Changes of Serum Glycoproteins in Lung Cancer Patients

Tran The Thanh, Nguyen Thi Minh Phuong, Nguyen Bich Nhi & Phan Van Chi*

Institute of Biotechnology (IBT), Vietnamese Academy of Science & Technology (VAST),
18 Hoang Quoc Viet, Hanoi, Vietnam

*Corresponding author: Phan Van Chi, Institute of Biotechnology (IBT),
Vietnamese Academy of Science & Technology (VAST), Tel: (+84-4) 7913657; E-mail: chi@ibt.ac.vn

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Abstract

Lung cancer (LC) is one of the leading causes of cancer deaths worldwide and approximately 3 million individuals die from this disease each year. Therefore, detecting LC at its early stages is very important to achieve decreased LC mortality. While gene expression profiling has been successfully used to classify various tumors and assess tumor stages, the gene-based prediction for LC is not yet entirely dependable. In serum, glycosylation is one of the most common post-translational modifications, and glycoproteins play a core role in a diversity of biological processes and pathogenesis. The development of an analytical approach for the study of variations of human serum glycoproteins has been limited by the structural heterogeneity of the post-translational modifications and the complexity of glycomics. Thus, in this report we present a strategy of using Concavanalin A (Con A) as an affinity agent combined with two-dimensional electrophoresis (2-DE) and nanoLC ESI-MS/MS to enrich and characterize the N-linked glycoproteins that are the most common glycosylation motifs in serum. By comparison of serum samples between LC patients and the healthy, we found that 8 glycoproteins were significantly up regulated in LC serum and 3 glycoproteins were down regulated. The useful information related to LC was discovered while we investigated changes of glycosylation in LC serum. The results suggested that the combination of proteomic techniques could be used for mining protein biomarkers for LC.

Keywords: 2-DE; Con A; Glycomics; Glycosylation; Lung cancer; NanoLC-ESI-MS/MS

Introduction

Lung cancer (LC) is one of the most common causes of cancer deaths throughout the world, and approximately 3 million individuals die from this disease annually. According to American Cancer Society, LC was the cause of nearly 162,460 cancer deaths in the United States in 2006, accounting for nearly 29% of all deaths from cancer (Jemal et al., 2006). The 5- and 10-year survival rates for LC remain very low at 14% and 8%, respectively (Chen et al., 2003). This is largely due to the late stage of diagnosis and the lack of effective treatments. Therefore, early detection is considered crucial for successful clinical therapy, an improved prognosis, and increased survival rate. In serum, glycosylation is one of the most common post-translational modifications and glycoproteins occupy nearly 50% of protein profile. Glycoproteins and glycosylation play a fundamental role in a series of biological processes and pathogenesis. They appear to serve many functions such as stabilizing protein, structure, protecting proteins from degradation, changing protein solubility, affecting the transport of proteins in cells, determining the half-life of proteins in the blood, controlling cellular processes and immunizing (Rudd et al., 2001). The changes of glycosylation are considered to involve in pathogenesis. It has been shown that glycosylation is correlated with signal pathways associated with the transformation of a normal cell to a cancer cell and glycosylation has been intimately associated with cancer (Alper J, 2003). Several groups have earlier carried out proteomic studies of LC (Kikuchi and Carbone, 2007; Ting et al., 2005) A number of proteins with the expression levels and their alterations were identified.

Proteomic analysis is a powerful and promising technology developed to enhance our study on the diagnosis, treatment and prevention of human diseases (Hunt, 2002). By comprehensively examining the different protein expression profiles, proteomics may provide useful information on new biomarkers, disease-associated targets and the processes of pathogenesis. Up to now, this technique has been extensively employed to investigate cancers and other diseases, especially in mining of protein markers for early diagnosis. Specific markers used for diagnosis and treatments have been seeking for many past decades. Some of them, CA125, CEA and AFP are the most widespread markers (Pauld et al., 2004). While glycoproteins occupy a major part of serum proteome, there is currently a lack of global methods for characterizing them and their changes in glycosylation. Thus, the development of an analytical approach for the study of variations of human serum glycoproteins is necessary to provide more useful information related to pathogenesis.

