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Tandem ^{18}O Stable Isotope Labeling for Quantification of N-Glycoproteome

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A new strategy using tandem ^{18}O stable isotope labeling (TOSIL) to quantify the N-glycosylation site occupancy is reported. Three heavy oxygen atoms are introduced into N-glycosylated peptides: two ^{18}O atoms are incorporated into the carboxyl terminal of all peptides during a tryptic digestion, and the third ^{18}O atom is incorporated into the N-glycosylation site of asparagines-linked sugar chains specifically via a N-glycosidase F (PNGase F)-mediated hydrolysis. Comparing samples treated in $H_2{}^{18}O$ and samples treated in $H_2{}^{16}O$, a unique mass shift of 6 Da can be shown for N-glycosylated peptide with single glycosylation site, which could be easily distinguished from those nonglycosite peptide pairs with a mass difference of 4 Da only. The relative quantities of N-glycosylated and its parent protein-levels were obtained simultaneous by measuring the intensity ratios of $^{18}O/^{16}O$ for glycosylated (6 Da) and for nonglycosylated (4 Da) peptides, respectively. Thus, a comparison of these two ratios can be utilized to evaluate the changes of occupancy of N-glycosylation at specific sites between healthy and diseased individuals. The TOSIL approach yielded good linearity in quantitative response within 10-fold dynamic range with the correlation coefficient $r^2 > 0.99$. The standard deviation (SD) ranged from 0.06 to 0.21, for four glycopeptides from two model glycoproteins. Furthermore, serums from a patient with ovarian cancer and healthy individual were used as test examples to validate the novel TOSIL method. A total of 86 N-glycosylation sites were quantified and N-glycosylation levels of 56 glycopeptides showed significant changes. Most changes in N-glycosylation at specific sites have the same trends as those of protein expression levels; however, the occupancies of three N-glycosylation sites were significantly changed with no change in proteins levels.

Keywords: ^{18}O-labeling • N-glycosylation • Quantitative proteomics • Mass spectrometry • Ovarian cancer

1. Introduction

Glycosylation is a common and important form of post-translational modification. It has been reported that more than 50% of human proteins are likely conjugated with glycans.[1] Glycosylation, especially N-linked glycosylation, is prevalent in proteins destined for extracellular environments,[2] including serum. The diagnostic and therapeutic potentials in decoding patterns of protein N-glycosylation within serum have been recognized.[3,4] Thus, the study of glycoproteins has been arousing considerable attention and substantial advances have been achieved during recent years.[5,6] To date, thousands of N-glycosylation sites have been identified;[4,7] however, little attention has been paid to the change of occupancy of N-glycosylation at specific sites in most of these investigations, especially the relationship between changes in glycosylation levels and protein expression levels.

The aggressiveness of cancer cells such as tumor cell invasion, metastasis, has been shown to correlate with the changes of N-glycosylation level.[3,8,9] It is believed that glycoproteins play important roles in the pathogenesis. Thus, method innovations in quantitative profiling of the N-glycosylation sites occupancy and analyzing the relationship between the change of N-glycosylation sites occupancy and proteins expression are of important clinical significance. Two-dimensional electrophoresis (2-DE) with specific stains applied in series can determine glycoprotein expression levels and glycosylation patterns,[10] while the occupancy of particular N-glycosylation site will not be retrieved. In a first attempt to characterize alterations in protein glycosylation at specific sites, Zhang et al.[11] utilized deuterium labeling for N-linked glycosylated peptides followed by enrichment of glycopeptides hydrazide chemistry and by LC-MS analysis. This approach has also been combined with other labeling method, such as derivation with iTRAQ reagents, to analyze quantitatively N-linked glycoproteins in tear fluid of Climatic Droplet Keratopathy.[12] Andreas et al. developed MRM protocol to quantify

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the N-glycosylation site occupancy of the serum glycoproteins transferrin and α1-antitrypsin.[13] MRM has demonstrated its specificity, accuracy and sensitivity in quantification; however, it could not be used for the unknown sequence. ^{18}O labeling is a relatively simple and convenient stable isotope-coding approach, and this ^{18}O labeling can produce a general and sequence-independent labeling of tryptic peptides at the C terminal carboxylic acids.[14,15] Recently, it has been reported that ^{18}O-labeling method could be more precise than ICAT method for identification of proteins.[16] Kaji et al. described a strategy termed isotope-coded glycosylation-site-specific tagging (IGOT) for quantification.[17] Unfortunately, the isotope envelopes of the ^{16}O- and ^{18}O-tagged peptides were partly overlapping, and interfered with the accuracy of quantification. In a subsequent study, Kristy et al. referred that parallel hydrolysis sequentially with Glu-C and PNGase F in $H_2{}^{16}O$ or $H_2{}^{18}O$ can generate 6 Da difference between a glycopeptide pair, which would overcome isotope distributions overlapping.[18] However, the quantitation accuracy of this method and its application in characterizing glycoproteome have not been assessed in detail.

Here, we developed a tandem ^{18}O stable isotope labeling (TOSIL) method to quantify accurately changes in levels of N-glycosylation and to distinguish changes of protein expression and individual N-glycosylation site occupancy. The enzyme-catalyzed ^{18}O labeling in the C-terminal carboxylic acid is combined in tandem with PNGase F hydrolysis in $H_2{}^{18}O$. The spectra of glycopeptides with single N-glycan site shift +1 and +7 Da after treatment in $H_2{}^{16}O$ and $H_2{}^{18}O$, respectively, and in contrast, the nonglycopeptides pairs are separated only by 4 Da. Therefore, glycopeptides and nonglycopeptides can be easily distinguished and quantified in parallel in a single process. We also showed an example with TOSIL method that the analyses of N-glycosylation levels in serums from one healthy individual and one patient with ovarian cancer yield quantitative information of 86 unique glycosylation sites on 49 proteins. The TOSIL method is considered to be a potential useful tool for high-throughput quantitative analysis of N-glycosylation site occupancy.

2. Materials and Methods

2.1. Chemicals and Materials. Bradford assay reagent was obtained from Bio-Rad. The 3000 Da MWCO spin column were from Millipore. Sequencing grade modified trypsin was from Promega. Immobilized trypsin beads were purchased from Applied Biosystems. Micro spin column was from PIERCE. $H_2{}^{18}O$ (97%) was from Cambridge Isotope Laboratories, Inc. (CIL). PNGase F was from New England Biolabs. ProteoExtract Albumin/IgG removal kit was obtained from Calbiochem. Sepharose CL-4B was purchased from Amersham Bioscience. Nitrocellulose membranes were from Millipore and blotter was from Bio-Rad. Rabbit polyclonal antibody to alpha 2 Macroglobulin, Rabbit polyclonal antibody to Zinc Alpha 2 Glycoprotein, Mouse monoclonal antibody to alpha 1 Antitrypsin were from Abcam; rabbit polyclonal antibody to Antithrombin-III and rabbit polyclonal antibody to Alpha-1-antichymotrypsin were from Santa Cruz Biotechnology. ECL Advance/plus Western Blotting Detection Kit was from Amersham Biosciences. The water used was Milli-Q grade. All protein standards and other chemicals were purchased from Sigma.

2.2. Tryptic Digestion and Tandem Isotope Labeling of Fetuin and Invertase with ^{18}O-Water. The model protein (Fetuin or Invertase) in solution were denatured by incubating at 100 °C for 10 min. After cooling down to room temperature, the samples were treated with 10 mM dithiothreitol (DTT) for 30 min at 57 °C and alkylated with 20 mM iodoacetamide (IAA) at room temperature for 1 h in the dark. Prior to adding trypsin, the solution of model protein was diluted 10-fold with 50 mM NH_4HCO_3 buffer. Trypsin was added at an enzyme-to-substrate ratio of 1:30 (w/w). The hydrolysis was allowed to proceed overnight at 37 °C. Twenty micrograms (1 μg/μL) of tryptic peptides from model protein was divided into two identical aliquots, and dried in a vacuum centrifuge. The two aliquot were redissolved with 100 μL of acetonitrile (ACN; 20% v/v) prepared in $H_2{}^{16}O$ or $H_2{}^{18}O$, respectively. Two microliters (20% slurry v/v) of immobilized trypsin beads was added into each tube to catalyze the labeling of tryptic peptides C-terminally at 37 °C for 24 h. The immobilized trypsin beads were removed postlabeling using MicroSpin column. The samples within flowthrough were dried with a vacuum centrifuge. The peptides were then dissolved in 100 mM NH_4HCO_3 buffer prepared with $H_2{}^{16}O$ or $H_2{}^{18}O$ (corresponding with the labeling ahead). PNGase F (500 units/μL) was added to the peptide solution at a concentration of 1 μL PNGase F/mg of crude proteins, and the deglycosylation and specific labeling of glycosylated peptide were proceeded overnight at 37 °C. The ^{16}O- and ^{18}O-labeled samples were pooled at designated ratios (10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10), and then lyophilized.

