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Direct-S: A Directed Mass Spectrometry Method for Biomarker Verification in Native Serum

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Abstract

Serum has been the logical choice and most-used bio−specimen for monitoring biomarkers. However, direct analysis of low-abundance biomarkers in serum is still a problem. Here, we established a directed mass spectrometry (inclusion list driven MS) method, Direct-S, for direct quantification of protein biomarkers in native serum samples without high-abundance protein depletion or pre-fractionation. In Direct-S, ^{18}O-labeling technique was used to produce internal standards of the targeted peptides, and only targeted peptides were selected for tandem mass (MS/MS) fragmentation to increase sensitivity and efficiency. The ^{16}O/^{18}O ion pairs of target peptides and the elution time/fragmental pattern of the internal standards were used to facilitate the identification of the low-abundance peptides. Using Direct-S, three candidate biomarkers, α1-antitrypsin (A1AT), galectin-3 binding protein (LG3BP) and cathepsin D (CTSD), which represent different abundance levels, were quantified in serum samples of colorectal cancer (CRC) patients and healthy candidates. Direct-S exhibited good linearity of response from 20 fmol to 0.5 nmol ($r > 0.9845$). Reliable quantification across five orders of magnitude and as low as 71 pg/μL was achieved in serum samples. In conclusion, Direct-S is a low cost, convenient and accurate method for verifying serum biomarkers.

Keywords: Serum, biomarkers, quantification, targeted mass spectrometry, colorectal cancer

Introduction

Protein biomarkers, which undergo changes in concentration or state in association with a biological process or disease, play important roles in disease diagnosis, evaluation of treatment progression, and prognosis[1]. Serum has been the logical choice and most-used bio specimen for monitoring biomarkers to date[2]. Serum proteins are present at concentrations ranging across 12 orders of magnitude[3]. A few dominant proteins, such as serum albumin, immunoglobulins and so on, account for approximately 99% of the protein content, which complicates the detection of other proteins[4]. To improve detection of low-abundance proteins, approaches including depletion of highly abundant proteins, protein or peptide-level fractionation or selective immune-enrichment of target peptides/proteins have been applied[5]. Different strategies have been developed for the depletion of highly abundant proteins in serum analysis[,,,], such as immunoaffinity depletion[6], centrifugal ultrafiltration[7], solid-phase extraction[8], protein precipitation[9] and organic-solvent extraction[10]. On the other hand, strong cation exchange chromatographic (SCX)[11], size exclusion chromatography (SEC)[12], free-flow electrophoresis[13], isoelectric focusing[14] and gel-based fractionation[15] have been used to fractionate serum proteins/peptides. However, depletion and fractionation strategies generally suffer from low sample throughput and potentially poor recovery. Particularly when using depletion strategies, some lower-abundance proteins might also be depleted because of nonspecific interactions[4]. Accordingly, strategies with high sensitivity that can be used to directly analyze

native serum samples are needed for biomarker verification.

Advances in proteomic technologies have allowed the systematic identification of biomarkers in serum samples. Although enzyme-linked immune absorbent assay (ELISA) is considered the "gold standard" in clinical assays, the development of antibodies and assays for a large number of candidate proteins is time consuming and expensive[16]. Alternatively, high-resolution MS provides a high throughput and relatively economic method for targeted analysis. Advances in MS have also enabled high-sensitivity and accurate protein quantification[5]. By using these MS-based methods, native serum can be analyzed without pretreatment. A widely used method is based on multiple reaction monitoring (MRM) with liquid chromatography (LC). In one LC-MS run, a number of candidate biomarkers can be quantified simultaneously using MRM. Reliable quantification at the ng/μL level was achieved in direct analysis of native serum samples[17]. MRM–MS methods must first be developed and then validated on a case-by-case basis in the clinical setting. Alternatively, directed MS methods using inclusion lists of precursor ions from target molecules to guide MS/MS fragmentation can be easily applied in quantitative analysis[18]. By considering the mass-to-charge ratio (m/z) and elution time, directed MS methods can be used to detect low-abundance precursors with good precision and accuracy even in complex samples[19]. Recently, Ranish developed a directed MS method, iMSTIQ, using mTRAQ-labeled "index" peptides to trigger the acquisition of full MS/MS spectra for targeted peptides independent of their ion intensities[20]. The index-ion triggered method enhanced detection by MS-directed approaches at the sub-fmol level in yeast

extracts. Combining iMSTIQ with hydrazide-based solid-phase capture of N-glycosylated peptides enabled quantification of low fmol amounts of target N-glycosylated peptides in serum samples. However, direct analysis of serum without any pretreatment has not been performed.

CRC is the third most common malignancy worldwide[21]. By taking advantage of MS techniques, many candidate biomarkers have been identified. However, only a few protein biomarkers have been applied in clinical treatment and assessment of CRC[22], which indicates the need for high-throughput and convenient verification techniques. In this study, a method named Direct-S was developed to verify candidate biomarkers in CRC serum samples without any pretreatment. In Direct-S, we produced heavy-labeled standard peptides using an ^{18}O-labeling technique because of the simple reaction process, high labeling efficiency and low cost[23,24]. To increase the detection sensitivity and efficiency, only predetermined precursor ions of biomarker candidates were selected for fragmentation. For proteins with different abundances, specific identification rules were established. Three candidate biomarkers for CRC[25,26], A1AT, LG3BP and CTSD, representing high-, medium- and lower-abundance proteins, respectively, were selected. Using Direct-S, they were successfully identified and quantified in native CRC serum samples.

