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A novel quantitative proteomics workflow by isobaric terminal labeling

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ABSTRACT

Quantification by series of b, y fragment ion pairs generated from isobaric-labeled peptides in MS2 spectra has recently been considered an accurate strategy in quantitative proteomics. Here we developed a novel MS2 quantification approach named quantitation by isobaric terminal labeling (QITL) by coupling ^{18}O labeling with dimethylation. Trypsin-digested peptides were labeled with two ^{16}O or ^{18}O atoms at their C-termini in $H_2^{16}O$ or $H_2^{18}O$. After blocking all ε-amino groups of lysines through guanidination, the N-termini of the peptides were accordingly labeled with formaldehyde-d_2 or formaldehyde. These indistinguishable, isobaric-labeled peptides in MS1 spectra produce b, y fragment ion pairs in the whole mass range of MS2 spectra that can be used for quantification. In this study, the feasibility of QITL was first demonstrated using standard proteins. An accurate and reproducible quantification over a wide dynamic range was achieved. Then, complex rat liver samples were used to verify the applicability of QITL for large-scale quantitative analysis. Finally, QITL was applied to profile the quantitative proteome of hepatocellular carcinoma (HCC) and adjacent non-tumor liver tissues. Given its simplicity, low-cost, and accuracy, QITL can be widely applied in biological samples (cell lines, tissues, and body fluids, etc.) for quantitative proteomic research.

1. Introduction

Systematically and accurately detecting the quantitative changes in protein profiles of different cell lines, tissues, or body fluids is becoming increasingly important in biological and biomedical research. “Shotgun” mass spectrometry (MS) coupled with stable isotope labeling [1,2] has become a more powerful tool in quantitative proteomics over the past decade, compared with traditional two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)-based methods [3]. Generally, MS-based quantitative methods can be divided into two categories corresponding to the stage at which the mass spectra peptides are quantified. The first category is MS1 quantification, which uses MS1 data for protein quantification and MS2 data for protein identification. Alternatively, MS2 quantification uses MS2 data for simultaneous protein identification and quantification.

In the MS1 quantification method, quantitative ratios of protein are obtained by comparing the intensities or areas of the lightly and heavily labeled peptides from different samples, such as: stable isotope labeling by amino acids in cell culture (SILAC)/amino acid coded mass tagging (AACT) [4,5]; proteolytic ^{18}O-labeling [6]; isotope-coded affinity tags (ICAT) [7]; and dimethyl labeling [8]. These methods, however, feature noteworthy drawbacks. First, MS1 spectra are often filled with background noise and unassigned peaks, signifi-

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cantly decreasing the signal-to-noise ratio and limiting the accuracy and dynamic range of quantification. Second, MS1 signals of the same peptides from different samples split into two or more peaks, leading to increased complexity and reduced sensitivity of MS1 spectra as well as aggravation of the undersampling problem. Furthermore, co-eluting isobaric peptides or contaminants can heavily interfere with the detection of target peptides when samples are highly complex.

The aforementioned drawbacks associated with MS1 quantification are successfully addressed in MS2 quantification methods. MS2-based quantitative approaches can be specifically classified into two subtypes corresponding to how the peptides are labeled. The first subtype is mass-difference MS2 quantification, wherein lightly and heavily labeled peptides with distinct mass differences in the MS1 spectra are simultaneously selected to generate b, y fragment ion pairs in the MS2 spectra, allowing for protein identification and quantification through the application of a wide precursor isolation window in mass spectrometry performance. Metabolic ^{15}N labeling [9], SILAC [10], and ^{18}O-labeling [11] have been combined with wide isolation window to realize this strategy. Improvements in accuracy, precision, and dynamic range have been observed compared with MS1 quantification approaches. However, the enlarged precursor isolation window (10 *m/z*) in these methods also increases the possibility of interfering peptide ions and chemical noise inclusion in the collision cell, which adversely affects protein identification and quantification.

The second subtype is isobaric MS2 quantification, which not only effectively overcomes the drawbacks of MS1 quantification but also addresses the issues of mass-difference MS2 quantification. These methods produce isobaric precursor peaks in MS1 spectra, but produce specific mass tags or b, y fragment ion pairs in MS2 spectra through several ingenious experimental designs. For example, isobaric tagging methods of iTRAQ [12,13] and TMT [14,15], have the same precursor peaks but specific mass tags (113–119, and 121 for iTRAQ or 126–131 for TMT) in low mass range of MS2 spectra, which can be used for protein quantification of up to 8 samples, reducing overall analysis time and experimental variance. However, costly TMT and iTRAQ reagents and the "one-third rule" of ion trap mass spectrometry [16] limit wide application of these methods. Even worse, the repression effect on specific mass tags observed in low mass range dramatically affects the accuracy and dynamic range of these methods [17].

A novel isobaric MS2 quantification strategy using b, y fragment ion pairs in the whole mass range of the MS2 spectra for quantification – instead of mass tags in low mass range of the MS2 spectra – was recently established. This strategy primarily aims to achieve isobaric labeling of peptides that show the same molecular weight in MS1 spectra, yet exhibit b, y fragment ion pairs in MS2 spectra. For example, isobaric peptide termini labeling (IPTL) was developed to realize complementary labeling of the N- and C-termini of the Lys-C-digested peptides through a two-step reaction with isotopic chemical reagents [18,19]. However, compared with trypsin-digested peptides, the obtained peptides ending with lysine in IPTL were longer, highly charged, and poorly identified by collision-induced dissociation (CID) in mass spectrometry [20]. In addition, those peptides derived from the missed cleavage of Lys-C cannot be used for quantification. In vivo termini amino acid labeling (IVTAL), combining a set of heavy amino acid $^{13}C_6$-arginine and $^{13}C_6$-lysine in cell culture and enzymes of Lys-N and Arg-C, was also used to yield isobaric peptides [21]. However, IVTAL is limited to cell culture systems. Index-ion triggered MS2 ion quantification (iMSTIQ), based on cell culture with $^{15}N_4$-arginine and $^{13}C_6^{15}N_2$-lysine and peptide labeled by mTRAQ reagents, was developed for target quantitative proteomics analysis [22]. It is also limited to cell samples, and the expensive mTRAQ reagents prohibit its wide application.