2.3. Preparation and Labeling of Serum Proteins. Serum samples were collected at the Gynecology and Obstetrics Hospital of Fudan University (Shanghai, China). Informed consents were obtained from a patient and a healthy control. The patient was 65 years old, diagnosed with serous ovarian cancer, stage IIb. The cancer metastatic site (diameter > 2 cm) could be observed on the front wall of rectum and pelvic peritoneum. A healthy individual served as control. A volume of 2 mL of blood was drawn from each woman, and the obtained blood was kept in two 5-mL glass tubes (BD Co. Ltd.) at room temperature for 2 h for clotting. Subsequently, the serum was centrifuged at 1000 rpm, then transferred to 1.5-mL Eppendorf tube and centrifuged again at 12 000 rpm at 4 °C. The serum collected in the supernatant was immediately aliquoted and stored at −70 °C. Each aliquot was allowed to thaw once only. The clinical protocol of sample collection was supervised by a senior researcher to ensure each operational step was carried out strictly and correctly.

The top two most abundant proteins (Albumin and IgG) in serum were removed by ProteoExtract Albumin/IgG removal kit according to vendor instruction (Calbiochem). The resulting sera were desalted by a 3000 Da MWCO spin column in 1.5-mL Eppendorf tube. Protein concentrations of the desalted sera were measured by the Bradford assay. The samples were then subjected to digestion as described above.

Because of the high complexity of the digested serum, the glycopeptides were enriched by hydrophilic affinity extraction as previous described.[4,19,20] Briefly, 100 μg of the digest was mixed with 20 μL of Sepharose CL-4B resin and 1 mL organic solvent composed of 1-butanol/ethanol/H_2O (4:1:1, v/v/v) in 1.5-mL Eppendorf tube. The mixture was incubated with gentle shaking for 1 h followed by a quick spin. The supernatant was discarded, and the resin was washed three times with the aforementioned organic solvent. The glycopeptides were eluted with 1 mL of 50% ethanol aqueous solution for 45 min. After a quick spin, the enriched glycopeptides within the supernatant were transferred to another 1.5-mL Eppendorf tube. The same extraction procedure was repeated once to ensure quantitative

sample recovery. The glycopeptides from both supernatants were combined, lyophilized, and labeled using the method mentioned above. To avoid the bias of a particular labeling combination, cross-labeling was carried out in parallel, and the averaged ratios were used in the final analysis. Aliquots of ^{16}O-labeled peptides and ^{18}O-labeled peptides were mixed at a 1:1 ratio, and subsequently analyzed by LC-MS.

2.4. Nano-LC-ESI-MS/MS and MALDI-TOF-MS Analysis. The lyophilized peptide samples were resuspended with 5% ACN in 0.1% formic acid, separated by nanoLC and analyzed by online electrospray tandem mass spectrometry. The experiments were performed on a Nano Aquity UPLC system (Waters Corporation, Milford, MA) connected to a LTQ Orbitrap XL mass spectrometer (Thermo Electron Corp., Bremen, Germany) interfaced with an online nano electrospray ion source (Michrom Bioresources, Auburn, CA). The separation of the peptides was performed in a Symmetry C18, 5 μm, 180 μm i.d. $\times$ 2 cm trap-column and a BEH300 C18, 1.7 μm, 75 μm i.d. $\times$ 15 cm reverse phase column (Waters Corporation, Milford, MA).

Model glycoprotein digests (0.5 μg) were injected onto the trap-column with a flow of 15 μL/min for 3 min. The mobile phases were 5% ACN with 0.1% formic acid (phase A and the loading phase) and 95% ACN with 0.1% formic acid (phase B). To achieve sufficient separation, a 60-min linear gradient from 5 to 45% phase B was employed. The flow rate of the mobile phase was set at 300 nL/min and column temperature was maintained at 35 °C. The electrospray voltage was used at 1.2 kV. In the analyses of the serum samples, the length of the linear gradient was adjusted to 90 min; other parameters were not changed.

LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. The survey full-scan MS spectra with two microscans (m/z 400−2000) were acquired in the Obitrap at a resolution of 100 000 (at m/z 400), followed by eight MS/MS scans in the LTQ trap. Dynamic exclusion was set to initiate a 60-s exclusion for ions analyzed twice within a 10-s interval.

Model glycoprotein digestion was also analyzed by 4700 Proteomics Analyzer (Applied Biosystems) using the method described previously.[21]

2.5. Data Analysis. The mass spectra acquired by Nano-LC-ESI-MS/MS from human serum samples were searched against the human International Protein Index (IPI) database (IPI human v3.45 fasta with 71 983 entries) using the SEQUEST algorithm integrated into the Bioworks package (Version 3.3.1; Thermo Electron Corp.). The parameters for the SEQUEST search were as follows: enzyme, partial trypsin; missed cleavages allowed, two; fixed modification, carboxyamidomethylation (Cys); variable modifications, deamidation (Asn +0.98 Da), deamidation plus ^{18}O (Asn +2.98 Da), C-term (+4.01 Da) and oxidation (Met +15.99 Da); peptide tolerance, 10 ppm; MS/MS tolerance, 1.0 Da. The statistical significance of Database search results was evaluated with the aid of PeptideProphet.[22] A minimum PeptideProphet probability score (P) filter of 0.9 was elected as threshold to remove low-probability peptides as previous study.[23]

2.6. Western Blotting. To confirm the quantitative results from LC-MS, five model proteins were further validated with Western blotting. Depleted sera from normal and ovarian cancer were used. For depleted serum samples, no stably expressed protein can be used as internal control.[24] Total serum protein concentration was determined by the Bradford assay. Equal amounts of proteins were separated by 10% SDS-PAGE gels and then transferred onto nitrocellulose membranes. After blocking the membrane with 5% nonfat dry milk in TBST (150 mM NaCl, 0.1% Tween-20, 25 mM Tris, pH 7.5) for 2 h at room temperature, the membrane was incubated with a panel of primary antibodies in parallel analyses, that is, rabbit polyclonal antibody targeting alpha 2 Macroglobulin (Abcam, ab48555), rabbit polyclonal antibody targeting Zinc Alpha 2 Glycoprotein (Abcam, ab47116), mouse monoclonal antibody targeting alpha 1 Antitrypsin (Abcam, ab9399), rabbit polyclonal antibody targeting Antithrombin-III (santa cruz, sc-32887) and rabbit polyclonal antibody targeting Alpha-1-antichymotrypsin (santa cruz, sc-22747). Following incubation with primary antibody, the membranes were washed with TBST. The detection was performed using a horseradish peroxidase-conjugated secondary antibody (1 h at room temperature) and the ECL detection system. The quantitative signals were acquired and visualized via a LAS-3000 imager.

3. Results and Discussion

3.1. Strategy of Tandem ^{18}O Stable Isotope Labeling (TOSIL). The experimental strategy of TOSIL in quantitative glycoproteomics is shown in Figure 1. The two peptide mixtures to be compared were treated in tandem with trypsin and PNGase F to label the C-terminus of peptide and sites of N-glycolysation. One sample was treated in ^{16}O-water and the other was treated in ^{18}O-water. Three oxygen atoms were exchanged for glycosylated peptides, two at the C-terminus and one at the site of glycosylation. In contrast, only two oxygen atoms were exchanged for nonglycosylated peptides, both at the C-terminus. The two samples labeled with ^{16}O or ^{18}O were then mixed and analyzed by Nano-LC-ESI-MS/MS. Peptides were quantified by measuring the relative signal intensities of peptide ion pairs in the precursor scan. Relative concentration ratios were calculated assuming that at least one ^{18}O was incorporated during the enzymatic reaction.[25]

Equation 1 was used to calculate the ratio of glycopeptides:

$$\text{ratio}\left(\frac{^{16}\text{O}}{^{18}\text{O}}\right) = \frac{I_0}{I_2 + I_4 + I_6 - \left(\frac{M_2}{M_0}\right)I_4 - \left[\frac{M_2}{M_0} + \frac{M_4}{M_0} - \left(\frac{M_2}{M_0}\right)^2\right]I_2 - \left[\frac{M_2}{M_0} + \frac{M_4}{M_0} + \frac{M_6}{M_0} - \left(\frac{M_2}{M_0}\right)^2 + \left(\frac{M_2}{M_0}\right)^3 - 2\frac{M_2M_4}{{M_0}^2}\right]I_2} \quad (1)$$

Equation 2 was used to calculate the ratio of nonglycopeptides, similar to that previously reported.[25,26]

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

where I_0, I_2, I_4 and I_6 are the measured relative intensities of the monoisotope peak for the peptide, the peak with 2 Da increase in mass, the peak with 4 Da increase in mass, and the peak with 6 Da increase in mass, respectively; M_0, M_2, M_4 and M_6 are the corresponding theoretical relative intensities of the isotopic envelope of the peptide, which are calculated using MS-Isotope (http://prospector.ucsf.edu).