Results and Discussion

Overall Concept and Experimental Workflow of Direct-S

For biomarker verification, ^{18}O-labeled targeted peptides were used as internal standards to quantify the absolute amounts of candidate biomarkers in serum samples (Figure. 1). The ^{18}O-labeled standard peptides were spiked in until they could be detected by MS, which required 50 ng A1AT, 5 ng LG3BP, and 2 ng CTSD. Only the paired $^{16}O/^{18}O$-labeled target peptide ions were selected for CID, which saved time for MS/MS acquisition and also increased the detection sensitivity.

Depending on protein abundance, the identification of ^{16}O-containing target peptides in serum samples could be divided into three groups. For peptides whose abundance were too low to be selected for MS/MS analysis, the elution time, m/z, ^{16}O- and ^{18}O-labeled ion peaks of target peptides were used to determine the target peaks. For peptides with medium abundance and poor quality MS/MS spectra, the existence of these peptides was confirmed by comparing the MS/MS spectra with that of the ^{18}O-labeled internal standard peptides. For highly abundant peptides, the MS/MS spectra of ^{16}O-labeled and ^{18}O-labeled peptides could both be obtained and successfully identified. After identification, the absolute amounts of the target proteins were calculated based on the MS1 peak intensity ratios (light/heavy, $^{16}O/^{18}O$) of selected peptides. The $^{16}O/^{18}O$ ratio was calculated by equation 1[27], in which the contribution from incomplete (single) incorporation of ^{18}O is included.

$$Ratio\left(\frac{^{16}O}{^{18}O}\right)=\frac{I_0}{I_2+I_4-\left(\frac{M_2}{M_0}\right)I_2-\left[\frac{M_2}{M_0}+\frac{M_4}{M_0}-\left(\frac{M_2}{M_0}\right)^2\right]I_0} \quad (1)$$

I_0 is the intensity of the monoisotope peak for the serum peptide, I_2 represents the intensity of a single ^{18}O incorporated in the internal peptide, and I_4 is the intensity for the internal peptide with two incorporated ^{18}O, respectively. M_0, M_2, and M_4 are the corresponding theoretical relative intensities of the isotopic envelope of the peptide, which were calculated using MS-Isotope (http://prospector.ucsf.edu).

Using Direct-S, three proteins representing different abundance levels were quantified in serum digests without any pretreatment. We also used the MRM technique to analyze the three proteins in the serum sample by optimized transitions and corresponding collision energies (CE) as shown in supplemental table 1. For A1AT and LG3BP, every target peptide was detected by MRM, as shown in supplemental figure 1. However, no peak could be matched to a PCTSD peptide. In a recent MRM study[28], about 1000 yeast peptides can be quantified in one 60 min LC-MS experiment. Therefore, we can expect that, higher throughput and wider quantification range could be achieved using Direct-S.

Preparation of ^{18}O Heavy Isotope-Labeled Internal Standard Peptides

To obtain quantification references, the internal standard peptides were labeled with ^{18}O. The stable and completed incorporation of the heavy isotope are key factors for accurate quantification. The incorporation of ^{18}O is catalyzed by trypsin, and the stability is affected by trypsin-catalyzed back-exchange[29]. Therefore, the removal or

quenching activity of residual trypsin after labeling is important. In our study, immobilized trypsin was used to effectively reduce the back-exchange[29]. The stability of ^{18}O-labeled peptides was evaluated after the labeled peptides were stored at 4 ℃ for one week and -20 ℃ for one month. As shown in figure 2C and figure 2D, except for $P_{LG3BP-1}$ and $P_{LG3BP-2}$, which exhibited some back-exchange after being stored at -20 ℃ for one month, no significant back-exchange was observed in other conditions. As show in figure 2B, the MS peak of the unlabeled peptide was negligible, and only a few low-intensity peaks from peptides with one ^{18}O were observed, which indicated that the incorporation of ^{18}O was almost complete. The ^{18}O incorporation efficiencies $(1- I_0/(I_4 +I_2 +I_0))$ for P_{A1AT-1}, P_{A1AT-2}, $P_{LG3BP-1}$, $P_{LG3BP-2}$ and P_{CTSD} were 99.32%, 99.60%, 99.40%, 99.30% and 100%, respectively. Because of the impurity of the ^{18}O-labeled water, incomplete ^{18}O incorporation could not be avoided. The effect of incomplete ^{18}O incorporation was calculated as $I_2/(I_4 +I_2 +I_0)$. The values for P_{A1AT-1}, P_{A1AT-2}, $P_{LG3BP-1}$, $P_{LG3BP-2}$ and P_{CTSD} were 7.10%, 8.27%, 6.80%, 15.00% and 8.06%, respectively. As a result, the influence of incomplete labeling was weak (less than 15%). However, to ensure accurate quantification, we considered peptides with one ^{18}O in quantification as shown in equation 1. In conclusion, stable and high efficient ^{18}O incorporation qualified the reference peptides for use in quantification of samples.

