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iTRAQ plus ^{18}O: A new technique for target glycoprotein analysis

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ABSTRACT

A novel strategy combining iTRAQ with ^{18}O stable isotope labeling (iTRAQ plus ^{18}O) was established to identify N-glycosylation site, quantify the glycopeptides and non-glycosylated peptides, and obtain N-glycosylation site ratio on the target glycoprotein. In this approach, all peptides of four biological samples are labeled with four iTRAQ reagents in parallel, followed by PNGase F catalyzed labeling of N-glycosylation sites with $H_2{}^{16}O$ and $H_2{}^{18}O$. Two sample groups are labeled with $H_2{}^{16}O$ and the other two are labeled with $H_2{}^{18}O$. After the modification of MS precursor ion isolation window, tagged peptides are identified by LC–MS/MS, both glycopeptides and non-glycopeptides are quantified simultaneously using ProteinPilot™ Software. With four samples to be maximally analyzed in parallel, this workflow supports accurate identification and quantification of glycopeptides in a site-specific fashion. Furthermore, N-glycosylation site ratios on serum haptoglobin (Hp) β chain in healthy individuals as well as patients with hepatitis B virus (HBV), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) were quantified to validate the novel 'iTRAQ plus ^{18}O' method. Glycosite ratios of VVLHPN#YSQVDIGLIK were observed to change significantly in HCC patients compared with LC and HBV patients. This novel approach supports the screening of the target glycoproteins as biomarkers in clinical application.

1. Introduction

N-glycosylation is a common form of post-translational modifications. Glycosylation of a protein can modulate its structure, function, half-life and more [1]. Because of the important impacts of protein glycosylation in biology, it is necessary to optimize analytical strategies to identify and quantify events of protein glycosylation in a specific and accurate fashion. To date, there are two common approaches to quantify protein glycosylation: analysis of glycans deglycosylated from glycoprotein or glycopeptides directly [2]. In contrast to glycan analysis, glycopeptide analysis supports site-specific analysis of glycosylation and it distinguishes changes in protein expression or glycosylation stoichiometry [3,4]. For example, Zhang et al. [5] utilized deuterium labeling for N-linked glycosylated peptides after hydrazide chemistry enrichment. Kaji et al. [6] described a strategy termed isotope-coded glycosylation-site-specific tagging (IGOT) for quantification. Hulsmeier et al. [7] developed an MRM protocol to quantify the N-glycosylation site occupancy of ^{18}O labeled glycoproteins. Liu et al. [8] reported a novel approach TOSIL in which three heavy oxygen atoms are introduced into glycopeptides. These methods are very useful in detecting changes of glycosylation, however, the comparison are usually limited to two samples, a test group and a control group.

The advent of iTRAQ has permitted effective expression measurements of large sets of samples. Jung et al. [9] used iTRAQ reagent to label tryptic-digested proteins from lectin affinity chromatography fractions. In 2009, Zhou et al. [10] combined hydrazide-resin capture method and iTRAQ to analyze N-linked glycoproteins in tear fluid of climatic droplet keratopathy. In 2010, Ueda et al. [11] introduced a workflow which combined IGEL (isotopic glycosidase elution and labeling on lectin column chromatography) with 8-plex iTRAQ stable isotope labeling to identify and quantify sites of N-glycosylation. These workflows are significant steps forward in exploring novel glycoproteins, especially in the field of large-scale biomarkers mining.

Compared with the development of glycoproteomic technique, robust analytical methods are required to quantify N-glycosylation on the target glycoprotein. In our approach, a pair of isotopic labels was introduced at glycosylation sites, which enhanced the confidence of glycosylation identification. 'iTRAQ plus ^{18}O' is capable of supporting quantification of glycosite ratio (abundance ratio of

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Table 1
Characteristics of healthy individuals and patients with HBV, cirrhosis and HCC.