With the intensity ratios of ^{18}O/^{16}O for glycosylated (a/b) and for nonglycosylated (c/d) peptides, the change of N-glycosy-

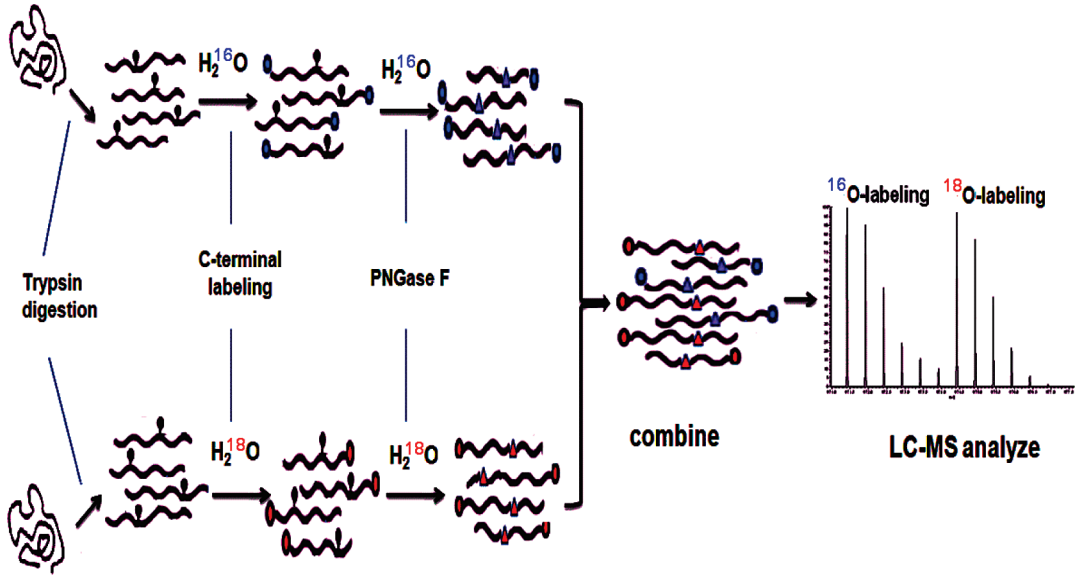

Figure 1. Analytical strategy of Tandem ^{18}O Stable Isotope Labeling (TOSIL) in quantitative proteomics. Protein mixtures were digested with trypsin and then with PNGase F in either ^{16}O- or ^{18}O-water. The two samples were combined and subjected to LC-MS. A pair of ^{18}O-labeled and unlabeled glycopeptides was easily detected because they had the same retention time and a 6 Da mass difference. The relative quantitation was determined by the ratio of peptide pairs.

lated protein level (just as a/b) and the change of its parent protein level (as c/d) associated with pathogenesis can be obtained simultaneously. As a consequence, a comparison (e.g., ratio of $(a/b)/(c/d)$) of these two changes can be utilized to evaluate the changes of N-glycosylation site occupancy[12] $[(a/c)/(b/d)]$ from control (b/d) to disease (a/c). With such a comparison, we might avoid experimental difficulties in a direct measurement of N-glycosylation site occupancy $(b/d$ or $a/c)$.

3.2. TOSIL for Model Glycoproteins. To demonstrate the effectiveness of TOSIL strategy in characterizing protein glycosylation, we analyzed an N-glycosylated model protein, fetuin. The MS spectra of a deglycosylated glycopeptide (LCPDCPLLAPLN#DSR) via TOSIL strategy were acquired both by MALDI-TOF (Figure 2A) and ESI-MS (Figure 2B). Characteristic 6 Da shift in mass were observed. On the other hands, the labeling procedure with PNGase F only introduced a 2 Da shift in mass and overlapping of isotopic envelope was evident (Figure 2C). Thus, TOSIL strategy will significantly enhance the accuracy of quantitative protein glycosylation analyses.

To investigate the dynamic range of the method, ^{16}O- and ^{18}O-labeled glycopeptides were pooled at various ratios including 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10. As a result, the measured ratios for the four glycopeptides from model glycoprotein, invertase and fetuin, were found to be in close agreement with the expected ratios. A dual-logarithm plot between the theoretical ratio and the corresponding measured ratios was generated, exhibiting a correlation coefficient $r^2 > 0.99$ (Supporting Information Figure 1). Moreover, six nonglycopeptides were also analyzed, showing a good linearity between the theoretical ratio and the corresponding measured ratios (Supporting Information Figure 2). These results supported the TOSIL method had good linearity within the 10-fold dynamic range, and the resulting quantitative information, was accurate and reliable.

For the model glycoprotein, fetuin, the same glycosylation site was observed to be in different glycopeptides due to partial enzymatic digestion, such as LCPDCPLLAPLN#DSR, CPLLAPLN#DSR, KLCPDCPLLAPLN#DSR and PLLAPLN#DSR. We examined the linearity of all four forms within the 10-fold dynamic range. The glycopeptide LCPDCPLLAPLN#DSR showed close correlation between experimental and expected ratios, as well as a good linearity. This glycopeptide was identified with the most spectrum counts and the highest intensity; the ratio calculated from this glycopeptide was very close to the theoretical value. To accurately delineate the changes in N-glycosylation levels from biological samples, the peptide with highest abundance and most spectrum counts would be selected to calculate the ratio.[27,28]

3.3. Criteria for TOSIL Quantification. We have established a set of criteria for glycopeptide quantification in order to quantify glycoproteins from healthy and diseased human samples. The standard deviation (SD) was first evaluated. In six replicate analyses of the glycopeptides from model glycoproteins mixed at 1:1 ^{16}O/^{18}O ratios, the SD ranged from 0.06 to 0.21 (Supporting Information Table 1). As suggested in references, the accuracy of ^{18}O-labeling method was relevant to the resolution of mass spectrometer. For example, the mass spectrometer with relatively low resolution such as LCQ ion trap mass spectrometer showed that more than 97% of peptide pairs were within 2-fold changes $(1.0 + 1.0/-0.50)$ and more than 81% were within 1.5-fold $(1.0 + 0.5/-0.33)$, in the analysis of two equivalent samples.[16] With LC-FTICR, the average ratio for all peptide pairs was improved to 1.02 ± 0.23.[29] In this study, the LTQ Orbitrap provided a mass resolution of 100 000 at m/z of 400, and resulted a maximal SD values of 0.21 for model glycoprotein digests. In general, we have taken more than 3-fold of the maximal SD apart from the abundance ratio at 1.0, to estimate the N-glycoslylation levels being changed significantly from the control experiment in a 99% confidence level. Statistically, we have utilized 1.63-fold of expected 1:1 ratio for both up and low limit (either >1.63 or <0.61), and similarly in the cross labeling. If the changes are just from a SD to limit (1.21−1.63 or 0.83−0.61), these changes of N-glycosylation levels should be considered as minor.

3.4. TOSIL Quantification of Glycoproteins in Normal and Ovarian Carcinoma Serum Samples. To test the practicality of TOSIL method in complicated samples, we quantified glycopeptides to screen glycopeotome quantitatively in two