Dynamic Range and Reproducibility of Direct-S

To assess the dynamic range of the quantification based on isotopic peak intensity ratios, 8 calibration mixtures with $^{16}O/^{18}O$ ratios ranging from 0.01 to 10 were

prepared. The amounts of ^{18}O-labeled peptides were fixed. The amounts of ^{16}O-labeled peptides were varied according to their endogenous concentration. For A1AT peptides, the concentration range was from 0.5 pmol to 0.5 nmol. For LG3BP peptides, the concentration range was from 50 fmol to 50 pmol. For CTSD peptides, the concentration range was from 20 fmol to 20 pmol. To construct a blank serum matrix without these three proteins, albumin, IgG, and transferrin, which are estimated to account for 82% of the protein in serum[24], were mixed in corresponding proportions as a serum analog. By replicate LC-MS/MS analyses of the calibration mixtures, the accuracy, reproducibility, and linear dynamic range were evaluated. The $^{16}O/^{18}O$ ratios were calculated with equation 1 above. The calibration curves for these five peptides are shown in figure 3. P_{A1AT-1} and $P_{LG3BP-1}$ had a linear response range from 0.1 to 10, likely either because of lower signal intensities or interference by the unlabeled peptides at lower spiked levels. P_{A1AT-2}, $P_{LG3BP-2}$, and P_{CTSD} exhibited a linear response in relative $^{16}O/^{18}O$ concentration ratios from 0.01 to 10. To evaluate the quantification accuracy of Direct-S, the quantitative deviations of the calibration mixtures were calculated by the formula "|(measured value – expect value) /expect value|". As a result, all the quantitative deviations were below 20.75%, which indicated that Direct-S could accurately quantify standard sample even in lower fetomole level.

The reproducibility of the quantification was evaluated among the replicates. The RSD of every ratio was calculated (Supplemental Table 2), and most of them were less than 10%, which indicated good quantification reproducibility of Direct-S.

Protein Biomarker Verification in CRC Serum Samples

The abundance of A1AT, LG3BP and CTSD was quantified in 15 cases of normal serum and 15 cases of CRC serum. The three proteins were reported with a significant expression difference between CRC and normal samples[25,26]. A1AT is a high-abundance protein found at the μg/μL level; LG3BP is a medium-abundance protein found at the ng/μL level; the abundance of CTSD is lower and found at the pg/μL level in serum[30]. The ^{18}O-labeled standard peptides were added to each serum sample at a fixed concentration. The amounts of ^{18}O-labeled peptides of A1AT were 50 ng, the amounts of ^{18}O-labeled peptides of LG3BP were 5 ng, and the amounts of ^{18}O-labeled peptide of CTSD were 2 ng. As shown in figure 4, the intensities of ^{18}O-labeled and ^{16}O-labeled P_{A1AT} were both high, while the intensities of ^{16}O-labeled were low for LG3BP and CTSD peptides, especially P_{CTSD}, which was too low to be selected for CID. As expected, the A1AT ^{18}O-labeled and ^{16}O-labeled peptides were both successfully identified by the database search (Supplemental Figure 2). However, for LG3BP and CTSD, not all of the ^{18}O-labeled and ^{16}O-labeled peaks could be identified because of their low abundance (Supplemental Figure 3 and Supplemental Figure 4). For LG3BP, the MS/MS spectra of serum peptides (^{16}O-labled) were poor quality (Supplemental Figure 3), and could not pass the threshold required for the database search. The distribution of high-intensity fragmented ion peaks was consistent with the MS/MS spectra of ^{18}O-labeled peptides. If the threshold for the database search was not set as high as described in experimental section, the top-ranking peptides of the unidentified spectra were assigned to the corresponding

LG3BP ^{16}O peptides (Supplemental Figure 5). This information could help to confirm the existence of the peptide. For CTSD, only the MS/MS spectra of the internal standard peptide (^{18}O-labeled) could be obtained. In this case, the elution time (15-18 min), paired $^{16}O/^{18}O$ ions (^{16}O, 410.72; single ^{18}O, 411.72; two ^{18}O, 412.72) was used to determine the peak of serum P_{CTSD}. To further confirm the identification of P_{CTSD}, the ion chromatographs of native (^{16}O-labeled) and ^{18}O-labeled standard peptides were extracted and are shown in supplemental figure 6. In addition, during the elution, the pattern (^{16}O, 410.72; single ^{18}O, 411.72; two ^{18}O, 412.72) was present in multiple consecutive MS1 scans as shown in supplemental figure 7. Using this information, ^{16}O-labeled peaks of P_{CTSD} were successfully identified in the MS spectra of 23 of the 30 serum samples. After identification, the peak intensity was extracted, and the absolute amounts of target proteins were calculated by equation 1. The absolute amounts were 0.57~2.44 μg/μl for A1AT, 14.16~30.99 ng/μl for LG3BP and 71-662 pg/μl for CTSD, which were the same as previously described[30]. Accordingly, Direct-S can reliably quantify serum proteins at the pg/μL level. A1AT, a member of the serpin protein superfamily, is a secreted protein and the most abundant circulating protease inhibitor in the serum. In a previous study[26], A1AT was demonstrated to be decreased and inactivated in CRC patients compared to that of healthy people. A1AT is highly abundant in serum and can be easily detected. Therefore, it would be a good choice as a candidate biomarker for CRC. LG3BP, also known as Mac-2-binding protein, is a secreted glycoprotein and a galectin-3 ligand. Increased levels of LG3BP have been reported in colon cancer[25,31]. The extracellular interactions between LG3BP

and galectin-3 could be governed by cell-type-specific glycosylation, which might contribute to enhanced malignancy[27]. CTSD is a lysosomal acid proteinase mainly involved in the metabolic degradation of intracellular proteins[26]. It has been reported to correlate with the progression and lymph node metastasis of CRC[32,33]. Consistent with previous reports[25,26,] A1AT was down-regulated while CTSD and LG3BP were up-regulated in CRC serum samples compared to healthy serum samples (Figure 5). These results further supported the reliability of Direct-S.