Group	N	HBV	LC[c]	HCC[c]
Number	5	5	5	5
Sex (F/M)	2/3	2/3	1/4	2/3
Age (years)	26.8 ± 3.1	22.6 ± 4.5	49.0 ± 5.2	43.0 ± 6.7
HBV DNA (copy)[a]	NA	5.2×10^5–4.5×10^6	1.8×10^3–1.2×10^5	1.3×10^3–1.0×10^5
AFP (ng/mL)[b]	NA	2.1 (0.9–3.1)	12.9 (5.3–38.3)	1817.2 (67.0–35350.0)
$HBsAg^+$ (%)	0	100	100	100
AST (U/L)	18.6 (13–25)	69.4 (59–118)	69.2 (59–90)	152.6 (45–296)
ALT (U/L)	15.6 (13–25)	100.4 (41–206)	41.2 (23–75)	138.6 (63–350)

[a] HBV DNA was detected with fluorescent quantitative PCR (FQ-PCR) and has a detection limit of sensitivity of approximately 1×10^3 genome equivalents per mL.
[b] AFP (alpha fetoprotein) was determined using standard kits (Abbott Labs) and 20 ng/mL was considered the upper limit of normal.
[c] LC and HCC diagnosis was confirmed by ultrasound imaging and biopsy.

glycopeptides/abundance ratio of protein) on the target glycoprotein simultaneously in four sample sets. Commercial Hp β chain was acquired to evaluate the accuracy and precision of quantification. The utility of this approach was further validated with serum samples extracted from patients suffering liver diseases and healthy individuals. 'iTRAQ plus ^{18}O' is a promising strategy for the target glycoprotein analysis with the potential of clinical application.

2. Experimental

2.1. Preparation of specimens

The serum specimens used in this study were obtained from the First Affiliated Hospital of Guangxi Medical University in 2008. Informed consent was obtained from each patient and this study was approved by the First Affiliated Hospital of Guangxi Medical University Research Ethics committee and the Institutional Review Board of the National Cancer Center. The pathological data of the patients were provided in Table 1. All serum samples were collected using standard protocol from whole blood and stored at −80 °C until used.

2.2. Purification of Hp β chain from sera

500 μL of sera was centrifuged at 12,000 × g for 10 min and Albumin and IgG were depleted by Albumin/IgG Removal Kit (Merck KGaA, Darmstadt, Germany). The depleted sera were applied to a HiTrap (GE Healthcare, Uppsala, Sweden) [12] packed with NHS-activated resin coated with 300 μL of anti-human haptoglobin antibody (4.5 mg). The haptoglobin bound to the column was eluted and then concentrated by acetone precipitation. Purified haptoglobin was displayed on a 12% SDS-PAGE to separate β chain and then stained with coomassie brilliant blue.

2.3. Tryptic digestion and iTRAQ reagents labeling

The SDS-PAGE gels were destained, reduced, alkylated and digested by trypsin at an enzyme-to-substrate ratio of 1:30 (w/w). The tryptic peptides from commercial Hp β chain (100 μg) were divided into aliquots at designated ratios 1:1:1:1 and 1:2:4:6. The four iTRAQ reagents (obtained from iTRAQ® Reagent–8plex kit) were prepared by adding 50 μL of isopropanol to each vial. The reconstituted reagents were then transferred to individual sample tubes and incubated at room temperature for 2 h.

2.4. Stable isotope tagging of glycopeptide

iTRAQ reagents-114 labeled sample and iTRAQ reagents-116 labeled sample were pooled and then processed by PNGase F (New England Biolabs, Ipswich, MA) digestion in $H_2{}^{16}O$. iTRAQ reagents-115 labeled sample and iTRAQ reagents-117 labeled sample were pooled and processed by PNGase F in $H_2{}^{18}O$ (97%, Cambridge Isotope Laboratories, Inc.). The ^{16}O- and ^{18}O-labeled samples were combined and then lyophilized.

2.5. LC–MS analysis and data mining

The lyophilized peptide samples were resuspended with 5% ACN in 0.1% formic acid and separated on HPLC systems (Shimadzu Corporation, Kyoto, Japan). The mobile phases were 5% acetonitrile with 0.1% formic acid (phase A) and 95% acetonitrile with 0.1% formic acid (phase B). To achieve sufficient separation, a 70-min linear gradient from 5% to 50% phase B was employed. For the ESI-MS/MS systems, the following parameters were used: ion spray voltage, 2200 V; curtain gas, 10. Samples acquired on Qstar XL mass spectrometer (Qstar XL Pulsar™, Applied Biosystems-MDS Sciex) were in informative-depended acquisition mode with 1 MS scan (400–1800 m/z) followed by 4 MS/MS scans (50–2000 m/z) using corresponding software Analyst version 1.1. Precursor ions were chosen based on intensity (above 30 counts) and charge states (from 2+ to 4+). Dynamic exclusion was set to a period of 120 s and automatic rolling collision energy was applied for better peptide fragmentation. The tolerance settings for both MS and MS/MS were 0.2 Da. The mass spectra acquired by Qstar XL were searched using ProteinPilot™ Software (Version 3.0, Applied Biosystems). The parameters for this search were as follows: sample type: iTRAQ 8plex (peptide labeled); Cys alkylation: methyl methanethiosulfonate (MMTS); digestion, trypsin; ID focus: biological modification; database: non-redundant international protein index (IPI) human sequence database version 3.45 (with 71,979 protein entries); search effort: through ID; result quality: detected protein threshold [Unused ProtScore (confidence)] > 1.3 (95%).