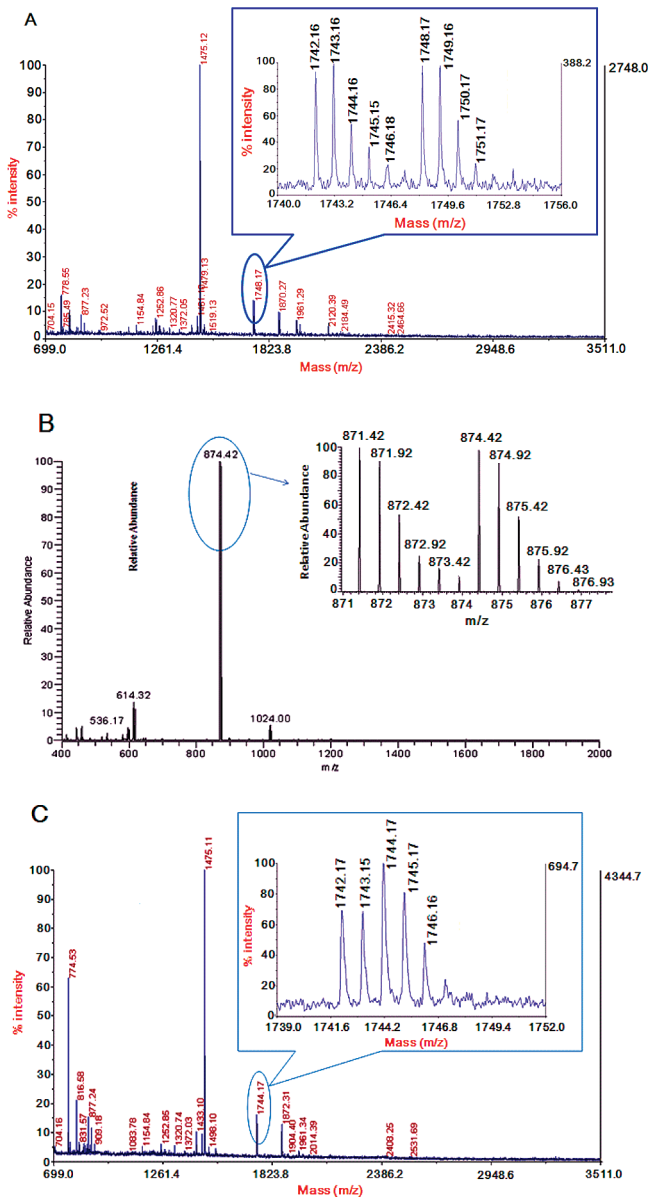

Figure 2. The glycopeptide LCPDCPLLAPLN#DSR from model protein fetuin was examined by TOSIL with MALDI-TOF MS (A) and ESI-MS (B) at expected ratio of 1:1; and by only PNGase F step in $H_2{}^{18}O$ against in normal $H_2{}^{16}O$ with MALDI-TOF MS (C). The isotope distributions of the ^{16}O- and ^{18}O-tagged peptide overlapped due to 2-mass unit difference. The # in LCPDCPLLAPLN#DSR denotes the residue site of N-glycosylation.

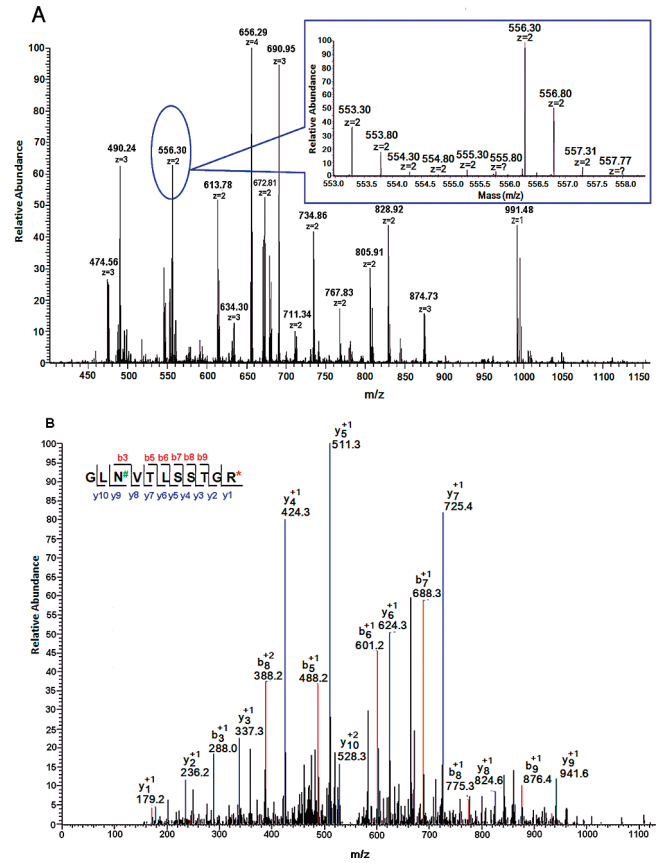

Figure 3. TOSIL quantitative analysis of deglycosylated peptide GLN#VTLSSTGR* [$(M + 2H)^{2+}$ at m/z 553.30] from Complement C4-A precursor by Nano-LC-MS: (A) the PMF spectrum of glycopeptide with an initial MS scan to quantify the occupancy of N-glycosylated site, showing a calculated $^{18}O/^{16}O$ ratio of 2.899 as indicated in the inset enlarged-spectrum; (B) the CID spectrum of glycopeptide at m/z 556.30 (^{18}O-labeling). y1-y8 ions from this glycopeptide displayed a 4.00 Da mass shift indicating that two ^{18}O atoms are localized at the carboxyl terminus, and y9-y10 ions showed a mass increase of 6.98 Da (4.00 Da plus 2.98 Da) confirming that the site of N-glycosylation was labeled by the third ^{18}O atom. The # denotes the residue site of N-glycosylation in GLN#VTLSSTGR. The * denotes the C terminal carboxylic acid was labeled by two ^{18}O atoms.

human samples of ovarian carcinoma serum and normal serum, and then the results were confirmed by Western blot.

A representative nano-LC-ESI-MS/MS spectrum of a paired signal at expected 1:1 ratio after labeling with TOSIL was shown in Figure 3A, which represented a peptide fragment of GLN#VTLSSTGR [$(M + 2H)^{2+}$ at m/z 553.30] from complement C4-A precursor in serum samples. The pair of doubly charged peptides has monoisotopic peaks at m/z 553.30 and 556.30 with a 3 Da shift, indicating glycosylation. To sequence the peptide and site of N-glycosylation, the precursor ion at m/z 556.30 from ^{18}O-labeled cancer sample was analyzed by MS/MS (Figure 3B). A mass difference of 117 Da between the y8 and y9 ion corresponds to the mass of aspartic acid labeled by one ^{18}O atom and, therefore, confirms a deamidation of asparagine in the peptide. Furthermore, the series of y ions from this peptide displayed a mass shift of 4 Da indicating that the C-terminal was labeled by two ^{18}O atoms.

A total of 86 unique sites of N-glycosylation from 49 glycoproteins were quantified from both normal and ovarian carcinoma serum samples. We confirmed that all identified N-linked glycopeptides have the consensus motif Asn-X-Thr/Ser (X ≠ P), among which 10 glycosites are novel identifications (no annotation in Swiss-Prot). To eliminate the bias caused by labeling efficiency and to ensure the reliability of the results, cross-labeling was carried out twice. As a result, 69 in total 86 N-glycosites exhibited consistent quantity changes in either labeling combination, and the rest 17 glycosites were quantified in one of cross-labeling experiments. These results demonstrated the excellent reliability of our method.

The set of glycopeptides with the change of N-glycosylation level for ovarian carcinoma serum is provided in Table 1, including 56 significant and 13 minor changes. For the ovarian serous

Table 1. Relative Abundance Changes for N-Glycosylation Levels in Ovarian Cancer (^{18}O Labeling) Serum Compared to Control (^{16}O Labeling)