Experimental

Chemicals

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

Serum Samples, Standard Proteins and Synthetic Peptides

Serum samples from patients diagnosed with colorectal cancer and from healthy donors were supplied by the Shanghai Cancer Center of Fudan University, China. Serum samples were collected as previously described[34]. All candidates gave informed consent for the study, and samples were handled according to ethical and legal standards. The three standard proteins used in this study were obtained from Sigma-Aldrich, *i.e.*, serum albumin, human IgG, and human transferrin. In the experiment to quantify endogenous protein concentrations, target peptides were selected according to following rules. First, these peptides are unique peptides of the target proteins. Second, there are no post-translational modification sites and missed cleavage sites in the peptides, and should not include amino acids which may easily be modified during processing. Third, the peptides are easy to be ionized and fragmented and can be detected by mass spectrometry. Fourth, peptides should have nearly completed 18O-labeling. In addition, because the serum samples are too complicated, there should be no interference peptides to influence the detection of target peptides. Accordingly, five standard peptides were used, two (P_{AIAT-1}:

SASLHLPK, P_{AIAT-2}: LQHLENELTHDIITK) of which were for A1AT, two ($P_{LG3BP-1}$: ELSEALGQIFDSQR; $P_{LG3BP-2}$: ASHEEVEGLVEK) of which were for LG3BP, and one (P_{CTSD}: VGFAEAAR) was for CTSD. All peptides were purchased from Shanghai ChinaPeptides Co., Ltd. with over 99% purity.

Preparation of Internal Standard Peptides with ^{18}O Stable Isotope Labeling

Internal standard peptides were labeled with immobilized trypsin. The immobilized trypsin was prepared as previously described[35]. The peptide samples (10 μg) were lyophilized to dryness and reconstituted in 20 μl of 50 mM NH_4HCO_3 in $H_2{}^{18}O$ (97%; Cambridge Isotope Laboratories, Inc. USA) containing 10 mM $CaCl_2$ and 20% acetonitrile (ACN). The immobilized trypsin was added at a 1:10 trypsin/peptide ratio (w/w). The combined solution was incubated for 24 h at 37 ℃ in the dark. The reaction was stopped by the removal of immobilized trypsin and boiling the sample in a water bath for 10 min. To test the labeling efficiency, the labeled peptides were mixed with α-cyano-4-hydroxycinnamic acid, spotted on a matrix-assisted laser desorption ionization (MALDI) target plate, and analyzed with a 5800 mass spectrometer (Applied Biosystems, Framingham, USA).

Protein Digestion

Serum samples were initially denatured with 8 M urea, 50 mM NH_4HCO_3. And then reduced with 10 mM dithiothreitol in 50 mM NH_4HCO_3 buffer for 1 h at 37 ℃. They were then alkalized with 20 mM iodoacetamide for 0.5 h at 37 ℃ in the dark.

Following a 5-fold dilution with 50 mM NH_4HCO_3, each sample was digested separately with trypsin at a protein-to-trypsin ratio of 30:1 (w/w) for 16 h at 37 ℃. The digestion reaction was stopped by adding 0.1% trifluoroacetic acid (TFA). The digested peptides were desalted on a C18 column (Waters Milford, USA) and lyophilized. ^{18}O-labeled internal standard peptides were added to serum peptides before LC-MS/MS analysis.

Preparation of Calibration Mixtures

Serum analogs of a peptide mixture of bovine serum albumin, human IgG, and human transferrin were used as the matrix to prepare calibration mixtures. ^{18}O-labeled and -unlabeled standard peptides mixed at ratios of 100:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10 (w/w) were spiked into the matrix to generate the calibration mixtures. To ensure the lower-abundance proteins were in the linear range, a 100:1 mixture was prepared. The amounts of ^{18}O-labeled peptides were fixed (peptides of A1AT: 50 ng, peptides of LG3BP: 5 ng, peptide of CTSD: 2 ng). The ratios were achieved by varying the amount of unlabeled peptides. All mixtures were analyzed by LC-MS/MS with three times.

LC-MS/MS Analysis

All samples were analyzed on a nanospray reverse-phase LC system coupled online with a high-accuracy mass analyzer linear ion trap quadrupole (LTQ)-Orbitrap (Thermo Electron Corporation, San Jose, USA). The LC system consisted of 2

LC-20AD nano-flow LC pumps and 1 LC-20AB micro-flow LC pump (all from Shimadzu Corporation, Tokyo, Japan). Sample injection was performed via an SIL-20 AC auto-sampler (Shimadzu Corporation, Tokyo, Japan) and loaded onto a CAPTRAP column (0.5 x 20 mm, MICHROM Bioresources Inc., Auburn, USA) over 5 min at a flow rate of 60 μL/min. The sample was subsequently separated by a PICOFRIT C18 reverse-phase column (0.075 x 100 mm, New Objective Inc., Woburn, USA) at a flow rate of 500 nL/min. The mobile phases consisted of 5% ACN with 0.1% formic acid (FA) (phase A and the loading phase) and 95% ACN with 0.1% FA (phase B). To achieve proper separation, a 120-min linear gradient from 5 to 45% phase B was employed. The separated sample was introduced into the mass spectrometer via a 15 μm silica tip (New Objective Inc., Woburn, USA) adapted to a DYNAMIC nano-electrospray source (Thermo Electron Corporation, San Jose, USA). The spray voltage was set at 1.9 kV and the heated capillary at 220 ℃. The mass spectrometer was operated in data-independent mode, and each cycle of duty consisted of one full-MS survey scan at the mass range of 350~1800 Da with resolution power of 60,000 using the Orbitrap section followed by MS/MS experiments for predefined m/z values, which were put in the “parent mass list”, using the LTQ section. The Automatic Gain Control expectation during full-MS and MS/MS was 500000 and 10000, respectively. Peptides were fragmented in the LTQ section using collision-induced dissociation with helium, the normalized collision energy value was set at 35%, and previously fragmented peptides were excluded for 30 s.