3. Results and discussion

3.1. Strategy of 'iTRAQ plus ^{18}O' and MS detection window modification

As shown in Fig. 1A, any combination of two labeled samples (for example, iTRAQ reagents-114 labeled sample and iTRAQ reagents-116 labeled sample) was mixed and treated with PNGase F in $H_2{}^{16}O$. The other two labeled samples were mixed and treated with PNGase F in $H_2{}^{18}O$. All samples were mixed together and analyzed by LC–MS/MS. In 'iTRAQ plus ^{18}O' approach, iTRAQ reagents were used to label lysine side chain and N-terminus of a peptide. PNGase F specifically cleaves N-glycans from the polypeptide backbones [13]. It converts asparagine to aspartic acid during this reaction, which leads to a 1 Da mass shift if done in $H_2{}^{16}O$ [14] or a 3 Da mass shift if done in $H_2{}^{18}O$. That is, the glycopeptides in the pool of iTRAQ reagents-114 and 116 labeled samples incorporated a '1 Da' mass shift at their glycosylation sites, while the pool of iTRAQ

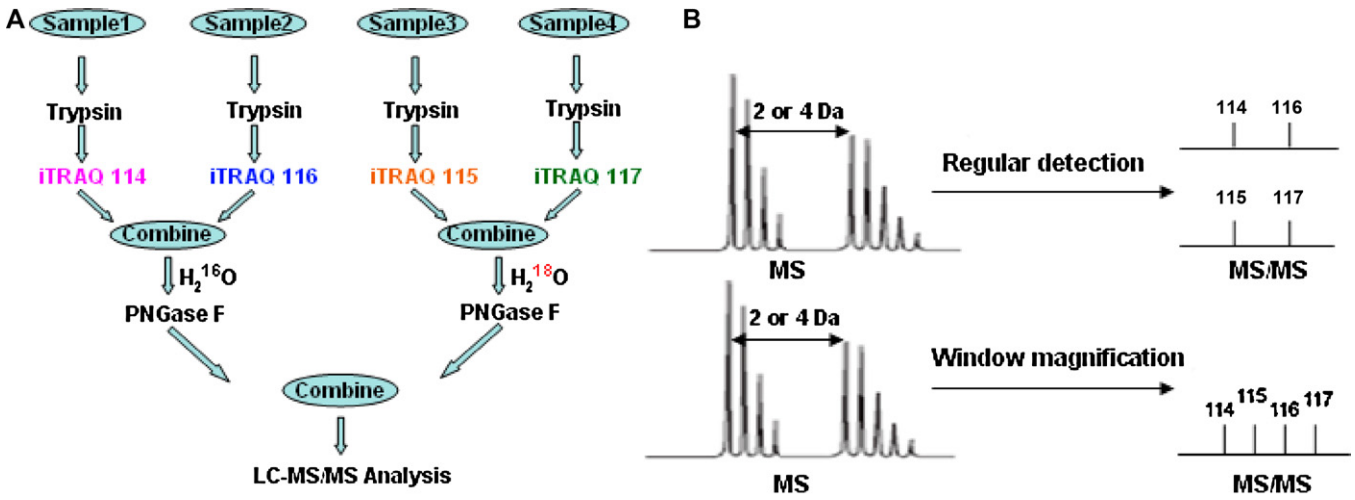

Fig. 1. (A) Schematic representation of 'iTRAQ plus ^{18}O' strategy. Four samples were digested with trypsin and labeled with iTRAQ reagents in parallel. Any combination of two labeled samples was mixed and treated with PNGase F in $H_2{}^{16}O$. The other two labeled samples were mixed and treated with PNGase F in $H_2{}^{18}O$. All samples were combined and subjected to LC–MS/MS. (B) Modification of precursor ion isolation window to quantify glycopeptides in four samples simultaneously.

reagents-115 and 117 labeled glycopeptides incorporated a '3 Da' mass shift at the glycosylation sites. In the end, all samples were combined and analyzed by LC–MS/MS together.