protein IPI[a]	protein name	peptide sequence[b,c]	N-sites[d]	$^{18}O/^{16}O$ ratio
		N-Glycosylation Sites with Significant Changes in Ovarian Cancer Serum		
IPI00017696.1	Complement C1s subcomponent	K.NCGVN#CSGDVFTALIGEIASPNYPK.P	Y,N174	2.618
IPI00019568.1	Prothrombin	R.YPHKPEIN#STTHPGADLQENFCR.N	Y,N143	2.155
IPI00019943.1	Afamin	*R.DIENFN#STQK.F*	Y,N33	0.466
IPI00020091.1	Alpha-1-acid glycoprotein 2	F.FYFTPN#KTEDTIFLR.E	Y,N72	2.114
IPI00020986.2	Lumican	*K.LHINHNN#LTESVGPLPK.S*	Y,N127	0.346
IPI00020986.2	Lumican	K.AFEN#VTDLQWLILDHNLLENSK.I	Y,N88	0.455
IPI00021727.1	C4b-binding protein alpha chain	H.ASISCTVEN#ETIGVWRPSPPTCEK.I	Y,N221	2.445
IPI00022229.1	Apolipoprotein B-100	*Y.SN#ASSTDSASYYPLTGDTR.L*	Y,N983	2.212
IPI00022229.1	Apolipoprotein B-100	R.FN#SSYLQGTNQITGR.Y	Y,N1523	2.058
IPI00022229.1	Apolipoprotein B-100	R.FEVDSPVYN#ATWSASLK.N	Y,N3895	1.698
IPI00022371.1	Histidine-rich glycoprotein	R.VIDFN#CTTSSVSSALANTK.D	Y,N125	0.218
IPI00022392.1	Complement C1q subcomponent subunit A	R.NPPMGGNVVIFDTVITNQEEPYQN#HSGR.F	Y,N146	2.793
IPI00022395.1	Complement component C9	R.AVN#ITSENLIDDVVSLIR.G	Y,N415	2.364
IPI00022417.4	Leucine-rich alpha-2-glycoprotein	F.N#LTHLPANLLQGASK.L	Y,N79	2.096
IPI00022418.1	Fibronectin	R.DQCIVDDITYNVN#DTFHK.R	Y,N528	6.411
IPI00022418.1	Fibronectin	K.LDAPTNLQFVN#ETDSTVLVR.W	Y,N1007	7.463
IPI00022418.1	Fibronectin	*F.LYNNHN#YTDCTSEGR.R*	Y,N430	5.319
IPI00022429.3	Alpha-1-acid glycoprotein 1	N.LVPVPITN#ATLDQITGK.W	Y,N33	2.375
IPI00022429.3	Alpha-1-acid glycoprotein 1	R.QDQCIYN#TTYLNVQR.E	Y,N93	2.321
IPI00022431.1	Alpha-2-HS-glycoprotein	K.AALAAFNAQNN#GSNFQLEEISR.A	Y,N176	0.524
IPI00022431.1	Alpha-2-HS-glycoprotein	K.VCQDCPLLAPLN#DTR.V	Y,N156	0.562
IPI00022463.1	Serotransferrin	H.LFGSN#VTDCSGNFCLFR.S	Y,N630	0.288
IPI00022463.1	Serotransferrin	*K.CGLVPVLAENYN#K.S*	Y,N432	0.559
IPI00022488.1	Hemopexin	W.SFDATTLDDN#GTMLFFK.G	Y,N64	0.424
IPI00023673.1	Galectin-3-binding protein	R.ALGFEN#ATQALGR.A	Y,N69	3.788
IPI00023673.1	Galectin-3-binding protein	K.GLN#LTEDTYKPR.I	Y,N398	3.534
IPI00032179.2	Antithrombin-III	K.LGACN#DTLQQLMEVFK.F	Y,N128	0.404
IPI00032179.2	Antithrombin-III	K.SLTFN#ETYQDISELVYGAK.L	Y,N187	0.559
IPI00032258.4	Complement C4-A	R.GLN#VTLSSTGR.N	Y,N1328	2.899
IPI00032258.4	Complement C4-A	R.FSDGLESN#SSTQFEVK.K	Y,N226	2.762
IPI00032328.2	Kininogen-1	K.LNAENN#ATFYFK.I	Y,N294	0.358
IPI00032328.2	Kininogen-1	*Y.SIVQTN#CSK.E*	Y,N205	0.564
IPI00061977.1	IGHA1 protein	L.HRPALEDLLLGSEAN#LTCTLTGLR.D	N,N291	0.582
IPI00163207.1	N-acetylmuramoyl-L-alanine amidase	R.LEPVHLQLQCMSQEQLAQVAAN#ATK.E	Y,N367	0.324
IPI00163207.1	N-acetylmuramoyl-L-alanine amidase	W.SLN#ATELDPCPLSPELLGLTK.E	Y,N77	0.213
IPI00163207.1	N-acetylmuramoyl-L-alanine amidase	*R.GFGVAIVGN#YTAALPTEAALR.T*	Y,N485	0.366
IPI00166729.4	Zinc-alpha-2-glycoprotein	K.DIVEYYNDSN#GSHVLQGR.F	Y,N109	1.647
IPI00218192.2	Interalpha-trypsin inhibitor heavy chain H4	*F.MTNQLVDALTTWQN#K.T*	Y,N207	2.481
IPI00218413.2	Biotinidase	*K.NPVGLIGAEN#ATGETDPSHSK.F*	Y,N329	0.551
IPI00218732.3	Serum paraoxonase/arylesterase 1	K.VTQVYAEN#GTVLQGSTVASVYK.G	Y,N324	0.151
IPI00291262.3	Clusterin	K.MLN#TSSLLEQLNEQFNWVSR.L	Y,N354	0.487
IPI00291262.3	Clusterin	K.ELPGVCN#ETMMALWEECKPCLK.Q	Y,N103	0.475
IPI00291866.5	Plasma protease C1 inhibitor	R.VLSN#NSDANLELINTWVAK.N	Y,N253	3.215
IPI00291867.3	Complement factor I	Y.LFQPN#DTCIVSGWGR.E	Y,N464	2.625
IPI00305461.2	Factor VII active site mutant immunoconjugate	*R.EEQYN#STYR.V*	N,N529	0.174
IPI00384952.1	Putative uncharacterized protein DKFZp686K04218	C.LVQGFFPQEPLSVTWSESGQN#VTAR.N	N,N185	0.172
IPI00384952.1	Putative uncharacterized protein DKFZp686K04218	*K.TPLTAN#ITK.S*	N,N343	0.277
IPI00431645.1	HP protein	K.MVSHHN#LTTGATLINEQWLLTTAK.N	N,N59	5.747
IPI00431645.1	HP protein	K.VVLHPN#YSQVDIGLIK.L	N,N116	5.587
IPI00431645.1	HP protein	K.NLFLN#HSEN#ATAK.D	N,N82/N86	5.208
IPI00477090.6	IGHM protein	R.GLTFQQN#ASSMCVPDQDTAIR.V	N,N224	0.109
IPI00478003.1	Alpha-2-macroglobulin	F.SIN#TTNVMGTSLTVR.V	Y,N410	0.231
IPI00478003.1	Alpha-2-macroglobulin	K.SLGNVN#FTVSAEALESQELCGTEVPSVPEHGR.K	Y,N869	0.299
IPI00478003.1	Alpha-2-macroglobulin	K.VSN#QTLSLFFTVLQDVPVR.D	Y,N1424	0.254
IPI00550991.3	Alpha-1-antichymotrypsin	*R.TLN#QSSDELQLSMGNAMFVK.E*	Y,N127	1.789
IPI00553177.1	Alpha-1-antitrypsin	L.NFN#LTEIPEAQIHEGFQELLR.T	Y,N107	0.307
		N-Glycosylation Sites with Minor Changes in Ovarian Cancer Serum		
IPI00009793.4	Complement C1r subcomponent-like protein	*R.PVTPIAQN#QTTLGSSR.A*	Y,N242	0.816

Table 1. Continued

protein IPI[a]	protein name	peptide sequence[b,c]	N-sites[d]	$^{18}O/^{16}O$ ratio
IPI00019399.1	Serum amyloid A-4 protein	Y.LFGN#SSTVLEDSK.S	Y,N94	1.531
IPI00019591.1	Complement factor B	Y.LVLDGSDSIGASN#FTGAK.K	Y,N285	1.536
IPI00020091.1	Alpha-1-acid glycoprotein 2	N.LVPVPITN#ATLDR.I	Y,N33	1.395
IPI00022229.1	Apolipoprotein B-100	*R.VNQNLVYESGSLN#FSK.L*	Y,N2982	1.325
IPI00027482.1	Corticosteroid-binding globulin	R.AQLLQGLGFN#LTER.S	Y,N96	0.652
IPI00291262.3	Clusterin	R.LAN#LTQGEDQYYLR.V	Y,N374	0.749
IPI00291262.3	Clusterin	*R.QLEEFLN#QSSPF.Y*	Y,N145	0.73
IPI00292530.1	Interalpha-trypsin inhibitor heavy chain H1	F.FAPQN#LTNMNK.N	Y,N285	0.803
IPI00298971.1	Vitronectin	K.NN#ATVHEQVGGPSLTSDLQAQSK.G	Y,N86	0.789
IPI00305461.2	Interalpha-trypsin inhibitor heavy chain H2	*F.ISN#FSMTVDGK.T*	Y,N118	0.677
IPI00550991.3	Alpha-1-antichymotrypsin	F.LSLGAHN#TTLTEILK.G	Y,N93	1.227
IPI00553177.1	Alpha-1-antitrypsin	K.YLGN#ATAIFFLPDEGK.L	Y,N271	0.655

[a] Protein accession number and protein name from IPI database, and glycosylation sites are annotated according to Swiss-Prot database. [b] The # denotes the residue site of N-glycosylation. [c] Italic refer to the glycopeptides detected by one cross labeling. [d] Y, found in Swiss-Prot database; N, not found in Swiss-Prot database. When the abundance changes were more than 1.63-fold from the expected 1:1 ratio, the N-glycosylation level was considered to have significant changes in ovarian cancer serum. When the changes were just from a SD to limit (1.21−1.63 or 0.83−0.61), the change of N-glycosylation level was minor.

carcinoma sample, the N-glycosylation level of 56 glycopeptides significantly changed (with up-regulated glycosylation levels of 28 sites, while down-regulated levels of 29), 13 sites showed minor changes, and 16 sites almost not changed. We quantified three peptides with 2-sites of N-glycosylation, for which the calculated ratios represented the change of precursor peptides rather than individual site occupancy.

In parallel, we have also analyzed the total level of parent proteins in sera from the ovarian cancer patient and the healthy individual. The quantification was based on their nonglycosite peptide pairs with a 4 Da mass differences. This analysis was possible because nonglycopeptides were not completely removed during the glycopeptides enrichment procedure. Accordingly, the expression levels of 23 out of the 49 glycoproteins were quantified. Glycopeptides with both significant changes in N-glycosylation level and their parent protein level were summarized in Table 2. Subsequently, we investigated the correlation between the amount of glycosylated and parent proteins. The majority of glycosylated proteins exhibited minor or no changes in N-glycosylation site occupancies, with the same variation tendency as in both glycosylated and parent protein levels. But three proteins displayed 2-fold changes in glycosylation site occupancy (proteins highlighted in bold in Table 2), with a significant change in glycosylated protein level but no change in parent protein level.