Data Analysis

All spectra were searched against the human Uniprot database (release 2009–02 with 20,331 entries) augmented with reversed sequences using SEQUEST (v.28 (revision 12), Thermo Electron Corp.). The search parameters were as follows: full tryptic cleavage with two missed cleavage sites; fixed modification of cysteine carboxymethylation (+57.0215 Da), variable modifications of methionine oxidation (+15.9949 Da) and double incorporation of ^{18}O at the peptide C terminus (+4.0085 Da); mass tolerance of 50 ppm for precursor ions and 1.0 Da for fragment ions. Following the database search, Trans Proteomic Pipeline software (revision 4.2) (Institute of Systems Biology, Seattle, WA) was utilized to identify peptides and proteins based on Peptide Prophet probability with a p value over 0.90 and Protein Prophet probability with a p value over 0.95. The false discovery rate was limited to less than 1%.

Conclusion

Direct-S, which combines ^{18}O-labeling with high-sensitivity and high-resolution mass spectrometry (LTQ-Orbitrap), was used to quantify the absolute amounts of candidate biomarkers in native serum samples. In Direct-S, ^{18}O-labeled peptides exhibited good stability and high labeling efficiency. Repeatable and good quantification linearity over three orders of dynamic range was achieved. Using Direct-S, three candidate biomarkers representing different abundance levels, A1AT, LG3BP and CTSD, were reliably quantified in CRC serum samples. Consistent with previous reports, A1AT was demonstrated to be downregulated while CTSD and LG3BP were upregulated in CRC serum samples, which further confirmed the quantification reliability of Direct-S. The application in CTSD indicated that Direct-S could reliably quantify serum proteins down to the pg/μL level. In conclusion, Direct-S is a low cost, reliable and convenient preparation strategy for verifying serum biomarkers.

Conflict of interest

All authors have declared no conflict of interest.

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Figure

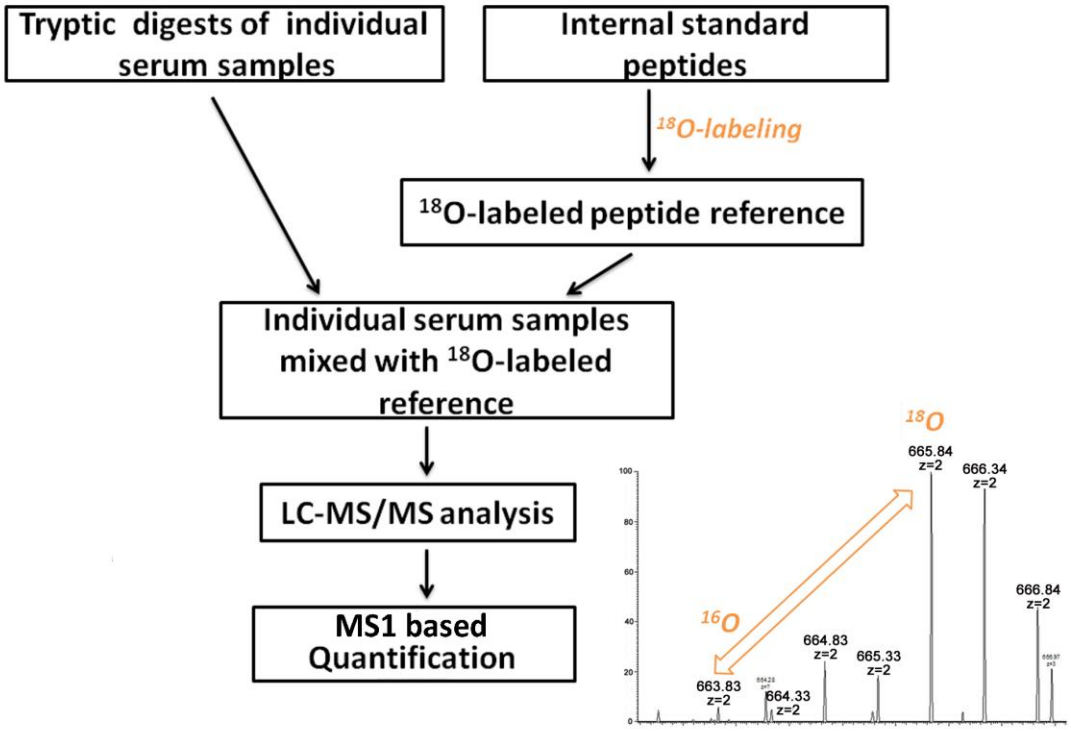

Figure 1. The scheme of Direct-S. Serum samples were digested directly without any pretreatment. ^{18}O-labeled standard peptides were added as a reference for quantification. The high-sensitivity and high-resolution LTQ-Orbitrap mass spectrometer was used for MS analysis. The amounts of serum peptides (^{16}O peptides) were determined by comparison with ^{18}O-labeled standard peptides.