In the LC–MS/MS analysis, the non-glycosylated peptides were labeled only with iTRAQ reagents, which could be quantified by comparing the peak area of one reporter ion versus another. In iTRAQ technology, the ratio of one peak area to another is indicative of the relative amount of a given peptide in each of the corresponding sample digests and an additional series of peptide y- and b-ions are used for protein identification [15,16].

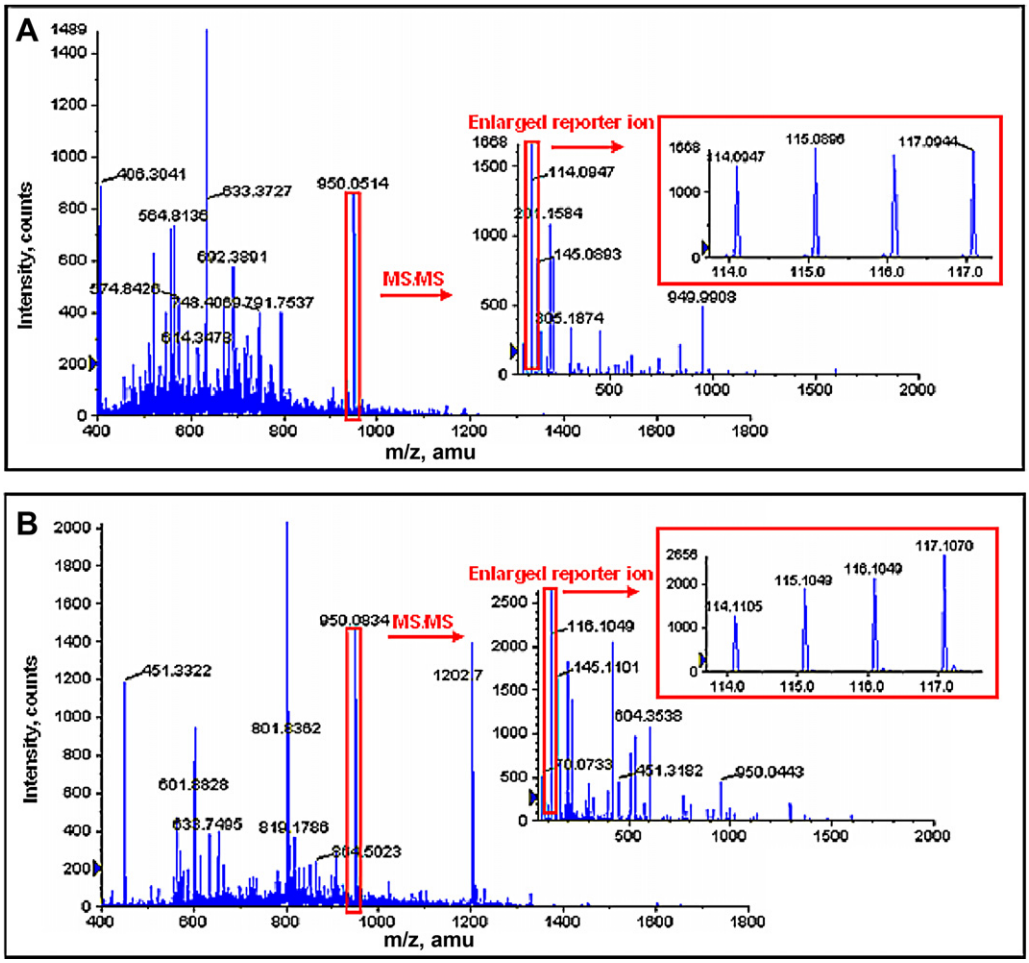

Fig. 2. (A) LC–MS and MS/MS of the peptide DIAPTLTLYVGK. This peptide exhibited ratio 1:1.03:1.00:1.09 was in agreement with theoretical ratio 1:1:1:1. (B) LC–MS and MS/MS of the peptide DIAPTLTLYVGK. An experimental ratio of 1:1.95:2.66:3.64 was observed for the formula of 1:2:4:6.