In an attempt to confirm the quantitative results obtained by TOSIL method for parent proteins, Western blot was performed by using specific antibodies targeting five representative proteins in the serum samples. The TOSIL method showed the ratios of Alpha-1-antitrypsin, Alpha-2-macroglobulin, Antithrombin-III, Zinc-alpha-2-glycoprotein and Alpha-1-antichymotrypsin were 0.451, 0.177, 0.484, 1.566 and 1.003, comparing ovarian cancer patients and healthy individuals, respectively. On the basis of chemiluminescence intensity measurement, Western blot confirmed the quantity changes observed in the TOSIL method independently: the down-regulation of Alpha-1-antitrypsin, Alpha-2-macroglobulin, Antithrombin-III, the up-regulation of Zinc-alpha-2-glycoprotein, and no change in Alpha-1-antichymotrypsin in the sera of ovarian cancer patients (Figure 4). Thus, the results certify that the TOSIL strategy is an accuracy method for complicated samples.

3.5. Analysis of Glycosite Occupancy Change for Pathological Glance. We paid particular attention to these three proteins, that is, Alpha-1-acid glycoprotein 2, Hemopexin and Interalpha-trypsin inhibitor heavy chain H4. The overall levels of these proteins showed no appreciable changes between control and ovarian cancer serum, but the occupancies of three N-glycosylation sites from these proteins were significantly changed: FYFTPN#KTEDTIFLR from Alpha-1-acid glycoprotein 2, SFDATTLDDN#GTMLFFK from Hemopexin, MTNQLVDALTTWQN#K from Interalpha-trypsin inhibitor heavy chain H4.

Alpha-1-acid glycoprotein 2 (AGP), also known as orosomucoid (ORM), is an acute phase protein, synthesized mainly by hepatocytes. Human AGP is a glycoprotein of 41−43 kDa in molecular mass, which consists approximately of 45% carbohydrate attached in the form of five complex-type of N-linked glycans.[30,31] AGP consists of 183 amino acid residues and a 22 amino acid difference is detected between the two alternative splicing variants, AGP-1 and AGP-2.[32,33] In a number of pathophysiological states, for example, inflammation, rheumatoid arthritis, and cancer, change in AGP protein levels and alterations of N-linked glycans have been observed.[34−36]

AGP-1 in our study showed a significant up-regulation both in protein level and N-glycosylation site occupancy. For AGP-2, there was almost no change of the protein level, but the occupancies of one N-glycosylation site was significantly changed (FYFTPN#KTEDTIFLR). Several studies showed that the constitutive level of AGP-1 is 5-fold higher than AGP-2 and that an increase in AGP-1 mRNA but not AGP-2 mRNA accounts for most of the change in the mouse AGP mRNA pool during the acute phase response.[37] In human ovarian, lymphoma, and melanoma cancer, Budai et al. also found that AGP-1 is predominantly responsible for the acute-phase property of AGP. Clearly, the change of protein level obtained by TOSIL method is in agreement with that in reference, but the change in N-glycosylation site occupancy verified by TOSIL

Table 2. The Changes in N-Glycosylation Levels and Corresponding Proteins in Ovarian Cancer Serum (^{18}O Labeling) Compared to Control (^{16}O Labeling)

protein IPI[a]	protein name	peptide sequence[b]	N-sites	$^{18}O/^{16}O$ ratio for glycopeptide	average $^{18}O/^{16}O$ ratio for protein	change in N-glycosylation site occupancy	no. of peptides quantified/SD
IPI00020091.1	**Alpha-1-acid glycoprotein 2**	**F.FYFTPN#KTEDTIFLR.E**	**Y,N72**	**2.114**	**0.917**	**2.31**	**2/0.16**
IPI00021727.1	C4b-binding protein alpha chain	H.ASISCTVEN#ETIGVWRPSPPTCEK.I	Y,N221	2.445	3.485	0.7	3/0.56
IPI00022229.1	Apolipoprotein B-100	*Y.SN#ASSTDSASYYPLTGDTR.L*	Y,N983	2.212	1.67	1.32	2/0.11
IPI00022229.1	Apolipoprotein B-100	R.FN#SSYLQGTNQITGR.Y	Y,N1523	2.058	1.67	1.23	2/0.11
IPI00022229.1	Apolipoprotein B-100	R.FEVDSPVYN#ATWSASLK.N	Y,N3895	1.698	1.67	1.02	2/0.11
IPI00022418.1	Fibronectin	R.DQCIVDDITYNVN#DTFHK.R	Y,N528	6.411	8.692	0.74	3/1.50
IPI00022418.1	Fibronectin	K.LDAPTNLQFVN#ETDSTVLVR.W	Y,N1007	7.463	8.692	0.86	3/1.50
IPI00022418.1	Fibronectin	*F.LYNNHN#YTDCTSEGR.R*	Y,N430	5.319	8.692	0.61	3/1.50
IPI00022429.3	Alpha-1-acid glycoprotein 1	N.LVPVPITN#ATLDQITGK.W	Y,N33	2.375	1.806	1.32	2/0.56
IPI00022429.3	Alpha-1-acid glycoprotein 1	R.QDQCIYN#TTYLNVQR.E	Y,N93	2.321	1.806	1.28	2/0.56
IPI00022431.1	Alpha-2-HS-glycoprotein	K.AALAAFNAQNN#GSNFQLEEISR.A	Y,N176	0.524	0.505	1.04	2/0.15
IPI00022431.1	Alpha-2-HS-glycoprotein	K.VCQDCPLLAPLN#DTR.V	Y,N156	0.562	0.505	1.11	2/0.15
IPI00022463.1	Serotransferrin	H.LFGSN#VTDCSGNFCLFR.S	Y,N630	0.288	0.263	1.1	5/0.04
IPI00022463.1	Serotransferrin	*K.CGLVPVLAENYN#K.S*	Y,N432	0.559	0.263	2.13	5/0.04
IPI00022488.1	**Hemopexin**	**W.SFDATTLDDN#GTMLFFK.G**	**Y,N64**	**0.424**	**0.837**	**0.51**	**4/0.16**
IPI00032179.2	Antithrombin-III	K.LGACN#DTLQQLMEVFK.F	Y,N128	0.404	0.484	0.84	3/0.09
IPI00032179.2	Antithrombin-III	K.SLTFN#ETYQDISELVYGAK.L	Y,N187	0.559	0.484	1.15	3/0.09
IPI00032258.4	Complement C4-A	R.GLN#VTLSSTGR.N	Y,N1328	2.899	3.152	0.92	2/0.19
IPI00032258.4	Complement C4-A	R.FSDGLESN#SSTQFEVK.K	Y,N226	2.762	3.152	0.88	2/0.19
IPI00061977.1	IGHA1 protein	L.HRPALEDLLLGSEAN#LTCTLTGLR.D	N,N291	0.582	0.351	1.66	3/0.01
IPI00166729.4	Zinc-alpha-2-glycoprotein	K.DIVEYYNDSN#GSHVLQGR.F	Y,N109	1.647	1.566	1.05	2/0.21
IPI00218192.2	**Inter-alpha-trypsin inhibitor heavy chain H4**	***F.MTNQLVDALTTWQN#K.T***	**Y,N207**	**2.481**	**0.905**	**2.74**	**2/0.27**
IPI00291866.5	Plasma protease C1 inhibitor	R.VLSN#NSDANLELINTWVAK.N	Y,N253	3.215	2.217	1.45	2/0.60
IPI00431645.1	HP protein	K.MVSHHN#LTTGATLINEQWLLTTAK.N	N,N59	5.747	4.557	1.26	4/0.83
IPI00431645.1	HP protein	K.VVLHPN#YSQVDIGLIK.L	**N,N**116	5.587	4.557	1.23	4/0.83
IPI00431645.1	HP protein	K.NLFLN#HSEN#ATAK.D	N,N82/86	5.208	4.557	1.14	4/0.83
IPI00477090.6	IGHM protein	R.GLTFQQN#ASSMCVPDQDTAIR.V	N,N224	0.109	0.103	1.06	3/0.01
IPI00478003.1	Alpha-2-macroglobulin	F.SIN#TTNVMGTSLTVR.V	Y,N410	0.231	0.177	1.31	5/0.06
IPI00478003.1	Alpha-2-macroglobulin	K.SLGNVN#FTVSAEALESQELCGTEVPSVPEHGR.K	Y,N869	0.299	0.177	1.69	5/0.06
IPI00478003.1	Alpha-2-macroglobulin	K.VSN#QTLSLFFTVLQDVPVR.D	Y,N1424	0.254	0.177	1.44	5/0.06
IPI00553177.1	Alpha-1-antitrypsin	L.NFN#LTEIPEAQIHEGFQELLR.T	Y,N107	0.307	0.451	0.68	3/0.10

[a] Numbers highlighted in bold are the changes in the occupancy of N-glycosylation sites inconsistent with the glycoproteins. [b] Italic refer to the glycopeptides detected by one cross-labeling.