To overcome the aforementioned drawbacks, we developed a novel strategy named quantitation by isobaric terminal labeling (QITL) to obtain b, y fragment ion pairs for quantification. This strategy was applied to profile proteome of hepatocellular carcinoma (HCC) and adjacent non-tumor liver tissues, showing a promising future in quantitative proteomics with its high accuracy, unlimited samples, low cost, and easy manipulation.

2. Experimental section

2.1. Materials and reagents

All reagents, as well as standard proteins of myoglobin, cytochrome c, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Water was obtained from a Milli-Q Integral water purification system (Millipore, Billerica, MA).

2.2. Sample preparation

An adult male Sprague–Dawley rat was obtained from the laboratory animal housing of Shanghai Medical College, Fudan University (Shanghai, China). Paired HCC and adjacent non-tumor liver tissues were obtained from ten HCC patients who underwent surgical resection at the Zhongshan Hospital of Fudan University (Shanghai, China). All patients were provided informed consent for their participation, and Institutional Review Board of the hospital approved this study. Detailed pathological information of these samples is shown in Table S1.

Protein extraction from rat liver and HCC samples was performed as follows. The livers were promptly removed and placed in an ice-cold phosphate-buffered saline (PBS). After repeated mincing with scissors and washing to remove blood, the livers were lysed using lysis buffer of: 7 M urea, 2 M thiourea, 1 mM phenylmethanesulfonyl fluoride, 50 mM dithiothreitol (DTT), as well as phosphatase and protease inhibitors (Complete tablets; Roche Diagnostics). The lysate was sonicated for three cycles of 5 s each and centrifuged at 14,000×*g* for 30 min to collect the supernatant. The supernatant protein concentration was determined through Bradford assay.

2.3. Protein digestion and peptide labeling of QITL

Standard proteins, proteins extracted from rat liver or human liver, were suspended in solution and denatured at 100 °C for

10 min, respectively. After being cooled down to room temperature, the sample was reduced by 10 mM DTT at 37 °C for 1 h, then alkylated by 25 mM iodoacetamide in the dark at room temperature for 45 min, and finally precipitated by ice acetone overnight. After centrifugation and supernatant removal, the pellet was re-dissolved and digested with trypsin with a ratio of 1:50 (w/w) in 25 mM ammonium bicarbonate at 37 °C for 18 h.

Then, $^{18}O_2$-labeling of the tryptic peptides was performed using a previously described protocol with a slight modification [23]. Two equal aliquots of trypsin-digested peptides were lyophilized in a vacuum centrifuge (Eppendolf, Hamburg, Germany), then re-suspended in $H_2^{16}O$- or $H_2^{18}O$-prepared reaction buffer containing ammonium acetate (100 mM, pH 6.0) and trypsin (1:25, w/w). For HCC samples, the aliquot of trypsin-digested peptides of HCC tissues was suspended in $H_2^{18}O$ buffer and that of adjacent non-tumor tissues was suspended in $H_2^{16}O$ buffer. After incubation for 24 h at 37 °C, the two aliquots were boiled for 10 min at 100 °C and snap frozen at −80 °C briefly to deactivate residual trypsin.

The $^{16}O_2/^{18}O_2$-labeled sample was lyophilized and guanidinated following a previously described protocol [24]. The ε-amino groups of lysines in the $^{16}O_2/^{18}O_2$-labeled sample were blocked by adding O-methylisourea (2 M in 100 mM sodium bicarbonate), adjusted to pH 11 with sodium hydroxide (2 M), and incubated at 65 °C for 15 min. Then, 10% trifluoracetic acid (TFA) solution was added to terminate the reaction. Next, both aliquots were lyophilized for dimethyl labeling of the N-termini of these guanidinated peptides according to a previous protocol [25]. The lyophilized $^{16}O_2/^{18}O_2$-labeled peptides with blocked lysines were re-dissolved in sodium acetate buffer (100 mM, pH 5–6), mixed with freshly prepared 4% formaldehyde-d_2/formaldehyde solution, vortexed immediately, and then mixed with freshly prepared sodium cyanoborohydride (600 mM). Afterward, the mixtures were incubated in a fume hood for 1 h at room temperature. Then, 4% ammonium hydroxide was added and incubated to quench the reaction.

Finally, two differently labeled aliquots of standard proteins were mixed at varying ratios of 1:10, 1:8, 1:4, 1:2, 1:1, 2:1 4:1, 8:1, and 10:1, purified with μ-C18 ZipTips (Millipore, Billerica, MA), and analyzed using MALDI-MS. Differently labeled rat liver or HCC samples were desalted and separately analyzed by 1D low pH RPLC-ESI-MS/MS or 2D RPLC (a high pH in the first dimension and a low pH in the other)-ESI-MS/MS under the mixed ratio of 1:1.

All experiments were repeated thrice to guarantee technical reproducibility.