In this study, we present results of enrichment and isolation of glycoproteins by using Con A, a lectin from *Concanavalia ensiformis* as an affinity agent. It has been shown that Con A predominately recognizes alpha-mannose which is very common in N-linked glycans. These collected glycoproteins were then separated by 2-DE technique. The protein spots were further excised, trypsin-digested, and analyzed by nanoLC-ESI-MS/MS and identified by MASCOT v1.8 software.

Materials and Methods

Materials

Patient blood samples were supplied by the National Hospital K (Hanoi). Sera were collected at the time of diagnosis from 16 patients with LC, following informed consent. This group consisted of 10 males and 6 females. Patients with LC were diagnosed according to standard clinical criteria as provided by the hospital. The control group consisted of 18 serum samples (9 males and 9 females) were collected by the same procedure.

The protein contents of all serum samples were determined by the Bradford assay. Sera collected from LC patients and the healthy were divided and stored at $-80\ ^oC$ until use. All equipments and standard reagents used directly should be clean as necessary.

Methanol, Acetonitrile (ACN) were purchased from J.T Barker (Pittsburgh, USA); formic acid (FA), trifluroacetate (TFA) were obtained from Fluka (Fluka Chemie GmbH, Buchs, Switzerland); dithiothreitol (DTT), iodoacetamide (IAA), amonium bicarbonate (NH_4HCO_3), trypsin (sequence grade), methyl alpha-D-glycopyranoside were purchased from Sigma-Aldrich (St. Louis, MO, USA); Agarose bound Concanavalin A was obtained from Amersham Biosciences (Amersham Biosciences, Uppsala, Sweden). 2-D Starter Kit was purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA) and Coomassie® Brilliant Blue R250 was purchased from MP Biomedicals (MP Biomedicals, Eschwege, Germany).

Preparing con a affinity column

An immobilized Con A column was prepared by adding 1ml of corresponding agarose bound lectin to empty PD-10 disposable columns (Thiet et al., 2006). The agarose gel was then fixed between two frits. The columns were either immediately used or stored in an equilibration buffer (20 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM Ca^{2+}, 1 mM Mn^{2+}) at 4^oC. The flow through the column was gravitatively driven.

Isolating glycoproteins using con a-based affinity column

200 µl of human serum was diluted 10 times with the equilibration buffer for Con A lectin and loaded on the affinity column. After the 20 min reaction, the unbound proteins were washed with 10 ml of equilibration buffer and the flow-through was collected. The bound glycoproteins were released with 10 ml of elution buffer (0.5 mM methyl-α-D-glucopyranoside) specific for Con A and the eluted fraction was collected.

SDS-PAGE

The glycoprotein fractions isolated from the fragmentation of the serum on Con A affinity column were analyzed on the 12,6% SDS-PAGE gel with loading amount of 15 µg of total protein for each lane. The proteins were separated with SDS-PAGE running buffer in Bio-Rad MiniCell system (Bio-Rad, Hercules, CA, USA) at 140 V for 2.5 h. Proteins were visualized by staining with Coomassie® Brilliant Blue R250 (MP Biomedicals, LCC, Eschwege, Germany).

2-DE and quantitative analysis of protein expression level

2-DE was performed with the pH 4-7 ReadyStrip IPG strips in the PROTEAN IEF Cell (Bio-Rad, Hercules, CA, USA) using the protocol suggested by the manufacturer. Briefly, 125 µg of glycoprotein were mixed into 120 µl of ReadyPrep rehydration buffer (containing 8 M ure, 2% CHAPS, 50 mM DTT, 0.2% (w/v) Bio-Lyte 3/10 ampholyteand Bromophenol Blue). Initially, the rehydration step was carried out with precast 7 cm ReadyStrip IPG strip for 12 h at 50 V. Isoelectric focusing (IEF) step was run as following: 250 V for 20 min and 4,000 V for 3 h and then kept at 4,000 V until total 10,000 V-hr was reached. After IEF, the strips were subjected to two-step equilibration. The proteins were reduced by reduction buffer (containing 6 M urea, 20% glycerol, 2% SDS and 37.5 mM Tris-HCl (pH 8.8), 2% DTT w/v) for the first step, and then were alkylated by alkylation buffer (containing 6 M urea, 2% SDS 37.5 mM Tris-HCl pH 8.8, glycerol 20% and 40 mM IAA) for the second step. The equilibrated strips were then transferred onto the second-dimensional SDS-PAGE, which was run on 1.0 mm thick 12.6% polyacrylamide gels at 140 V for 2.5 hrs.