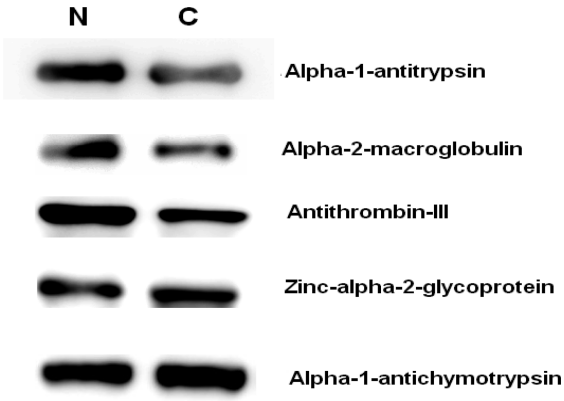

Figure 4. Validation of differentially expressed glycoproteins by Western blot. Depleted serum samples from normal (N) and ovarian cancer (C) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The immunoreactive band intensities were quantified and statistical analysis was performed. The result showed a lower expression levels for alpha-1-antitrypsin, alpha-2-macroglobulin and antithrombin-III, a higher expression levels for zinc-alpha-2-glycoprotein and nearly no change for alpha-1-antichymotrypsin in ovarian cancer patients (C) than those in normal controls (N).

method is new. The current data set suggested the alternation in N-glycosylation of AGP-2 may have significant pathogenesis relevance.

Hemopexin (HPX) is a 60-KDa plasma glycoprotein with the highest affinity to heme. It belongs to acute phase reactants expressed mainly in liver, and induced by inflammation. The binding affinity between heme and HPX and the presence of a specific heme-HPX receptor were able to catabolize the complex and to induce intracellular antioxidant activities. Hemopexin is a major vehicle for the transportation of heme in plasma, and prevents heme-mediated oxidative damage as well as the loss of heme-bound iron. Although HPX is a glycoprotein, previous reports showed that asialo-hemopexin also binds heme tightly, and heme−asialohemopexin complexes are recognized by the HPX receptors.[38] However, in our study, the occupancy of an N-glycosylation site (SFDATTLDDN#GTMLFFK) was significantly reduce with no change in protein level. We reason that the change in occupancy of this N-glycosylation site may impact the interaction between HPX and heme, thus, regulating heme-mediated oxidative stress and loss of heme-bound iron.

Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) is a glycoprotein mainly expressed in the liver tissue.[39] It is a member in intera-α-trypsin inhibitor family of serine protease inhibitors (ITIH1, 2, 3, and 4), which have diverse biological functions, including antiapoptotic and matrix-stabilizing activities. Kashyap et al. reported that the ITIH4 protein is completely absent in serum of patients suffering acute ischemic stroke (AIS), and the recovery of serum ITIH4 levels is indicative of condition improvement for AIS patients.[40] In human lung adenocarcinoma, ITIH4 was also proposed as a potential diagnostic serum marker.[41] In the present study, the protein level of ITIH4 showed no change in ovarian cancer serum compared to control, but N-glycosylation site occupancy was significantly increased at site N207 (MTNQLVDALTTWQN#K). We might hypothesize, thus, the glycosylation modification status of this particular site as a tentative marker for ovarian cancer diagnosis, which will be examined in our future studies.

Additional proteins involved in ovarian cancer were observed with the consistent changes between protein level and N-glycosylation site occupancy. As an example, fibronectin (FN) is a glycoprotein present in a soluble dimeric form in plasma, involved in cell adhesion, migration, wound healing, blood coagulation, host defense, and metastasis. N-linked glycan fragments attached to fibronectin found in sera of ovarian cancer patients were distinct from those in healthy individuals.[42] Ueda et al. have reported that some glycosylation levels in human serum fibronectin would increase in cancer.[43] In this study, we identified significant up-regulation in occupancy of three N-glycosylation sites in fibronectin. Antithrombin-III (ATIII), an extracellular plasma protein, is a crucial serine protease inhibitor that regulates the coagulation cascade. The levels of ATIII were significantly reduced in the patients suffering ovarian cancer.[44] Consistently, the analysis of the N-glycosylation levels in ATIII by TOSIL method revealed the occupancy of two N-glycosylation sites were down regulated.

Therefore, using TOSIL method to analyze the correlation between N-glycosylation site occupancy and corresponding protein, we would be able to discover important N-glycosylation sites of potential clinically significance in terms of disease etiology. The delineation of N-glycosylation codes will aid the characterization of molecular mechanism of pathogenesis as well as establishment of novel biomarkers.

4. Conclusions

We have developed a tandem ^{18}O stable isotope labeling (TOSIL) method for high-throughput quantitative analysis of N-glycosylation site occupancy. This method supports reliable and accurate quantification of the changes in N-glycosylation site occupancy, even with a complex protein mixture. Our strategy showed several unique strengths: TOSIL approach incorporates three atoms of ^{18}O in glycopeptides with one N-glycosylation, thus, introducing a 6 Da difference among differently labeled glycopeptides to support accurate quantification. A single process can be carried out not only for identifying the N-glycosylation sites, but also for quantifying the change both in glycosylation level of a certain glycosylation site and in protein expression level between two samples, for example, normal and diseased. The sites of N-glycosylation are characterized quantitatively in the context of corresponding protein expression levels, which empowers the estimation of N-glycosylation site occupancy. We believe the TOSIL strategy is a valuable addition to the proteomic toolbox bridging analytical proteomics and clinical application. However, the TOSIL demands a user-friendly software that can be used by all mass spectrometers and with all database search tools to quantify the occupancy of N-glycosylation sites automatically.

Abbreviations: TOSIL, tandem ^{18}O stable isotope labeling; PNGaseF, Peptide: N-Glycosidase F.

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Supporting Information Available: Dual-logarithm plot between the theoretical ratio and the corresponding measured ratios for four glycopeptides and for six nonglycopeptides; the SD for four glycopeptides from two model glycoproteins at 1:1 $^{16}O/^{18}O$ ratios. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Apweiler, R.; Hermjakob, H.; Sharon, N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta* **1999**, *1473*, 4–8.

(2) Roth, J. Protein N-glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control, and cell interactions. *Chem. Rev.* **2002**, *102*, 285–303.

(3) Dube, D. H.; Bertozzi, C. R. Glycans in cancer and inflammation potential for therapeutics and diagnostics. *Nat. Rev. Drug Discovery* **2005**, *4*, 477–488.

(4) Cao, J.; Shen, C. P.; Wang, H.; Shen, H. L.; Chen, Y. H.; Nie, A. Y.; Yan, G. Q.; Lu, H. J.; Liu, Y. K.; Yang, P. Y. Identification of N-glycosylation sites on secreted proteins of human hepatocellular carcinoma cells with a complementary proteomics approach. *J. Proteome Res.* **2009**, *8*, 662–672.

(5) Kalyan, R. Anumula high-sensitivity and high-resolution methods for glycoprotein analysis. *Anal. Biochem.* **2000**, *283*, 17–26.

(6) Morelle, W.; Canis, K.; Chirat, F.; Faid, V.; Michalski, J. C. The use of mass spectrometry for the proteomic analysis of glycosylation. *Proteomics* **2006**, *6*, 3993–4015.

(7) Chen, R.; Jiang, X. N.; Sun, D. G.; Han, G. H.; Wang, F. J.; Ye, M. L.; Wang, L. M.; Zou, H. F. Glycoproteomics analysis of human liver tissue by combination of multiple enzyme digestion and hydrazide chemistry. *J. Proteome Res.* **2009**, *8*, 651–661.

(8) Freeze, H. H. Update and perspectives on congenital disorders of glycosylation. *Glycobiology* **2001**, *11*, 129R–143R.

(9) Spiro, R. G. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* **2002**, *12*, 43R–56R.

(10) Schulenberg, B.; Beechem, J. M.; Patton, W. F. Mapping glycosylation changes related to cancer using the multiplexed mroteomics technology: a protein differential display approach. *J. Chromatogr., B* **2003**, *793*, 127–139.

(11) Zhang, H.; Li, X. J.; Martin, D. B.; Aebersold, R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol.* **2003**, *21*, 660–666.

(12) Zhou, L.; Beuerman1, R. W.; Chew, A. P.; Koh1, S. K.; Cafaro, T. A.; Urrets-Zavalia, E. A.; Urrets-Zavalia, J. A.; Li, S. F. Y.; Serra, H. M. Quantitative analysis of N-Linked glycoproteins in tear fluid of climatic droplet keratopathy by glycopeptide capture and iTRAQ. *J. Proteome Res.* **2009**, *8*, 1992–2003.