2.4. MALDI-TOF/TOF-MS

The MALDI-TOF/TOF™ 5800 System (AB SCIEX, Foster City, CA) equipped with a new 1 kHz OptiBeam™ on-axis laser was used. Acquisitions were performed in positive ion reflection mode. All mass spectra were obtained with an *m/z* scan range of 700 to 3600 (1000 shoots with a laser intensity of 3500). After selecting the top 20 precursors, MS2 spectra (3000 shoots with a laser intensity of 4500) were achieved by 2 kV CID using air as collision gas. Internal calibration was performed with trypsin-digested peptides of standard myoglobin protein with known molecular masses. For sample spotting, the peptide solution [0.5 μL in 50% acetonitrile (ACN)/0.1% TFA] was deposited onto the MALDI plate and dried, then the matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA, 0.5 μL) was deposited onto the peptide samples.

2.5. 2D high pH-low pH RPLC-ESI-MS/MS

The labeled peptides from the HCC samples were reconstituted in buffer A [10 mM ammonium formate (NH_4FA), pH 10] and injected onto Sepax PolyRP-300 column (5 μm, 300 Å, 2.1×150 mm) (Sepax Technologies, Newark, DE) using an LC-20AD high performance LC system (Shimadzu, Tokyo, Japan) for the first dimensional high pH-RP chromatography separation. Peptides were separated at room temperature with a flow rate of 200 μL/min and eluted from the column with a 40 min gradient from 0 to 50% buffer B (90% ACN/10% 10 mM NH_4FA, pH 10), followed by a 4 min gradient from 50% to 80% buffer B. A total of 24 fractions were collected based on UV absorbance at 214 nm. All fractions were lyophilized and reconstituted in 20 μL of 0.1% formic acid for the second dimensional low pH RPLC-ESI-MS/MS analysis.

The collected fractions of labeled HCC samples, as well as the labeled rat liver samples, were individually injected to a nano ACQUITY UPLC system (Waters Corporation, Milford) connected to an LTQ-Orbitrap XL mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with an online nano-electrospray ion source (Michrom Bioresources, Auburn). Peptide separation was performed in a Captrap Peptide column and a 100 μm i.d.×15 cm reverse phase column (Michrom Bioresources, Auburn). The peptide mixtures were injected onto the trap-column with a flow of 20 μL/min for 5 min, and subsequently eluted with a three-step linear gradient from 5% to 45% phase B in 95 min (phase A, water with 0.1% FA; phase B, ACN with 0.1% FA), then increasing to 80% phase B in 5 min, and finally maintained at 80% phase B for 5 min. The column was re-equilibrated at initial conditions for 15 min. Flow rate was maintained at 500 nL/min and column temperature was maintained at 35 °C. An electrospray voltage of 2.0 kV versus the mass spectrometer inlet was used. The LTQ-Orbitrap XL mass spectrometer was operated in data-dependent mode to switch automatically between MS1 and MS2 acquisition. MS1 scan with one microscan (*m/z* 400 to 1800) was acquired in the Orbitrap with a mass resolution of 60,000 at *m/z* 400, followed by eight sequential LTQ-MS/MS scans. Dynamic exclusion was used with two repeat counts: a 10 s repeat duration, and a 90 s exclusion duration. For MS2, precursor ions were activated using 35% normalized collision energy at the default activation *q* of 0.25.

2.6. Data analysis and interpretation

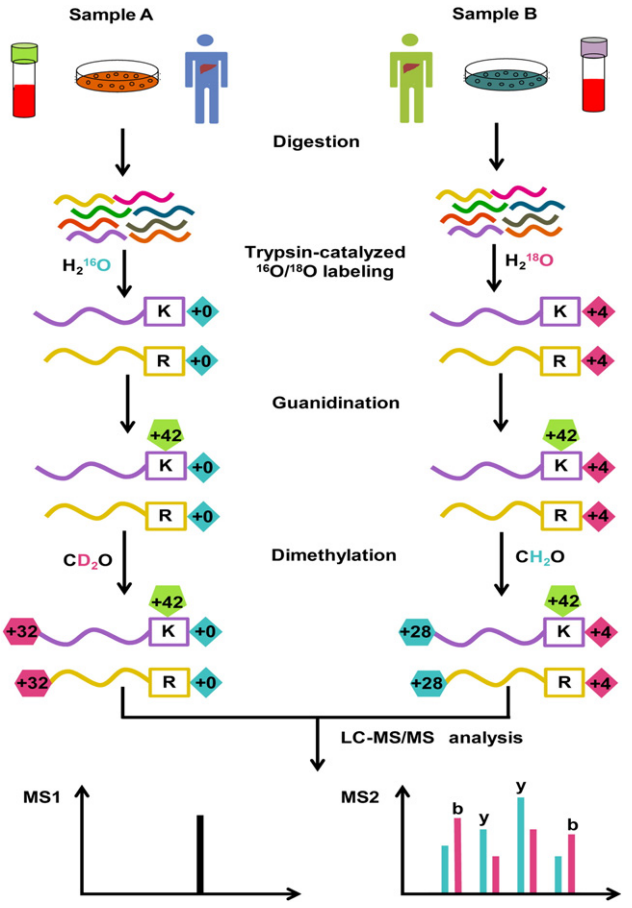
All MS2 spectra were searched twice against the rat UniProt database (release 2009–03 with 7296 entries) or human UniProt database (release 2009–02 with 20,331 entries) augmented with the reversed sequences using SEQUEST [v.28 (revision 12), Thermo Electron Corp.]. The same parameters of the two searches were set up as follows: partial tryptic cleavage with two missed cleavage sites; mass tolerance of

50 ppm for precursor ions and 1.0 Da for fragment ions; as well as fixed modification on cysteine (+57.0215 Da) and lysine (+42.0218 Da) and variable modification on methione (+15.9949 Da). For the first database search, N-termini (+32.0564 Da) for $^{16}O_2$- and formaldehyde-d_2-derivatized peptides were set up as fixed modification, whereas for the second database search, C- (+4.0085 Da) and N- (+28.0313 Da) termini for $^{18}O_2$- and formaldehyde-derivatized peptides were set up as fixed modification. After database searches, Trans Proteomic Pipeline software (revision 4.2) (Institute of Systems Biology, Seattle, WA) was utilized to identify peptides and proteins based on the Peptide Prophet probability with a *p*-value over 0.90 and the Protein Prophet probability with a *p*-value over 0.95. False discovery rate was limited to less than 1%. Results of the two database searches were combined to determine the identified peptides and proteins for the next quantification. Intensity values of b, y fragment ions of the identified peptides were extracted from the .DTA files generated by SEQUEST for ratios calculating using several in-house built scripts edited by Perl (version 5.10) and MatLab (version 7.10) [21]. The outlier data points of the ratios of b, y fragment ion pairs were removed using box plot. The ratio of a peptide was the mean of ratios of the assigned b, y fragment ion pairs in MS2 spectra, while the ratio of a protein was the mean of ratios of all quantified peptides.