2-DE gel images were scanned by Molecular Imager FX system (Bio-Rad, Hercules, CA, USA). Image analysis was carried out with PD Quest v 7.1 2D software package (Bio-Rad) including the quantitative analysis. Protein spots were initially detected, matched and then manually edited. Each spot intensity volume was processed by background subtraction and total spot volume normalization. Afterwards, the resulting spot volume was used for comparison. Only those significantly different spots (two-fold increase or decrease) were selected and excised by Spot Cutter (Bio-Rad, Hercules, CA, USA) for trypsin-digestion and analysis by using nanoLC-ESI-MS/MS.

Tryptsin in-gel digestion

Protein spots of interest were excised and transferred into 1.5 ml eppendorf tubes. Next step, gel pieces were washed and destained by using wash solution (50 mM NH_4HCO_3, pH 8.0, 50% ACN). After hydrating with ACN 100% and drying in a SpeedVac, the gel pieces were reduced by incubating with 5 mM DTT solution at 56^oC for 45 min and then alkylated for 1 h with 20 mM IAA solution in darkness at room temperature. Trypsin (1 µg enzyme per 50 µg substance) was added and incubated overnight at 37^oC. Finally, resulting peptides were extracted with extraction solution containing 60% ACN and 1% TFA (v/v). All extracts were saved and dried, and then redissolved in 0.1% TFA.

NanoLC-ESI-MS/MS process and protein identification

The trypsin-digested peptides were analyzed on nanoLC system (LC Packing, Dionex, Netherland). Peptide mixture was desalted and concentrated on C18 TRAP column (PepMap100, LC Packing, Dionex, Netherland), and separated onto C18 reverse phase column (GraceVydac, Hesperia, CA, USA). The flow rate was maintained at 0.2 µl/min. The sample was loaded onto capillary column with 0.1% TFA and eluted from C18 column with gradient from 0% to 100% of B solution (containing 85% ACN with 0.1% FA) for 90 min. After that, the resolved peptides were analyzed on the QSTAR® XL mass spectrometer (Apllied Biosystems, MDS SCIEX, Canada) with a nano-ESI ion source. MS and MS/MS spectra were obtained by the system operating in the IDE (Information Dependent Acquisition) mode. For identification, proteins were searched against the NCBInr protein database using Mascot v1.8 software (Matrix Science Ltd., London, UK). Species search was limited to *Homo sapiens*. Searches were performed without restriction of protein molecular mass (Mr) or p*I*, but with mandatory carbamidomethylation of cysteines and variable oxidation of methionine residues. One trypsin misscleavage was allowed. Peptide and MS/MS mass tolerance were set to ± 0.5 Da.

Results

Capturing of glycoproteins using affinity chromatography

In this study, we used Con A, a lectin which has a high affinity with alpha mannose to recognize and enrich N-linked glycoproteins. After the elution of bound proteins, concentrations of the whole serum, bound proteins and unbound proteins were measured by using the optical density (OD) at 280 nm. Amount of the bound protein was 22.12 µg/µl, accounting for nearly 29.49%. As described above, 12.6% SDS-PAGE was carried out to examine the ability of glycoprotein capture. The bound fractions (Fig. not shown) occupied a quite large amount and were clearly different from the unbound fractions. Especially, serum albumin, the most abundant protein, was removed from the bound fractions. The result indicated that the affinity chromatography reduced the complexity of analytical sample and facilitated the 2-DE.