(13) Hulsmeie, A. J.; Paesold-Burda, P.; Hennet, T. N-Glycosylation site occupancy in serum glycoproteins using multiple reaction monitoring liquid chromatography-mass spectrometry. *Mol. Cell. Proteomics* **2007**, *6*, 2132–2138.

(14) Sharon, N.; Grisaro, V.; Neumann, H. Pepsin-catalyzed exchange of oxygen atoms between water and carboxylic acids. *Arch. Biochem. Biophys.* **1962**, *97*, 219–221.

(15) Bender, M. L.; Kemp, K. C. Oxygen-18 studies of the mechanism of the α-chymotrypsin-catalyzed hydrolysis of esters. *J. Am. Chem. Soc.* **1957**, *79*, 111–116.

(16) Sakai, J.; Kojima, S.; Yanagi, K.; Kanaoka, M. ^{18}O-labeling quantitative proteomics using an ion trap mass spectrometer. *Proteomics* **2005**, *5*, 16–23.

(17) Kaji, H.; Saito, H.; Yamauchi, Y.; Shinkawa, T.; Taoka, M.; Hirabayashi, J.; Kasai, K.; Takahashi, N.; Isobe, T. Lectin affinity capture, isotope-coded tagging and mass spectrometry to identify N-linked glycoproteins. *Nat. Biotechnol.* **2003**, *21*, 667–672.

(18) Reynolds, K. J.; Yao, X. D.; Fenselau, C. Proteolytic 18O labeling for comparative proteomics: evaluation of endoprotease Glu-C as the catalytic agent. *J. Proteome Res.* **2002**, *1*, 27–33.

(19) Wada, Y.; Tajiri, M.; Yoshida, S. Hydrophilic affinity isolation and MALDI multiple-stage tandem mass spectrometry of glycopeptides for glycoproteomics. *Anal. Chem.* **2004**, *76*, 6560–6565.

(20) Tajiri, M.; Yoshida, S.; Wada, Y. Differential analysis of site-specific glycans on plasma and cellular fibronectins: application of a hydrophilic affinity method for glycopeptide enrichment. *Glycobiology* **2005**, *15*, 1332–1340.

(21) Wei, L. M.; Xue, Y.; Zhou, X. W.; Jin, H.; Shi, Q.; Lu, H. J.; Yang, P. Y. Nanodiamond MALDI support for enhancing the credibility of identifying proteins. *Talanta* **2008**, *74*, 1363–1370.

(22) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **2002**, *74*, 5383–5392.

(23) Zhou, Y.; Aebersold, R.; Zhang, H. Isolation of N-linked glycopeptides from plasma. *Anal. Chem.* **2007**, *79*, 5826–5837.

(24) Mao, L. M.; Dong, H. T.; Yang, P. Z.; Zhou, H. Y.; Huang, X. K.; Lin, X. M.; Kijlstra, A. MALDI-TOF/TOF-MS reveals elevated serum haptoglobin and amyloid A in Behcet's disease. *J. Proteome Res.* **2008**, *7*, 4500–4507.

(25) Yao, X. D.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenselau, C. Proteolytic ^{18}O labeling for comparative proteomics: model studies with two serotypes of adenovirus. *Anal. Chem.* **2001**, *73*, 2836–2842.

(26) Zang, L.; Toy, D. P.; Hancock, W. S.; Sgroi, D. C.; Karger, B. L. Proteomic analysis of ductal carcinoma of the breast using laser capture microdissection, LC-MS, and ^{16}O/^{18}O Isotopic Labeling. *J. Proteome Res.* **2004**, *3*, 604–612.

(27) Anderson, L.; Hunter, C. L. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics* **2006**, *5*, 573–588.

(28) Keshishian, H.; Addona, T.; Burgess, M.; Kuhn, E.; Carr, S. A. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* **2007**, *6*, 2212–2229.

(29) Qian, W. J.; Monroe, M. E.; Liu, T.; Jacobs, J. M.; Anderson, G. A.; Shen, Y. F.; Moore, R. J.; Anderson, D. J.; Zhang, R.; Calvano, S. E.; Lowry, S. F.; Xiao, W. Z.; Moldawer, L. L.; Davis, R. W.; Tompkins, R. G.; Camp, D. G.; Smith, R. D. Quantitative proteome analysis of human plasma following in vivo lipopolysaccharide administration using 16O/^{18}O labeling and the accurate mass and time tag approach. *Mol. Cell. Proteomics* **2005**, *4*, 700–709.

(30) Schmid, K.; Nimerg, R. B.; Kimura, A.; Yamaguchi, H.; Binette, J. P. The carbohydrate units of human plasma α1-acid glycoprotein. *Biochim. Biophys. Acta* **1977**, *492*, 291–302.

(31) Yoshima, H.; Matsumoto, A.; Mizuochi, T.; Kawasaki, T.; Kobata, A. Comparative study of the carbohydrate moieties of rat and human plasma α1-acid glycoproteins. *J. Biol. Chem.* **1981**, *256*, 8476–8484.

(32) Fournier, T.; Medjoubi-N, N.; Porquet, D. α1-Acid glycoprotein. *Biochim. Biophys. Acta* **2000**, *1482*, 157–171.

(33) Dente, L.; Pizza, M. G.; Metspalu, A.; Cortese, R. Structure and expression of the genes coding for human α1-acid glycoprotein. *EMBO J.* **1987**, *6*, 2289–2296.

(34) Dijk, W. V.; Havenaar, E. C.; Brinkman-van der Linden, E. C. M. α1-Acid glycoprotein (orosomucoid): pathophysiological changes in glycosylation in relation to its function. *Glycoconjugate J.* **1995**, *12*, 227–233.

(35) Dijk, W. V.; Poland, D. C. Anti-inflammatory properties of specific glycoforms of human a1-acid glycoprotein. *Adv. Exp. Med. Biol.* **2003**, *535*, 251–256.

(36) Hochepied, T.; Berger, F. G.; Baumannc, H.; Libert, C. α1-Acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties. *Cytokine Growth Factor Rev.* **2003**, *14*, 25–34.

(37) Carter, K. C.; Post, D. J.; Papaconstantinou, J. Differential expression of the mouse alpha-1-acid glycoprotein genes (AGP-1 and AGP-2) during inflammation and aging. *Biochim. Biophys. Acta* **1991**, *1089*, 197–205.

(38) Smith A. Role of redox-reactive metals in the regulation of the metallothionein and heme oxygenase genes by heme and hemopexin. In *Iron Metabolism*; Ferreira, G. C., Moura, J. J. G., Franco R., Eds.; Wiley-VCH: Weinheim, 1999; pp 65−92.

(39) Nishimura, H.; Kakizaki, I.; Muta, T.; Sasaki, N.; Pu, P. X.; Yamashita, T.; Nagasawa, S. cDNA and deduced amino acid sequence of human PK-120, a plasma kallikrein-sensitive glycoprotein. *FEBS Lett.* **1995**, *357*, 207–211.

(40) Kashyap, R. S.; Nayak, A. R.; Deshpande, P. S.; Kabra, D.; Purohit, H. J.; Taori, G. M.; Daginawala, H. F. Inter-α- trypsin inhibitor heavy chain 4 is a novel marker of acute ischemic stroke. *Clin. Chim. Acta* **2009**, *402*, 160–163.

(41) Heo, S. H.; Lee, S. J.; Ryoo, H. M.; Park, J. Y.; Cho, J. Y. Identification of putative serum glycoprotein biomarkers for human lung adenocarcinoma by multilectin affinity chromatography and LC-MS/MS. *Proteomics* **2007**, *7*, 4292–4302.

(42) Li, B. S.; An, H. J.; Kirmiz, C.; Lebrilla, C. B.; Lam, K. S.; Miyamoto, S. Glycoproteomic analyses of ovarian cancer cell lines and sera from ovarian cancer patients show distinct glycosylation changes in individual protein. *J. Proteome Res.* **2008**, *7*, 3776–3788.

(43) Ueda, K.; Katagiri, T.; Shimada, T.; Irie, S.; Sato, T. A.; Nakamura, Y.; Daigo, Y. Comparative profiling of serum glycoproteome by sequential purification of glycoproteins and 2-nitrobenzensulfenyl (NBS) stable isotope labeling: a new approach for the novel biomarker discovery for cancer. *J. Proteome Res.* **2007**, *6*, 3475–3483.

(44) Koh, S. C. L.; Khalil, R.; Lim, F. K.; Ilancheran, A.; Choolani, M. The association between fibrinogen, von Willebrand factor, antithrombin III, and D-dimer levels and survival outcome by 36 months from ovarian cancer. *Clin. Appl. Thromb./Hemostasis.* **2006**, *12*, 3–8.

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