gastric cancer diagnosis by receiver operating characteristic (ROC) analysis. The results (Fig. 5) show that phosphopeptide F3 has higher sensitivity (90%) and specificity (90%) than F2 and F4. It has been recently reported that CEA at the recommended value of 5.0 ng mL^{-1} has a sensitivity of 46% and a specificity of 95%, and CA19-9 at 37 U mL^{-1} a sensitivity of 38% and a specificity of 91% for detection of GC [7]. Our studies herein demonstrate that as a potential biomarker for GC diagnosis F3 has higher sensitivity than CEA and CA19-9, while maintaining a comparable specificity (90%). Collectively, the diagnostic model based on phosphopeptide F3 was valuable and noninvasive alternatives for identifying gastric cancer.

4. Conclusion

With the efficient enrichment by TiO_2/MHMSM and stable isotopic labeling using light and heavy NAS, we developed an MS method for absolute quantification of endogenous phosphopeptides in human serum. The signal intensity ratios of the four identified phosphopeptides are linearly correlated with the molar ratios of light/heavy labeled peptides over a range from 0.1 to 4, allowing accurate determination of the phosphopeptides in serum. Among the four endogenous phosphopetides, three ones show remarkable difference in serum level between cancer and healthy groups. In particular, phosphopeptide DpSGEGDFLAEGGGVR (F3) in the serum samples of gastric cancer patients is significantly down-regulated in comparison to that in the sera of healthy group. These findings may help to the understanding of the pathogenesis process of tumors, implying that endogenous phosphopeptides in serum may be a group of promising biomarkers for cancer diagnosis. We anticipate that the developed approach could be widely applied for absolute quantitative analysis of phosphoproteins and phosphopeptides in large scale of clinic samples so as to provide fundamental information for biomarker discovery and clinical diagnosis.

Acknowledgement

We thank the Innovation Method Fund of China (2012IM030400), NSFC (Grant nos. 21020102039, 21135006, 21127901, 21275148 and 21321003), the 973 Program of MOST (2013CB531805) for support.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.03.025.

References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, CA. Cancer J. Clin. 61 (2011) 69–90.
[2] G.H. Liu, Z.G. Zhou, R. Chen, M.J. Wang, B. Zhou, Y. Li, X.F. Sun, Tumor Biol. 34 (2013) 2175–2181.
[3] J. Villanueva, D.R. Shaffer, J. Philip, C.A. Chaparro, H. Erdjument-Bromage, A.B. Olshen, M. Fleisher, H. Lilja, E. Brogi, J. Boyd, M. Sanchez-Carbayo, E.C. Holland, C. Cordon-Cardo, H.I. Scher, P. Tempst, J. Clin. Invest. 116 (2006) 271–284.