Protein separation

The serum glycoprotein patterns of LC patients and healthy individuals were compared each other based on 2-DE gel images using PQ Quest v7.1 software (Fig. 1). Of more than 200 detected spots, there were 11 significantly changed spots. The molecular weight of glycoproteins was ranged from 10 kDa to 200 kDa with p*I*s between 4 and 7. Many streaks of spots (isoforms) represent those glycoproteins with different levels of glycosylation or phosphorylation, resulting in changes of the p*I* and Mr. The comparison of spot volume was carried out between two types of samples. As shown in Fig. 1, the significantly changed or new spots were compared and found in at least 11 different spots.

Identification of glycoproteins

The spots of interest were excised and subjected to trypsin diges-tion, MS/MS mass spectra measurement, and database search-ing. By comparison of similarity of in-gel location, p*I* and Mr, 11 glycoproteins were identified in database searching. By compari-son of similarity of in-gel location, p*I* and Mr, 11 glycoproteins were identified in NCBInr database by using Mascot v1.8 soft-ware. Table 1 summarized the identified glycoproteins in 11 changed spots and their alterations between normal and LC se-rum.

The quantitative analysis of glycoproteins expression level

The expression level of glycoproteins (up/down) in 2-DE gel was calculated based on total spot volume represented by 3-D images and analyzed by PQ Quest v7.1 software. The total volume of changed spots was calculated and compared.
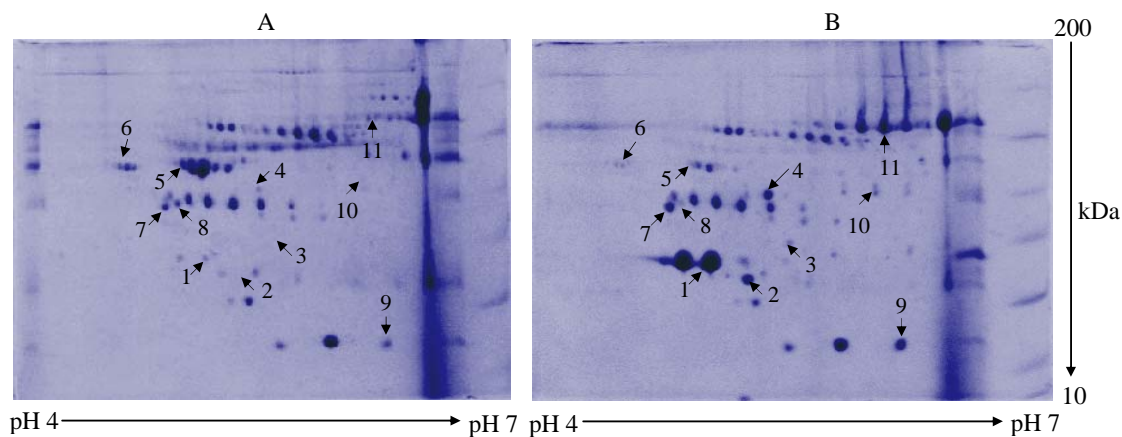

Figure 1: **2-DE gel images of serum glycoprotein samples from the healthy and LC patients**. (A) Normal sample, (B) LC sample. The identified protein spots: (1) Anti TNFα antibody light chain (ATAL); (2) Chain L, structure of Fab D3h44 (D3h44); (3) Transthyretin (TTR); (4) AIM/CD69; (5) Alpha1-Antitrypsin (AAT); (6) Alpha2-HS-glycoprotein (AHSG) (7) Complement C3; (8) Zinc-alpha2-glycoprotein (ZAG); (9) Haptoglobin alpha2 chain (HpA2); (10) Ig heavy chain mu (BOT); (11) IGHM protein.

The comparative data showed that Fab D3h44 is the most in-creased (thirteen-fold) while AAT is the most decreased (five-fold). The chart in Fig. 2 illustrated the comparative results of expression level of 11 spots between two types of samples.