For glycopeptides with one N-glycosylation site, ^{16}O and ^{18}O labeling introduces a mass difference of 2 Da. For glycopeptides with two N-glycosylation sites, the mass difference introduced was 4 Da. If precursor ion isolation window was set to 4 Da (for example, 2 amu, 2+), the peak pair of interest (either 2 Da or 4 Da mass differences) could be selected and analyzed simultaneously supporting both identification and quantification. Thus, the peak areas for peaks at m/z 114, 115, 116, 117 were indicative of the relative amount of glycopeptide (Fig. 1B).

3.2. Accuracy and precision of 'iTRAQ plus ^{18}O'

The accuracy and precision of this approach were examined by standard glycoprotein. Tryptic peptides derived from commercial Hp β chain were aliquot into four parts and labeled with iTRAQ reagents respectively. Two combinations were formulated: 1:1:1:1 or 1:2:4:6. Each combination was labeled in triplicate and analyzed by LC–MS/MS. An example of LC–MS and MS/MS spectra from Hp β chain peptide DIAPTLTLYVGK was shown in Fig. 2. An experimental ratio of 1:1.03:1.03:1.09 was observed for the formula of 1:1:1:1 and an experimental ratio of 1:1.95:2.66:3.64 was observed for the formula of 1:2:4:6. The observed 'suppression' in abundance ratio with isobaric tags such as iTRAQ was in agreement of previous report [17].

In this workflow, two different types of tags were introduced: iTRAQ reagent at lysine residue and N-terminus and $^{16}O/^{18}O$ label at the site of glycosylation. Mathematic formulas are available to calculate peptide abundance ratio based on $^{16}O/^{18}O$ incorporation. For example, the equation below was calculated to measure ratio of two ^{18}O atoms incorporated [18,19]:

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

where I_0, I_2, and I_4 are the measured relative intensities of the monoisotope peak for the peptide, the peak with 2 Da increase in mass and the peak with 4 Da increase in mass, respectively; M_0, M_2 and M_4 are the corresponding theoretical relative intensities of the isotopic envelope of the peptide, which are calculated using MS-Isotope (http://prospector.ucsf.edu).

Fig. 3 gives an example for glycopeptide NLFLN#HSEN#ATAK at expected abundance ratio of 1:1:1:1 and 1:2:4:6, respectively. The observed ratio 1:0.94:0.98:0.97 from MS/MS spectra was in agreement with theoretical 1:1:1:1 and the observed ratio of $^{16}O/^{18}O$ was the same as the expected ratio 1. These data also demonstrated labeling samples were stable without any observable cross-reaction. At formulated abundance ratio of 1:2:4:6, the observed ratio was 1:1.95:2.74:3.30, a significant 'suppression' from the formulated abundance ratio. However, the observed ratio of $^{16}O/^{18}O$ was 0.71 which was close to theoretical ratio 0.63 [(1 + 4)/(2 + 6)].

To evaluate the accuracy for this quantitative workflow, the standard deviation and coefficient of variance were calculated for peptides (confidence level >95%) and glycopeptides observed. The coefficient of variance was calculated for each of the iTRAQ reagents ratios which were expected to be 1:1:1:1 (Table 2) and 1:2:4:6 (Table 3). The average coefficient of variance for four reagents was 6.4% (1:1:1:1) and 15.8% (1:2:4:6). The coefficient of variance of the reagents is less than 20% which is consistent with iTRAQ instruction. To determine the reproducibility, three samples at expected ratio 1:1:1:1 were analyzed independently, the reproducibility was shown to be good (Table 4).

Table 2
Analysis of the peptides of commercial Hp β chain at expected ratio 1:1:1:1.

Non-glycosylated peptides (conf >95%)	115:114	116:114	117:114
VGYVSGWGR	0.91	0.99	0.96
GSFPWQAK	0.99	0.98	1.04
ILGGHLDAK	0.97	1.02	1.05
VTSIQDWVQK	0.99	1.02	1.03
SCAVAEYGVYVK	1.02	1.01	1.06
HYEGSTVPEK	1.15	1.13	1.11
HYEGSTVPEKK	1.01	1.01	1.04
DIAPTLTLYVGK	1.03	1.03	1.09
VMPICLPSKDYAEVGR	0.87	1.01	0.90
SPVGVQPILNEHTFCAGMSK	1.06	1.10	1.11
VMPICLPSK	1.09	1.11	1.11
KQLVEIEK	1.05	1.04	1.08
SPVGVQPILNEHTFCAGM	1.01	1.09	1.13
YVMLPVADQDQCIR	0.89	1.10	0.98
YVMLPVADQDQ	1.03	0.94	0.97
Average	1.01	1.04	1.04
Standard deviation	0.075	0.056	0.066
Coefficient of variance (%)	7.5	5.4	6.3
Glycopeptides with one or two N-glycosylation sites	115:114	116:114	117:114
NLFLN#HSEN#ATAK	0.94	0.98	0.97
VVLHPN#YSQVDIGLIK	1.01	1.06	1.04
MVSHHN#LTTGATLINEQWLLTTAK	1.10	1.17	1.17