3. Results and discussion

3.1. *Strategy of quantitation by isobaric terminal labeling*

The basic idea of QITL strategy is the complementary isotopic labeling of the C- and N-termini of the trypsin-digested peptides to obtain the isobaric-labeled peptides used to produce b, y fragment ion pairs in MS2 spectra for protein quantification. The outline is shown in Scheme 1. First, proteins extracted from samples A or B were digested using trypsin to generate peptides ending with lysine or arginine at their C-termini. These peptides were then respectively labeled in $H_2^{16}O$ or $H_2^{18}O$ to incorporate two ^{16}O or ^{18}O atoms at their C-terminus. After blocking all ε-amino groups on lysines in these peptides through guanidination with O-methylisourea, their N-termini were accordingly dimethylated with sodium cyanoborohydride and formaldehyde-d_2 or formaldehyde. Next, the labeled peptides were mixed and subjected to LC-MS/MS analysis. These labeled peptides from two different samples co-eluted during LC separation and exhibited isobaric peaks in MS1 spectra because of their identical physicochemical properties and molecular weights. After CID fragmentation, they yielded b, y fragment ion pairs with a mass difference of 4 Da in MS2 spectra, and their intensity ratios could be used for relative quantification of peptides and proteins. The lower mass ion of every b fragment ion pair was from formaldehyde-derivatized sample B and the higher mass ion was from formaldehyde-d_2-derivatized sample A, and vice versa for every y fragment ion pair. Based on multiple quantitative data points of b, y fragment ion pairs over the full mass range in MS2 spectra, QITL provides an accurate and reproducible quantification while remaining compatible with all varieties of mass spectrometers. Furthermore, trypsin-digested peptides of protein samples from any source can be easily labeled with commercially inexpensive isotopic reagents in QITL, making it an ideal choice for quantitative proteomics research.



Scheme 1 – Schematic of the QITL strategy. First, proteins extracted from different cell lines, tissues, or body fluids were digested with trypsin. Then, the C-termini of trypsin-digested peptides were separately incorporated into two ^{16}O or ^{18}O atoms by $^{16}O_2$ or $^{18}O_2$-labeling. After blocking all ε-amino groups of lysines of the $^{16}O_2$ or $^{18}O_2$-labeled peptides through guanidination, the N-terminal amino groups of the modified peptides were accordingly dimethylated with formaldehyde-d_2 or formaldehyde. The mixed labeled peptide counterparts showed the same molecular weight in MS1 spectra, but multiple b, y fragment ion pairs with a mass difference of 4 Da were exhibited over the full mass range in MS2 spectra. The intensity ratios of these b, y fragment ion pairs were calculated to provide accurate and reliable quantitative information for the peptides and proteins derived from sample A and B.

3.2. *Feasibility of QITL*

Two identical aliquots of trypsin-digested peptides derived from myoglobin were separately labeled as described in Scheme 1 and analyzed by MALDI-TOF/TOF-MS to demonstrate the feasibility of the proposed strategy. The MALDI-TOF

spectra and corresponding peptide sequences of each step are shown in Fig. 1 and Table S2. Guanidination of the $^{16}O_2$-labeled trypsin-digested peptides of myoglobin by O-methylisourea caused a mass shift of 42 Da for each lysine in these peptides (Fig. 1B vs. A). Aside from the sequence coverage of myoglobin increased from 64% to 99%, eight peptides that were previously unidentified were observed in the present MS1 spectra after guanidination (Table S2). Moreover, the intensities of the most guanidinated lysine-containing peptides increased, facilitating MS1 signal detection in our study. This phenomenon is consistent with the previous studies describing the basicity of modified peptides increasing along with lysine conversion into homoarginines in MALDI-MS [26]. The guanidinated $^{16}O_2$-labeled peptides were then dimethylated with formaldehyde-d_2, leading to an additional 32 Da mass shift (Fig. 1C). No significant changes in signal intensities and the sequence coverage were observed after dimethylation (Fig. 1B vs. C, Table S1), which may be ascribed to extremely minute changes in their ionic state [8]. Similarly, the MS1 spectra of the $^{18}O_2$-labeled peptides showed that almost all trypsin-digested peptides were incorporated into two ^{18}O atoms, resulting in a 4 Da mass shift when compared with their $^{16}O_2$-labeled counterparts (Fig. 1D vs. A). After guanidination of the $^{18}O_2$-labeled peptides and the subsequent mass shift of 42 Da to each lysine (Fig. 1E), dimethylation with