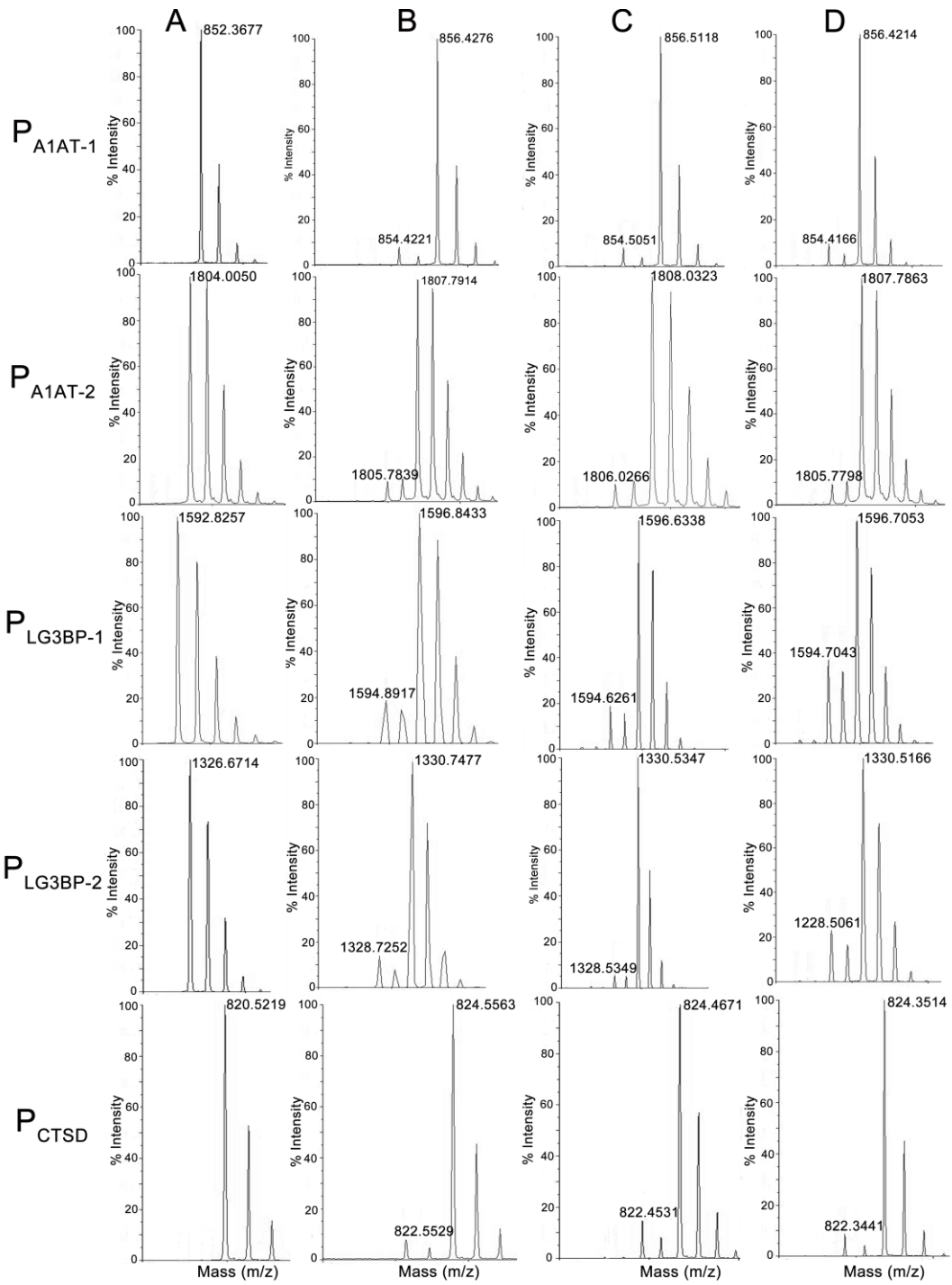

Figure 2. MS spectra of unlabeled and18O-labeled standard peptides in different storage conditions. (A) Unlabeled standard peptides; (B) ^{18}O-incorporated peptides sent for MS analysis immediately; (C) ^{18}O-incorporated peptides kept in buffer (50% ACN in 0.1% TFA) for one week at 4 ℃; (D) ^{18}O-incorporated peptides kept in buffer (50% ACN in 0.1% TFA) for one month at -20 ℃.