3.3. 'iTRAQ plus ^{18}O' quantification for Hp β chain in liver disease

In order to demonstrate the practicality of 'iTRAQ plus ^{18}O' method in biological samples, we quantified glycopeptides and non-glycosylated peptides derived from sera Hp β chain. Equal volume of sera pooled from 5 patients with HBV, 5 patients with LC, 5 HCC patients and 5 healthy individuals were used to purify Hp β chain. Fig. 4 shows the purified Hp β chain in sera pooled from 5 healthy individuals, 5 HBV patients, 5 LC patients and 5 HCC patients respectively. LC patients, healthy individuals, HBV patients and HCC patients were label with iTRAQ reagents-114, 115, 116 and 117 respectively. Then, Hp β chain of LC and HBV patients were mixed and digested in ^{16}O–water. The other two were mixed and digested in ^{18}O–water. List of glycopeptides and non-glycosylated

Table 3
Analysis of the peptides of commercial Hp β chain at expected ratio 1:2:4:6.

Non-glycosylated peptides (conf >95%)	115:114	116:114	117:114
VGYVSGWGR	1.62	2.57	2.83
GSFPWQAK	1.36	1.67	1.92
ILGGHLDAK	1.87	3.04	3.95
VTSIQDWVQK	1.71	2.29	2.79
SCAVAEYGVYVK	1.99	2.94	4.18
DIAPTLTLYVGK	1.95	2.66	3.64
VMPICLPSKDYAEVGR	1.86	2.37	3.00
SPVGVQPILNEHTFCAGMSK	1.79	2.68	3.44
SPVGVQPILNEHTFCAGM	1.92	3.00	4.22
VMPICLPSK	1.56	2.01	2.35
KQLVEIEK	2.01	2.80	3.69
YVMLPVADQDQCIR	1.87	2.83	3.41
PSKDYAEVGR	1.71	3.06	3.55
VGVQPILNEHTFCAGMSK	1.90	2.94	3.58
AVHDLEEDTWYATGILSFDK	2.12	3.27	4.27
Average	1.82	2.67	3.39
Standard deviation	0.196	0.433	0.692
Coefficient of variance (%)	10.8	16.2	20.4
Glycopeptides with one or two N-glycosylation sites	115:114	116:114	117:114
NLFLN#HSEN#ATAK	1.95	2.74	3.30
VVLHPN#YSQVDIGLIK	1.86	2.51	3.49
MVSHHN#LTTGATLINEQWLLTTAK	1.85	2.60	3.12