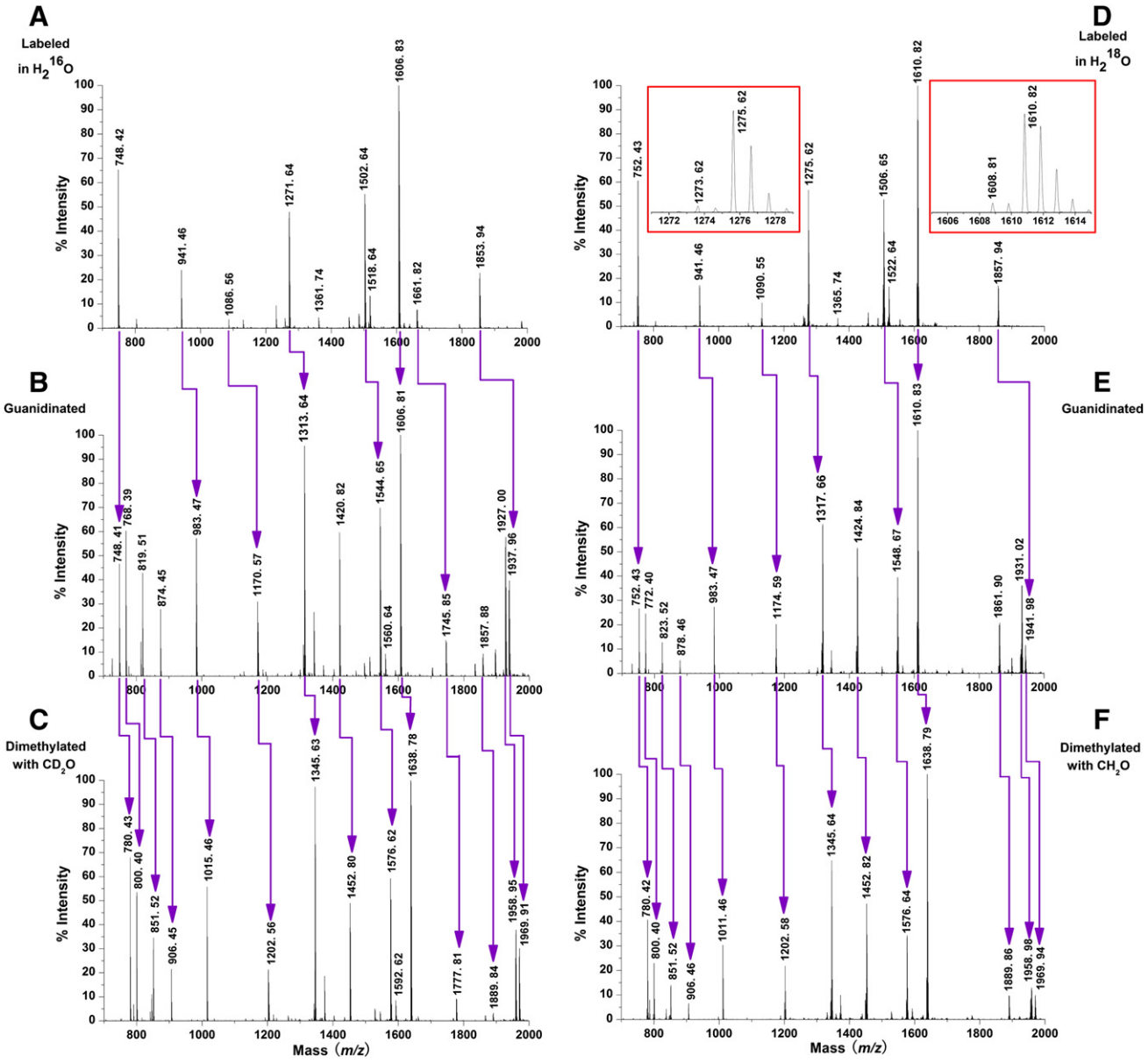


Fig. 1 – MALDI-TOF MS1 spectra of the myoglobin digest during different stages of the QITL method. The peptide mass fingerprint of the tryptic peptides labeled in $H_2^{16}O$ and $H_2^{18}O$ are shown in (A) and (D). The insets of Fig. 1D demonstrate the completeness of the $^{18}O_2$-labeling for the LFTGHPETLEK (*m/z* 1271.64) and VEADIAGHGQEVLIR (*m/z* 1606.83) peptides. Guanidination of these $^{16}O_2$ or $^{18}O_2$-labeled tryptic peptides caused a 42 Da mass shift for every lysine in the peptides (B and E). The N-termini of the modified peptides were dimethylated with formaldehyde-d_2 or formaldehyde, leading to an additional mass shift of 32 Da or 28 Da for all the peptides. The isobaric-labeled peptides were obtained after the QITL method (C and F).

formaldehyde caused another 28 Da mass shift (Fig. 1F). Finally, these two initial identical trypsin-digested peptides turned into isobaric-labeled peptides after derivatization (Fig. 1C vs. F). This strategy is also effective for peptides with missed cleavage sites, such as the HLKTEAEMK peptide (*m/z* 1086.56) that can be separately labeled to generate isobaric peptides of $H^{(+32)}LK^{(+42)}TEAEMK^{(+42)}$ and $H^{(+28)}LK^{(+42)}TEAEMK^{(+42+4)}$ (*m/z* 1202.58) with the same molecular weight in MS1 (Fig. 1C vs. F).

Completeness of reaction and elimination of side reactions are primary concerns for any chemical derivatization method. In the proposed strategy, $^{18}O_2$-labeling plays a key role in the workflow because of its high completeness and high specificity as well as the rarity of by-products during guanidination [24] and dimethylation [8]. Based on previous literature [27], an optimized condition of high enzyme-to-substrate ratio of 1:25 (w/w), weakly acidic buffer (100 mM ammonium acetate, pH 6), largely excessive $H_2^{18}O$ (>50 μl), longer incubation time (24 h), and rigorous trypsin inactivation were adopted to achieved full $^{18}O_2$ labeling. Two intense peaks assigned to the $^{18}O_2$-labeled peptide of $LFTGHPETLEK^{(+4)}$ and $VEADIAGHGQEVLIR^{(+4)}$ (*m/z* 1275.62 and *m/z* 1610.82) were labeled with an efficiency of 94% and 91%, respectively, and no unlabeled peaks were detected (*m/z* 1271.64 and *m/z* 1606.83) (Insets of Fig. 1D).