Overall, the expressions of 8 glycoproteins were elevated and 3 glycoproteins were suppressed in LC in comparison with normal one. Some of them, immunoglobins, C3, TTR, HpA2 and AIM are enhanced in the serum of LC patients. By contrast, AAT, AHSG and ZAG are reduced or even absent in some cases (data not shown). The alterations of glycosylations could be reflected the stage of disease in different organs and tissues.

Table 1: The identified glycoproteins using Mascot v1.8 software.

Spot No.	Protein name	Accession No.	Mw (2DE)/Mw (SwissProt)	Matched peptides	Recovery	Up/Down
1	Anti TNFα antibody light chain (ATAL)	Q6P5R5	23.6/25.8	25	42%	Up
2	Chain L, structure of Fab D3h44 (D3h44)	Q6GMW1	23.8/13.2	10	40%	Up
3	Transthyretin (TTR)	P02766	35.5/15.9	29	42%	Up
4	AIM/CD69	O43866	39.6/38.1	13	51%	Up
5	Alpha1-Antitrypsin (AAT)	P01009	53.4/46.7	65	63%	Down
6	Alpha2-HS-glycoprotein (AHSG)	P02765	53.2/39.3	5	13%	Down
7	Complement C3	P01024	41.0/40.9	10	31%	Up
8	Zinc-alpha2-glycoprotein (ZAG)	P25311	41.0/33.9	12	10%	Down
9	Haptoglobin alpha2 chain (HpA2)	P00738	16.9/16.0	2	10%	Up
10	Ig mu heavy chain disease protein (BOT)	P04220	48.5/43.1	12	13%	Up
11	IGHM protein	Q96AA6	58.9/68.6	11	10%	Up

(*) Abbreviation: Mw calculated from 2DE image; Mw (SwissProt) as defined in SwissProt

(∗) Abbreviation: Mw calculated from 2DE image; Mw (SwissProt) as defined in SwissProt

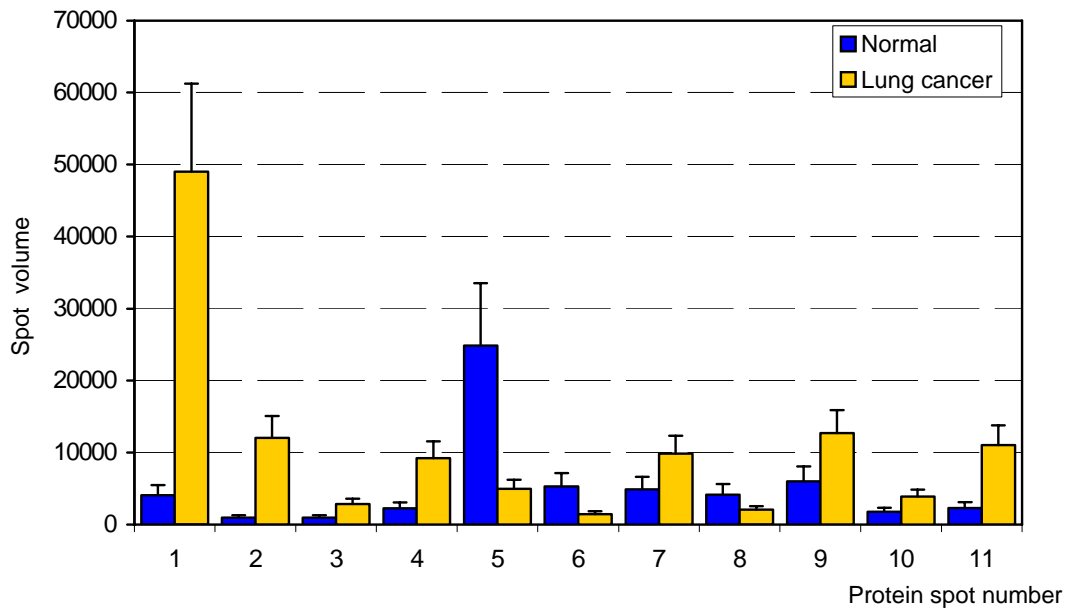N/L ratio   0.083   0.075   0.335   0.246   5.000   3.615   0.496   2.031   0.470   0.448   0.208

Figure 2: The comparative chart of spot level between two groups of samples (normal and lung cancer)

N/L ratio, normal/lung cancer ratio of total volume of each spot: (1) ATAL; (2) D3h44; (3) TTR; (4) AIM/CD69; (5) AAT; (6) AHSG; (7) C3; (8) ZAG; (9) HpA2; (10) BOT; (11) IGHM.