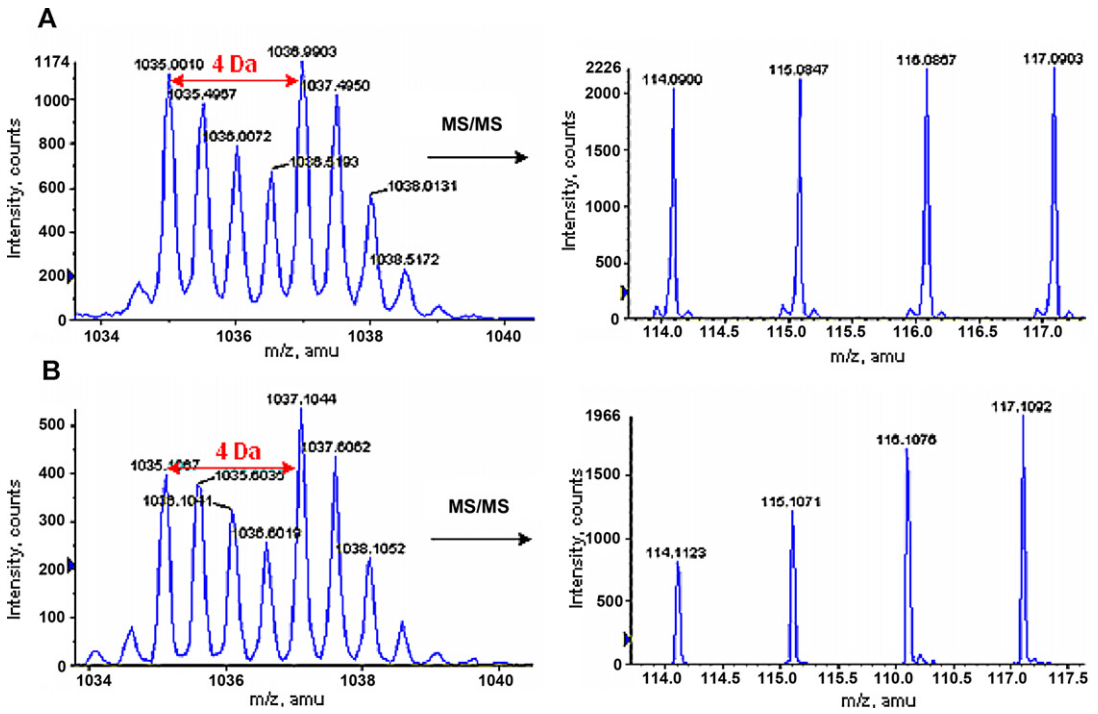

Fig. 3. (A) The glycopeptide NLFLN#HSEN#ATAK was examined by 'iTRAQ plus ^{18}O' at expected ratio of 1:1:1:1. 4 Da mass differences confirmed ^{18}O labeling of two N-glycosylation sites. It exhibited ratio 1:0.94:0.98:0.97 close to the expected ratio. The calculated $^{16}O/^{18}O$ ratio was the same as the expected ratio 1. (B) The glycopeptide NLFLN#HSEN#ATAK was examined by 'iTRAQ plus ^{18}O' at expected ratio of 1:2:4:6. It exhibited ratio 1:1.95:2.74:3.30 because of the limitation of iTRAQ method. The calculated $^{16}O/^{18}O$ ratio 0.71 was close to the theoretical ratio 0.63. The # in glycopeptides denotes the residue site of N-glycosylation.

Table 4
Analysis of three samples at expected ratio 1:1:1:1 independently.

Run	Non-glycosylated peptides (confidence level >95%)	NLFLN#HSEN#ATAK	VVLHPN#YSQVDIGLIK	MVSHHN#LTTGATLINEQWLLTTAK
1	1:1.03:1.04:0.97	1:0.90:1.01:0.89	1:1.03:1.06:0.94	1:1.14:1.19:1.06
2	1:0.97:0.92:0.92	1:0.92:0.91:0.88	1:0.96:0.93:0.94	1:1.09:0.94:1.01
3	1:1.01:1.04:1.04	1:0.94:0.98:0.97	1:1.01:1.06:1.04	1:1.10:1.17:1.17

peptides of Hp β chain in pooled sera from healthy people, HBV patients, LC patients and HCC patients via 'iTRAQ plus ^{18}O' was provided in Table 5. The protein expression of LC patients was found to be the lowest and it was used as the reference group for comparing conveniently. The average of non-glycosylated peptides (confidence level >95%) reflects the expression levels of Hp β chain and the ratio of expression is 1:6.43:2.28:2.84 (LC:N:HBV:HCC). A reduced protein expression level of Hp β chain was observed in all patients suffering liver diseases. Western blot results of Hp β chain for individual samples have been reported by us which were consistent with protein expression results of 'iTRAQ plus ^{18}O' [20].

In this workflow, only glycosylated peptide could be labeled in ^{18}O or ^{16}O by PNGase F. PNGase F deglycosylates Asn-linked glycopeptides and converts the corresponding Asn to Asp. For each glycopeptide, there will be a pair of precursors with characteristic mass shift of 2 Da or 4 Da, which significantly improves the specificity of glycopeptide identification. Using iTRAQ reporter ions to support quantification, Table 5 showed the abundance ratio of Hp β chain glycopeptides were 1:7.29:2.49:2.76 (LC:N:HBV:HCC), 1:9.11:2.37:4.84 (LC:N:HBV:HCC) and 1:6.73:2.47:3.19 (LC:N:HBV:HCC) for NLFLN#HSEN#ATAK, VVLHPN#YSQVDIGLIK and MVSHHN#LTTGATLINEQWLLTTAK, respectively.