3.3. *Accuracy and dynamic range of QITL*

Two identical aliquots of trypsin-digested peptides derived from BSA were separately labeled as described in Scheme 1 and mixed with a ratio of 1:1 before MALDI-TOF/TOF-MS analysis to assess the quantitative accuracy. The MS2 spectra of the $E^{(+28)}ACFAVEGPK^{(+42+4)}$ and $E^{(+32)}ACFAVEGPK^{(+42)}$ peptides with *m/z* 1181.62 is shown in Fig. 2A. Paired b, y fragment ions were observed and calculated to obtain a mean of 1.07 and a standard deviation of 0.13 for this pair of isobaric peptides, which can confirm the high accuracy and reliability of QITL. This high accuracy can be attributed to multiple quantitative data points for one peptide, reducing the impact of potentially interfering fragment ions.

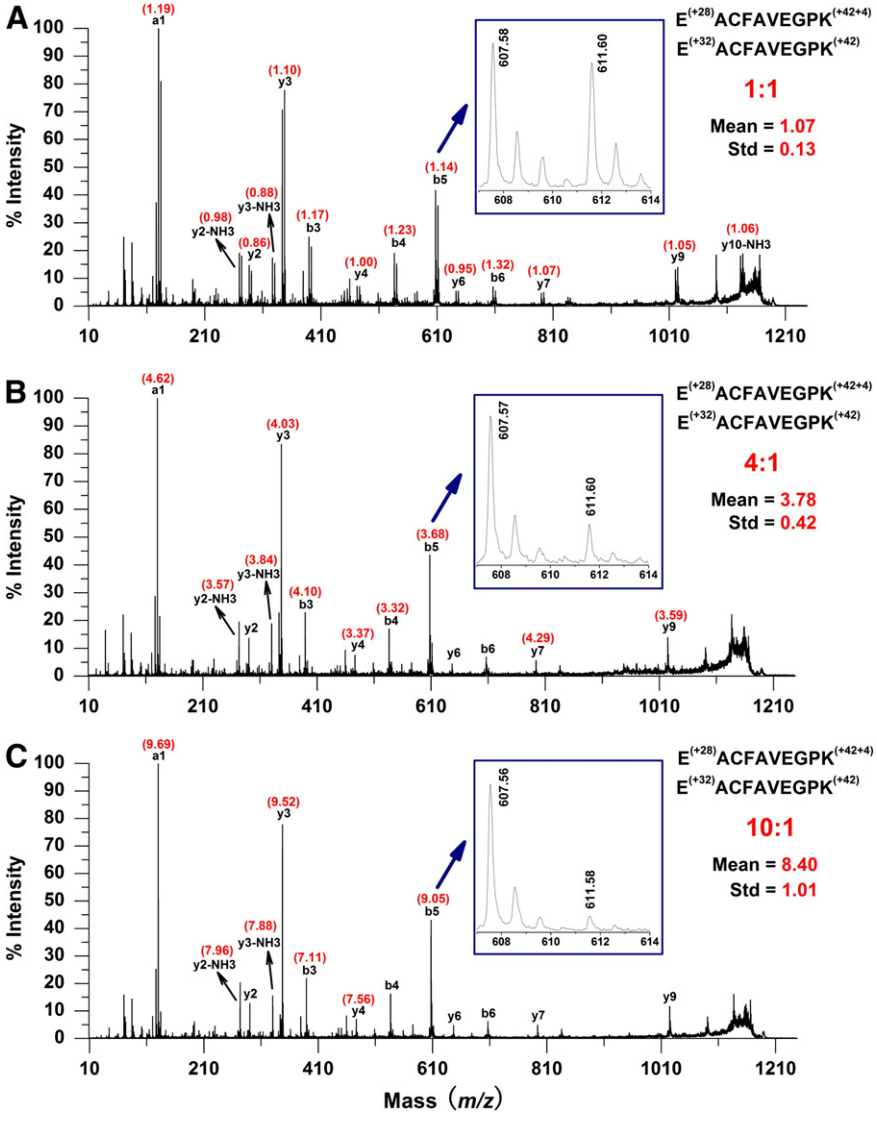


Fig. 2 – MALDI-TOF/TOF MS2 spectra of the labeled peptides $E^{(+28)}ACFAVEGPK^{(+42+4)}$ and $E^{(+32)}ACFAVEGPK^{(+42)}$ from BSA in the QITL method with the ratios of 1:1, 4:1, and 10:1, respectively.

The dynamic range as another important indicator was also evaluated. Trypsin-digested peptides derived from BSA, cytochrome c or myoglobin were labeled as described in Scheme 1, and mixed with different ratios of 10:1, 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:8, and 1:10 before MALDI-TOF/TOF-MS analysis. MS2 spectra of the peptides of $E^{(+28)}ACFAVEGPK^{(+42+4)}$ and $E^{(+32)}ACFAVEGPK^{(+42)}$ from BSA with the ratio of 1:1, 4:1, and 10:1 are displayed in Fig. 2, exhibiting good consistency with the true values for every ratio. The quantitative average ratios of myoglobin, cytochrome c and BSA were plotted against their expected ratios based on three replicates showing a good linearity across a 10-fold dynamic range with R^2 values larger than 0.99 (Fig. 3).