Discussion

Many serum proteins are glycosylated. When disease is present, subtle changes occur in the glycosylation and glycan composition. With serum glycoproteins, these changes provide useful information about the pathological processes (Turner, 1992). In this study, our goal is to investigate and compare the changes of glycoproteins between LC patients and the healthy. We used a Con A affinity column to capture glycoproteins from human serum and the results showed that the enriched method was specific, efficient and simultaneously depleted serum albumin. As mentioned above, the present of albumin, a high abundant protein, can affect the separation and identification of low abundant proteins. And it has been demonstrated that the removal of high abundant protein, such as albumin, can improve the ability of protein identification (Pieper et al., 2003). Therefore, affinity chromatography is a specific method for isolating glycoproteins, reducing the complexity of serum sample, and facilitating separation of proteins using 2-DE. In combination with mass spectrometry, 2-DE technique represents a unique tool of analyzing expression levels of a thousand proteins simultaneously and to compare the protein profiles of a given sample. In addition, the recent software developed for a cross-comparing gel image has offered the possibility to evaluate homologies and differences between comparative samples. By using dedicated software, accessing to Swiss-2DPAGE database and analyzing onnanoLC-ESI-MS/MS system, the changes of serum glycoproteins from LC patients were detected as following: AAT, ZAG, AHSG, ATAL, Fab D3h44, TTR, AIM/CD69, C3, HpA2, BOT, IGHM.

LC leads to unusual changes of immune system, which can increase or reduce the expression level or alter the function of unique protein (Mazzoccoli et al., 2003). In this study, we also described the high expression level of certain immunoglobins in LC serum such as: ATAL, Fab D3h44, BOT, and IGHM. It has been thought that their alterations are cause of pathological process (Okubo et al., 1990). The complement C3 that highly increases at inflammatory sites occupies nearly 70% of all complement proteins and plays a vital role in activation of classical and alternative pathways. In this report, C3 is two-fold higher in the serum of LC patients. These findings are in agreement with other literatures (Oner et al., 2004; Gminski et al., 1992), since C3 shows increased levels in the cancer serum. Some authors also demonstrated that Complement components (C3 and C4) levels were elevated in patients with LC compared with levels in the healthy group (Oner et al., 1994).

AIM/CD69 (Activating Inducer Molecular), an early activation marker, is a leukocyte receptor transiently induced after activation that is detected on small subsets of T and B cells in peripheral lymphoid tissues (Ikuko et al., 2001). The leukocyte activation marker - AIM/CD69 is a novel regulator of the immune response, inhibiting apoptosis and modulating the production of cytokines. Recently, it has been reported that AIM/CD69 unmasked a novel role as a negative regulator of anti-tumor responses (Esplugues et al., 2003). Here, the four-fold overexpresion level of AIM/CD69 in LC patients might involve in activation of B and T cells and inhibition of apoptosis, which are results of cancerous invasion and metastasis. It has thought that the high expression level of AIM/CD69 might correlate with lymphoma development (Giovanni et al., 2001). The function of AIM/CD69 during pathological process, however, remains unknown (Sancho et al., 2003). The discovery of change of this glycoprotein receptor in LC patients might consider as a novel maker for diagnosis and need to be further studied.