After quantification of protein and glycopeptides, the glycosite ratio was calculated by dividing abundance ratio of glycopeptides by abundance ratio of protein. The N-glycosylation site ratio of NLFLN#HSEN#ATAK, VVLHPN#YSQVDIGLIK and MVSHHN#LTTGATLINEQWLLTTAK were 1:1.13:1.10:0.97

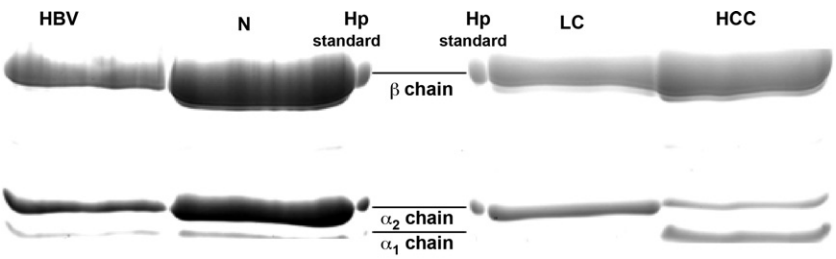

Fig. 4. Purification of Hp β chain from equal volume of 5 healthy, 5 HBV, 5 LC and 5 HCC pooling sera. The purified Hp were electrophoresed on SDS-PAGE and stained with coomassie brilliant blue. The commercial Hp helped confirm these protein gels correspond to Hp.

Table 5
Analysis of the glycopeptides and non-glycosylated peptides of sera Hp β chain.

Non-glycosylated peptides (conf >95%)	115:114	116:114	117:114
VGYVSGWGR	5.57	2.10	2.60
GSFPWQAK	4.51	2.09	2.28
ILGGHLDAK	5.03	2.33	2.46
VTSIQDWVQK	5.94	2.25	2.72
SCAVAEYGVYVK	6.07	2.36	2.97
HYEGSTVPEKK	7.42	2.05	2.83
DIAPTLTLYVGK	7.77	2.16	2.78
VMPICLPSKDYAEVGR	5.12	2.26	2.77
SPVGVQPILNEHTFCAGMSK	7.01	2.41	3.48
KQLVEIEK	7.81	2.76	3.69
AVHDLEEDTWYATGILSFDK	9.82	2.42	2.73
YQEDTCYGDAGSAFAVHDLEEDTWYATGILSFDK	6.11	2.13	2.91
YVMLPVADQDQCIR	5.40	2.27	2.75
Average	6.43	2.28	2.84
Glycopeptides with one or two N-glycosylation sites	115:114	116:114	117:114
NLFLN#HSEN#ATAK	7.29	2.49	2.76
VVLHPN#YSQVDIGLIK	9.11	2.37	4.84
MVSHHN#LTTGATLINEQWLLTTAK	6.73	2.47	3.19

(LC:N:HBV:HCC), 1:1.42:1.04:1.70 (LC:N:HBV:HCC) and 1:1.05:1.08:1.12 (LC:N:HBV:HCC). Based on specimens' complexity, the ratio of more than 1.5-fold was considered to be significant. In glycosite ratio, VVLHPN#YSQVDIGLIK changed significantly in HCC patients compared with LC patients (1.70/1 > 1.5) and HBV patients (1.70/1.04 > 1.5). This given example showed 'iTRAQ plus ^{18}O' was a valuable and simple tool to quantify glycopeptides of the target protein and this method has the potential for future clinic application.

4. Conclusion

We have developed 'iTRAQ plus ^{18}O' method, which combined iTRAQ with ^{18}O stable isotope labeling for quantitative analysis of N-glycosylation site ratio on the target glycoprotein in four samples, simultaneously. This strategy shows several unique strengths: it incorporates ^{18}O specifically in glycopeptides and iTRAQ reagent in both glycopeptides and non-glycopeptides. The pairwise 2 or 4 Da mass differences introduced to glycopeptides (one glycosite or two glycosites) support accurate identification and quantification of glycopeptides. Our workflow affords elevated specificity in glycopeptide identification in parallel with quantification analyses. With four samples to be maximally analyzed in parallel, this workflow expands the throughput of the target glycoprotein analysis.

Competing interests

No competing interests are declared.

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