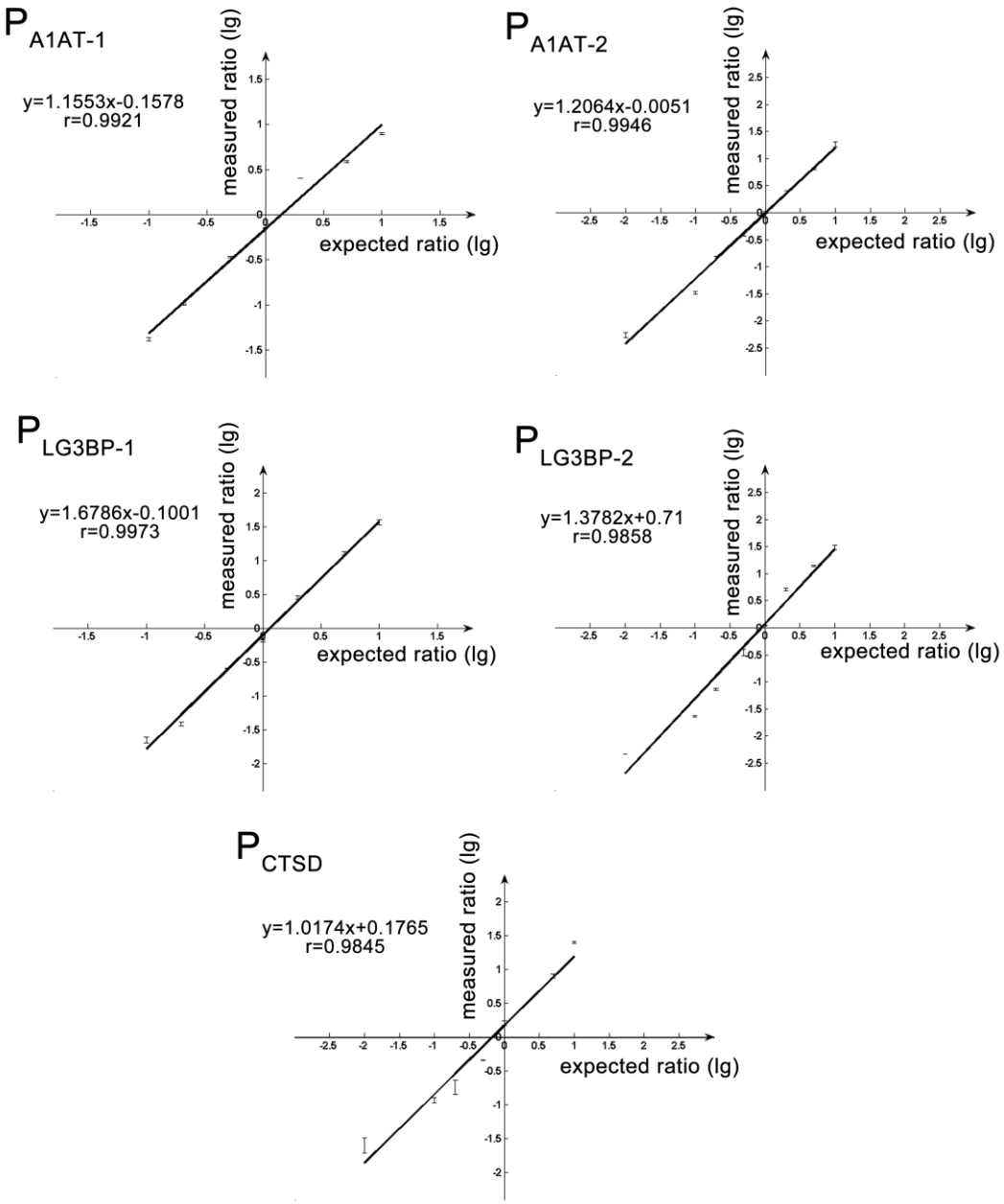

Figure 3. Calibration curves of five standard peptides. The average ratios were calculated and plotted with error bars representing standard deviation based on three replications.