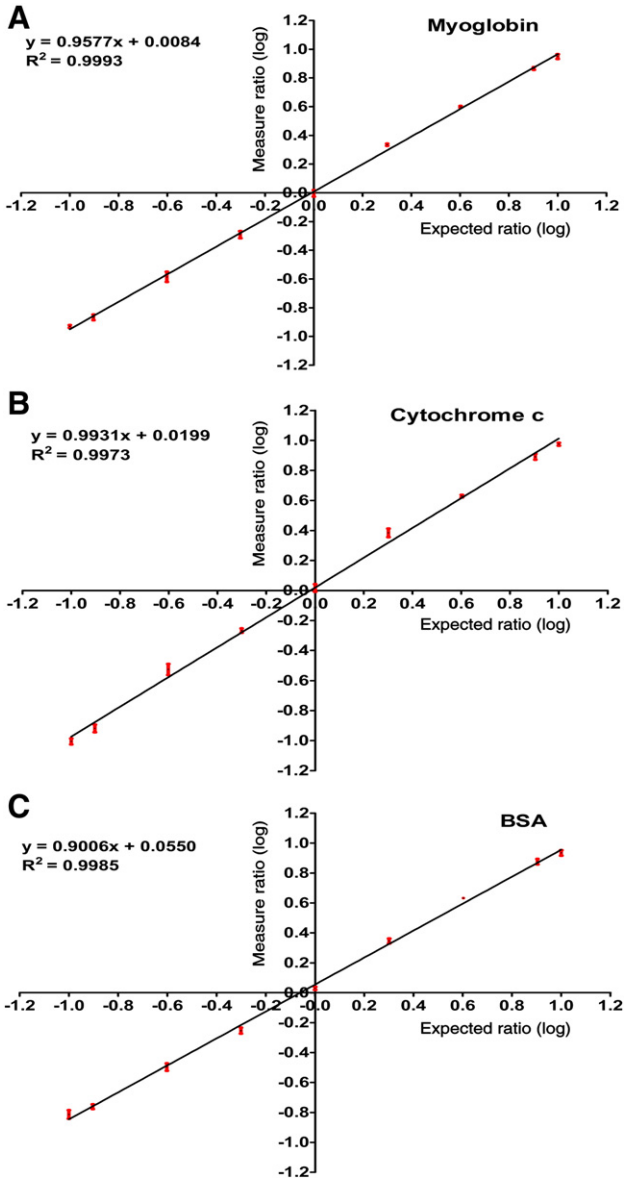


Fig. 3 – The linearity plot of the labeled standard proteins of myoglobin (A), cytochrome c (B), and BSA (C) by the QITL method with nine mixing ratios (1:10, 1:8, 1:4, 1:2, 1:1, 2:1, 4:1, 8:1, and 10:1). The average was calculated and plotted with error bars of standard deviation based on three replications.

3.4. *Isotope effect of QITL*

To examine the isotope effect of the differently labeled peptides by QITL, two identical aliquots of trypsin-digested peptides derived from BSA were separately labeled as described in Scheme 1 and mixed with a ratio of 1:1 before 1D-low pH-nano RPLC-ESI-MS/MS analysis. Extracting the selective ion chromatograms from the MS1 spectra to investigate the isotope effect is difficult in the QITL method due to the identical molecular weight of the isobaric peptides; however the selective ion chromatograms of the lightly and heavily labeled b, y fragment ions can be extracted from the MS2 spectra. Take the labeled peptide counterparts of $H^{(+28)}LVDEPQNLIK^{(+42+4)}$ and $H^{(+32)}LVDEPQNLIK^{(+42)}$ (m/z 1379.40) for example: selective ion chromatograms of the lightly labeled b4-ion ($H^{(+28)}LVD$, m/z 493.34) and the heavily labeled b4-ion ($H^{(+32)}LVD$, m/z 497.31) showed no chromatographic shift between the ^1H and ^2H-labeled fragment ions (Fig. S1-A vs. B) because the isotope effect of dimethyl labeling was minimized by grouping the deuterium atoms around polar functional groups [8,28]. Similarly, no chromatographic shift was detected between the ^{16}O and ^{18}O-labeled y fragment ions (Fig. S1-C vs. D) because ^{18}O atoms did not alter the retention time of labeled peptides on the RPLC [29]. Thus, co-elution of the isobaric-labeled peptides in QITL method further ensures accurate and reliable quantification of complex samples using the LC-ESI-MS/MS platform.

3.5. *Application of QITL to complex rat liver samples analyses*

Rat liver samples were prepared to verify the applicability of QITL for large-scale real biological samples analysis. Proteins extracted from rat liver tissues were digested, labeled and analyzed in triplicates as described in Scheme 1. Results on protein identification and quantification are summarized in Table 1. For three replicated analyses, 482, 476, and 407 non-redundant peptides corresponding to 203, 210, and 161 non-redundant proteins were identified. Then, 396, 375, and 316 non-redundant peptides corresponding to 174, 176, and 135 non-redundant proteins were quantified. The means and the standard deviations of the proteins for each run were 0.99 and 0.20, 1.01 and 0.19, and 0.96 and 0.17, respectively, indicating that the QITL strategy has high reproducibility and reliability for large-scale biological samples analysis. Moreover, 84.46% of the identified proteins were successfully quantified, a percentage higher than those of IVTAL (47.46%) [21] and IPTL (42.40%) [18]. This result is beneficial for quantitative analysis of complex biological samples and might be rooted in the higher efficiency of trypsin as well as the enhanced intensity of fragment ions after the QITL derivatization.

3.6. *Application of QITL to quantitative proteome profile of HCC and adjacent non-tumor liver tissues*

HCC, endemic to Asia and Africa with an increasing incidence in Western countries, is the fifth most frequent cancer and the third leading cause of cancer deaths worldwide [30,31]. Here, QITL, as a highly accurate and reliable quantitative method, was applied for HCC quantitative proteomic analysis.