[4] W.C.S. Cho, Mol. Cancer (2007), http://dx.doi.org/10.1186/1476-4598-1186-1125.
[5] Y.M. An, S. Bekesova, N. Edwards, R. Goldman, Dis. Markers 29 (2010) 11–20.
[6] Y. Xu, Z.Z. Shen, D.W. Wiper, M.Z. Wu, R.E. Morton, P. Elson, A.W. Kennedy, J. Belinson, M. Markman, G. Casey, JAMA-J. Am., Med. Assoc. 280 (1998) 719–723.
[7] L. Liu, B. Yan, J.L. Huang, Q.H. Gu, L.N. Wang, M. Fang, J.P. Jiao, X.Q. Yue, Plos One 8 (2013) e77821.
[8] N. Oue, K. Sentani, T. Noguchi, S. Ohara, N. Sakamoto, T. Hayashi, K. Anami, J. Motoshita, M. Ito, S. Tanaka, K. Yoshida, W. Yasui, Int. J. Cancer 125 (2009) 2383–2392.
[9] P. Cohen, Nat. Cell Biol. 4 (2002) E127–E130.
[10] P. Cohen, Nat. Rev. Drug Discov. 1 (2002) 309–315.
[11] C. Cirulli, G. Chiappetta, G. Marino, P. Mauri, A. Amoresano, Anal. Bioanal. Chem. 392 (2008) 147–159.
[12] N.L. Anderson, N.G. Anderson, Mol. Cell. Proteomics 1 (2002) 845–867.
[13] Z.D. Lu, J.C. Duan, L. He, Y.X. Hu, Y.D. Yin, Anal. Chem. 82 (2010) 7249–7258.
[14] B.T. Chait, Y. Oda, T. Nagasu, Nat. Biotechnol. 19 (2001) 379–382.
[15] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (1999) 2883–2892.
[16] S. Feng, M.L. Ye, H.J. Zhou, X.G. Jiang, X.N. Jiang, H.F. Zou, B.L. Gong, Mol. Cell. Proteomics 6 (2007) 1656–1665.
[17] H.J. Zhou, M.L. Ye, J. Dong, G.H. Han, X.N. Jiang, R.N. Wu, H.F. Zou, J. Proteome Res. 7 (2008) 3957–3967.
[18] X.Q. Xu, C.H. Deng, M.X. Gao, W.J. Yu, P.Y. Yang, X.M. Zhang, Adv. Mater. 18 (2006) 3289–3293.
[19] Y. Li, D.W. Qi, C.H. Deng, P.Y. Yang, X.M. Zhang, J. Proteome Res. 7 (2008) 1767–1777.
[20] Y. Li, X.Q. Xu, D.W. Qi, C.H. Deng, P.Y. Yang, X.M. Zhang, J. Proteome Res. 7 (2008) 2526–2538.
[21] M.W.H. Pinkse, P.M. Uitto, M.J. Hilhorst, B. Ooms, A.J.R. Heck, Anal. Chem. 76 (2004) 3935–3943.
[22] H.K. Kweon, K. Hakansson, Anal. Chem. 78 (2006) 1743–1749.
[23] L.H. Hu, H.J. Zhou, Y.H. Li, S.T. Sun, L.H. Guo, M.L. Ye, X.F. Tian, J.R. Gu, S.L. Yang, H.F. Zou, Anal. Chem. 81 (2009) 94–104.
[24] S.Y. Xu, J.C. Whitin, T.T.S. Yu, H.J. Zhou, D.Z. Sun, H.J. Sue, H.F. Zou, H.J. Cohen, R.H. Zare, Anal. Chem. 80 (2008) 5542–5549.
[25] S. Lemeer, M.W.H. Pinkse, S. Mohammed, B.van Breukelen, J.den Hertog, M. Slijper, A.J.R. Heck, J. Proteome Res. 7 (2008) 1555–1564.
[26] M.W.H. Pinkse, S. Mohammed, L.W. Gouw, B. van Breukelen, H.R. Vos, A.J.R. Heck, J. Proteome Res. 7 (2008) 687–697.
[27] T.E. Thingholm, O.N. Jensen, P.J. Robinson, M.R. Larsen, Mol. Cell. Proteomics 7 (2008) 661–671.
[28] M.R. Larsen, T.E. Thingholm, O.N. Jensen, P. Roepstorff, T.J.D. Jorgensen, Mol. Cell. Proteomics 4 (2005) 873–886.
[29] Y.H. Yan, J. Lu, C.H. Deng, X.M. Zhang, Talanta 107 (2013) 30–35.
[30] Y.H. Yan, Z.F. Zheng, C.H. Deng, X.M. Zhang, P.Y. Yang, Talanta 118 (2014) 14–20.
[31] J.H. Wu, X.S. Li, Y. Zhao, Q.A. Gao, L. Guo, Y.Q. Feng, Chem. Commun. 46 (2010) 9031–9033.
[32] J. Lu, M.Y. Wang, C.H. Deng, X.M. Zhang, Talanta 105 (2013) 20–27.
[33] C.A. Nelson, J.R. Szczech, C.J. Dooley, Q.G. Xu, M.J. Lawrence, H.Y. Zhu, Y. Ge, S. Jin, Anal. Chem. 82 (2010) 7193–7201.
[34] Y. Li, T.H. Leng, H.Q. Lin, C.H. Deng, X.Q. Xu, N. Yao, P.Y. Yang, X.M. Zhang, J. Proteome Res. 6 (2007) 4498–4510.
[35] G. Cheng, J.L. Zhang, Y.L. Liu, D.H. Sun, J.Z. Ni, Chem. Commun. 47 (2011) 5732–5734.
[36] Q.H. Min, X.X. Zhang, H.Y. Zhang, F. Zhou, J.J. Zhu, Chem. Commun. 47 (2011) 11709–11711.
[37] L.Y. Ji, J.H. Wu, Q. Luo, X.C. Li, W. Zheng, G.J. Zhai, F.Y. Wang, S. Lu, Y.Q. Feng, J.N. Liu, S.X. Xiong, Anal. Chem. 84 (2012) 2284–2291.
[38] S.E. Ong, B. Blagoev, I. Kratchmarova, D.B. Kristensen, H. Steen, A. Pandey, M. Mann, Mol. Cell. Proteomics 1 (2002) 376–386.
[39] E.B. Haura, A. Muller, F.P. Breitwieser, J.N. Li, F. Grebien, J. Colinge, K.L. Bennett, J. Proteome Res. 10 (2011) 182–190.
[40] P.J. Boersema, L.Y. Foong, V.M.Y. Ding, S. Lemeer, B. van Breukelen, R. Philp, J. Boekhorst, B. Snel, J. den Hertog, A.B.H. Choo, A.J.R. Heck, Mol. Cell. Proteomics 9 (2010) 84–99.
[41] J.A. Galan, L.L. Paris, H.J. Zhang, J. Adler, R.L. Geahlen, W.A. Tao, J. Am. Soc. Mass Spectrom. 22 (2011) 319–328.
[42] H.Q. Qin, F.J. Wang, P.Y. Wang, L. Zhao, J. Zhu, Q.H. Yang, R.A. Wu, M.L. Ye, H.F. Zou, Chem. Commun. 48 (2012) 961–963.
[43] J. Villanueva, J. Philip, C.A. Chaparro, Y.B. Li, R. Toledo-Crow, L. DeNoyer, M. Fleisher, R.J. Robbins, P. Tempst, J. Proteome Res. 4 (2005) 1060–1072.
[44] J.M. Kindy, J.A. Taraszka, F.E. Regnier, D.E. Clemmer, Anal. Chem. 74 (2002) 950–958.
[45] M. Kokubu, Y. Ishihama, T. Sato, T. Nagasu, Y. Oda, Anal. Chem. 77 (2005) 5144–5154.
[46] E.F. Petricoin, C. Belluco, R.P. Araujo, L.A. Liotta, Nat. Rev. Cancer 6 (2006) 961–967.
[47] C.J. Wu, Y.W. Chen, J.H. Tai, S.H. Chen, J. Proteome Res. 10 (2011) 1088–1097.