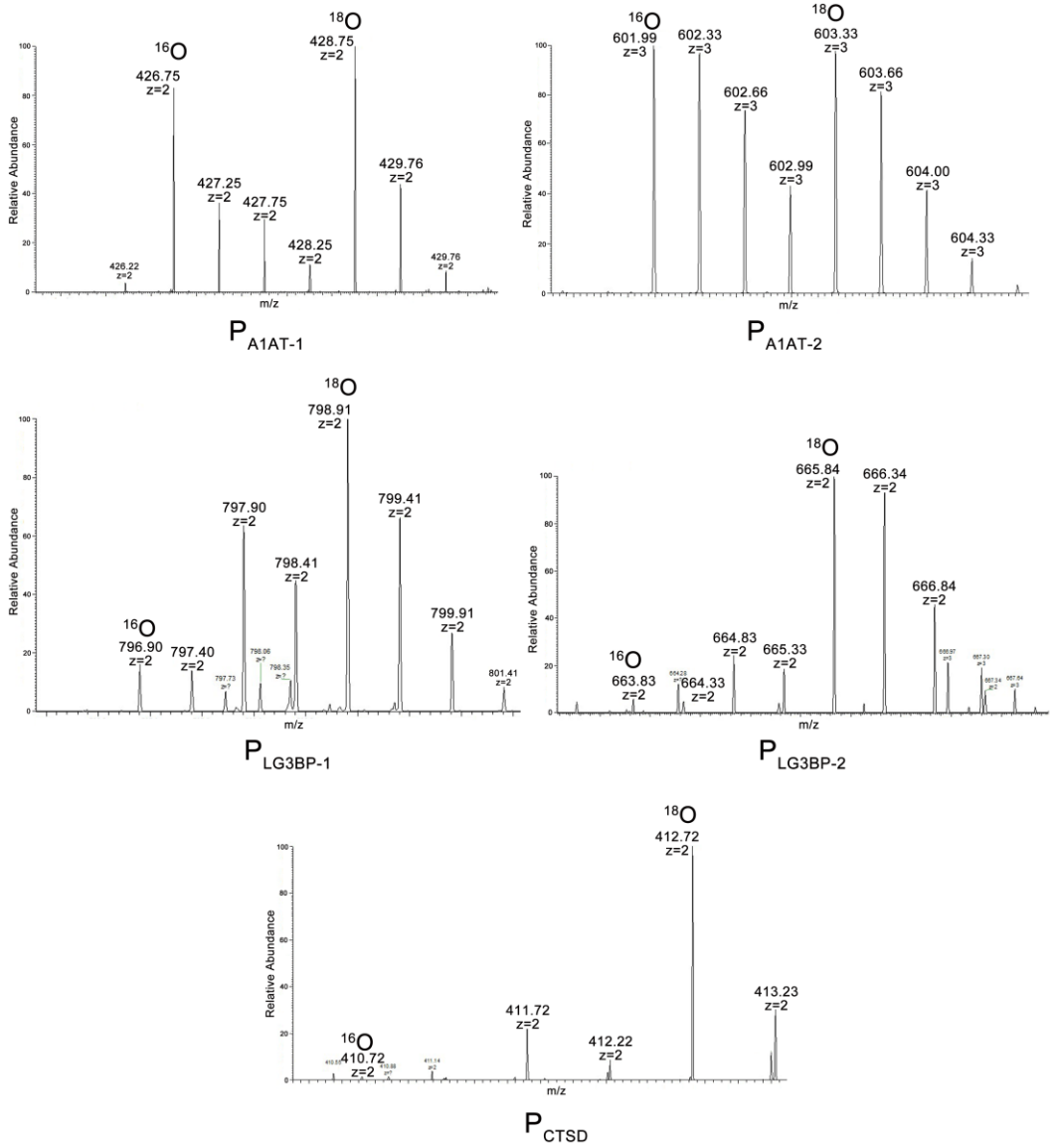

Figure 4. MS spectra of five target peptides in serum samples. Peaks marked with ^{16}O indicate monoisotopic peaks of serum peptides. Peaks marked with ^{18}O indicate monoisotopic peaks of standard peptides.

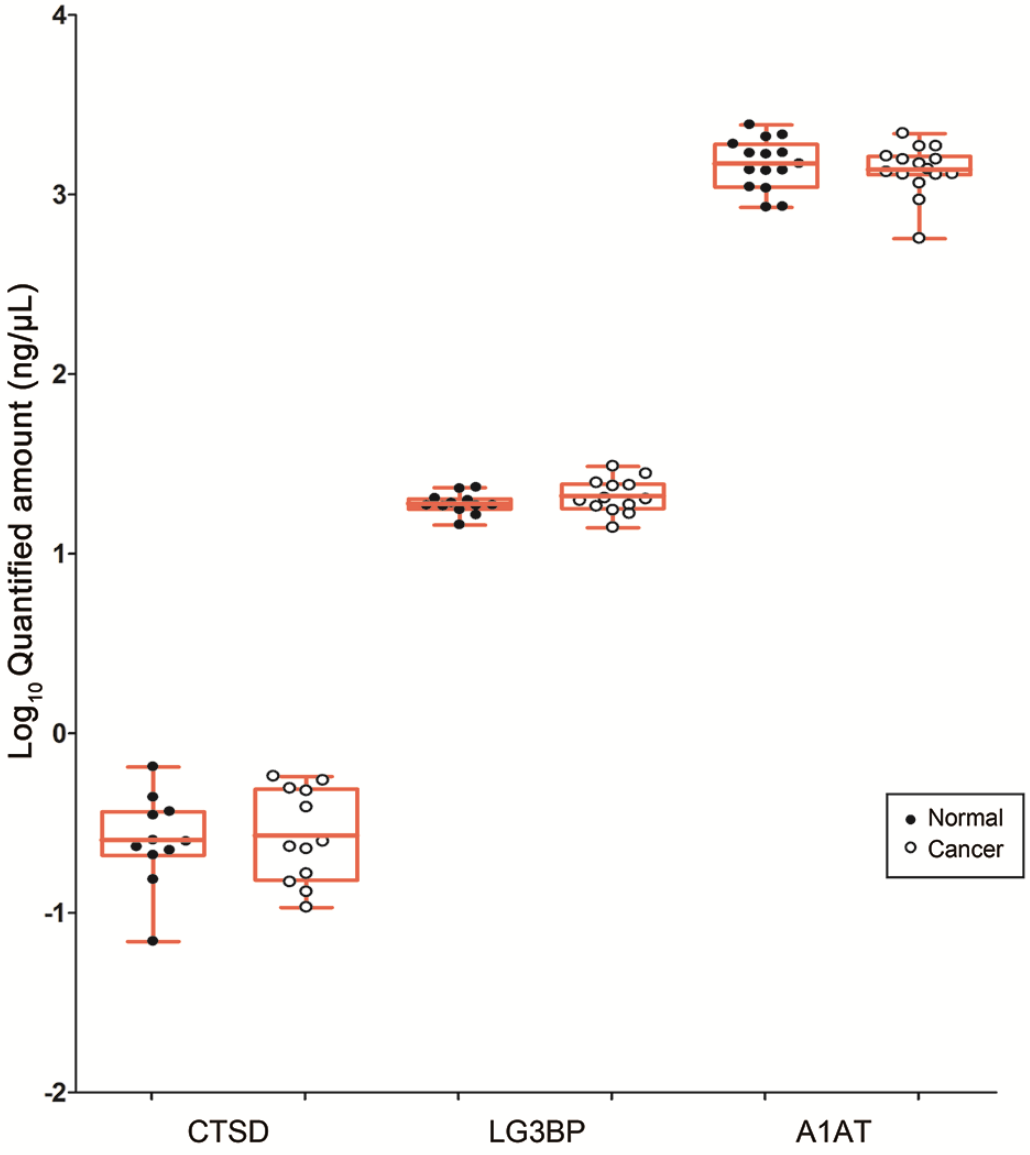

Figure 5. Concentration distribution of CTSD, LG3BP and A1AT in normal and CRC serum samples.