Table 1 – List of numbers of quantified proteins and peptides, mean, standard deviation, coefficient of variance of the quantified proteins, as well as numbers of identified proteins and peptides for the rat liver samples with a ratio of 1:1 in three runs by QITL.

Run no.	Number of quantified non-redundant peptides	Number of quantified non-redundant proteins	Mean	Standard deviation	Coefficient of variance	Number of identified non-redundant peptides	Number of identified non-redundant proteins
1	396	174	0.99	0.20	0.20	482	203
2	375	176	1.01	0.19	0.19	476	210
3	316	135	0.96	0.17	0.18	407	161

The same amount of proteins were extracted from HCC or adjacent non-tumor liver tissues, then digested, labeled and analyzed in triplicates as described in Scheme 1. We used high-pH RP rather than the popular SCX as the first dimension LC separation before the low pH RPLC-ESI-MS/MS to realize higher resolution, higher recovery, and minimal use of salt [32]. Finally, 1227, 1162, and 1130 non-redundant proteins were successfully quantified from 3314, 3059, and 3074 non-redundant peptides, respectively (Fig. 4A). The distribution of ratios of HCC samples to non-tumor samples in log scale for each replication at the protein level illustrates that the majority of the proteins remained unchanged in HCC tissues when compared with those in adjacent non-tumor tissues (Fig. 4B).

Differently regulated proteins between HCC and adjacent non-tumor liver tissues were obtained using a filter criterion [33]. When relative standard deviation (RSD)<50% in replicated analyses, the average ratio of quantified proteins must be larger than 2 or less than 0.5. When RSD>50% in replicated analyses, not only the average ratio of quantified proteins must be larger than 2 or less than 0.5, but they must also be simultaneously up- or down-regulated. Finally, a total of 124 proteins displayed more than 2-fold expression differences, 45 proteins exhibited up-regulation, and 79 proteins exhibited down-regulation in HCC tissues. Among these differently regulated proteins, approximately 50 proteins were also consistently quantified and validated in previous studies (Table S3). For example, cytokeratin 19 (CK19), the over-expression of which was reported to be related to metastatic behavior and might reflect pathological progression in some HCC patients, was found up-regulated by 4.60 fold in our study [34]. Epithelial cell adhesion molecule (EpCAM), a hepatic stem cell marker that was often highly elevated in premalignant hepatic tissues and in a subset of HCC, was also found up-regulated by 3.18 fold in our study [35]. Furthermore, 20 proteins involved in lipid metabolic process were down-regulated in HCC tissues (Table S3), as reported in previous studies, indicating that HCC-related lipid metabolic abnormalities may lead to hepatic steatosis and cancer development [36].

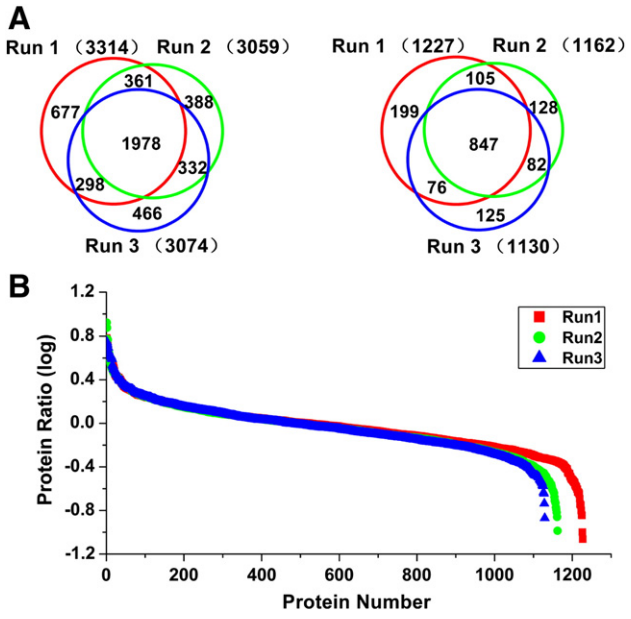


Fig. 4 – Quantitative proteomics profiling of the HCC and adjacent non-tumor tissues by the QITL method. Overlapping of the quantified peptides (A, left) and proteins (A, right) in three runs showed good reproducibility of QITL. The log diagram of quantitative protein ratios of HCC to adjacent non-tumor tissues in three runs (B) demonstrated high accuracy and reliability of QITL.

4. Conclusions

The proposed QITL method exhibits significant advantages in both efficiency and applicability. First, isobaric labeling of the peptides using QITL effectively reduced complexity and enhanced signal intensities of MS1 spectra. Second, a highly accurate and reliable quantification of each labeled peptide is enabled by multiple b, y fragment ion pairs in MS2 spectra. Third, quantitative data points over the full mass range instead of the limited low mass range in MS2 spectra enable QITL to be performed on all types of mass spectrometers. In terms of applicability, cost-effective and commercially available labeling reagents, as well as relatively fast and simple labeling procedures, make QITL applicable for regular proteomics analysis. Furthermore, the most widely used enzyme, trypsin, was applied for protein digestion, facilitating peptide identification by CID and full use of the digested peptides for protein quantification. The enzymes used for proteolytic ^{18}O labeling, such as endoproteinase Lys-C, can also be adopted in this method. Finally, and most importantly, QITL is applicable to any biological sample, including cell lines, tissues, body fluids, etc.

Based on the above advantages, the feasibility, accuracy, dynamic range, and reproducibility of QITL were demonstrated through the use of standard proteins and complex samples

of rat liver, as well as HCC and adjacent non-tumor tissues. About 124 up- or down-regulated proteins were found in HCC tissues, providing abundant information for proteomics analysis of HCC. Hence, QITL is a promising quantitative method in future proteomic research because of its universal suitability for any samples, low cost, and easy manipulation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.07.011.

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