Serum alpha1-antitrypsin (ATT) is a 52-kD protease inhibitor. As one of the powerful inhibitors of apoptosis and caspase activa-

tion, ATT can inhibit many of the proteases released from dying cells and thus protects normal tissues during periods of stress such as inflammation, emphysema disease, and cancerous metastasis (Novoradovskaya et al., 1998). The observed decrease of ATT level in LC patients may cause the failure in the response of self-protection of the lung. On the other hand, deficiency of ATT activity has been closely associated with LC. Our result showed that the expression level of ATT was decreased five-fold in LC patients compared with level in normal groups. Yang et al. (1999) (Yang et al., 1999) and Dabrowska et al. (1988) (Dabrowska et al., 1988) reported that the deficient level of ATT might be associated with tumor progression and prognosis. This deficiency can be inherited from mutant genes in some phenotype variants (Ortiz-Pallardo et al., 2000). We believe that the deficiency of this protease inhibitor may be due to the increased level of protease activity in malignant cells. Infiltration of granulocytes near tumor and released enzymes from them may exhaust proteolytic inhibitory capacity, too. Increased protease activity is associated with transformation and uncontrolled proliferation therefore antiproteases may be accepted as anticancerogenic factors. Further investigations are needed to bring us closer to understanding this question in LC.

Haptoglobin, a serum glycoprotein, is mainly produced by hepatic cells associated to hemoglobin for maintaining iron ions in blood. Recently, it has discoveried that serum haptoglobin increased branching of the oligosaccharide chains in the cancer (Turner, 1992). Six- and seven-fold increased fucosylation has also been reported for haptoglobin in ovarian and breast cancer (Thompson and Turner, 1987). Our results revealed that serum haptoglobin alpha2 chain (HpA2) content in LC patients is over two-fold higher than that of HpA2 from healthy individuals. This increase might relate to the inflammations, necrosis, and/or cancer. Interestingly, spot number 9 (isoforms) displays another dramatic change occurring in HpA2. A normal serum sample has three main HpA2 isoform spots. But in the LC serum, the pattern of HpA2 was altered. HpA2 isoform (number 9) was significantly up regulated while other two isoforms remained unchanged (Figure 1 & Figure 2). These alterations might be a result of changes in fucosylation in cancer that are correlated with an increase in the activity of α-(1-3)-fucosyl transferase in the blood (Thompson et al., 1991). Our findings indicate that the alterations of HpA2 occur not only in its level but also its whole pattern in the 2-D gel, which may play an important role in lung tumorigenesis (Maciel et al., 2005). It is therefore possible to develop monoclonal antibodies specific to the HpA2 isoforms for the serological assay of LC patients. This possibility is under investigation.

Human alpha2-HS-glycoprotein (AHSG), a negative acute phase protein, is synthesized and secreted by the liver into blood. Plasma concentrations of AHSG significantly decrease following infection, inflammation and malignancy (Daveau et al., 1990). AHSG stimulated the apoptosis of cancerous cells, thus it could resist cancer and its decreased concentration might progress the invasion and metastasis (Carol et al., 2004). In this report, we described 3.6-fold decrease of AHSG, which suggested being associated with the metastasis. Our results are in good agreement with other data (Madappa et al., 2005; Schweigert and Sehouli, 2004). Recent works reviewed by Madappa et al., (2005) (Madappa et al., 2005) has shown that the deficiency of AHSG correlated with many types of cancers including LC. Therefore, the measurement of changed level of AHSG in the patient serum is significant to diagnose and cure the disease.

Besides, transthyretin (TTR) or prealbumin, a known negative acute-phase protein, was found increased three-fold in LC patients. Higher levels of TTR have previously been reported in LC and ovarian cancer (Maciel et al., 2005). At present, no reports explain why TTR concentration enhances in the serum and its biological function in tumor progression is still unknown. In serum, Zinc alpha2-glycoprotein (ZAG) is a 41-kDa glycoprotein secreted by a variety of normal epithelia. ZAG was recently shown to stimulate lipolysis in adipocytes, leading to the development of cachexia in men with prostate cancer and oral tumors (Hale et al., 2001). Based on our results, the expression level of ZAG reduced in LC that may contribute to the development of LC. But the function of ZAG has remained unclear until recently. Thus, it is needed to further investigate for finding out real markers in LC

Conclusion

With the goal of investigating and comparing serum glycoproteome from LC patients and the healthy, the affinity chromatography was successfully used. The results showed that the enrichment method was specific and efficient, and the collected glycoproteins were well separated and identified. This study demonstrated the usefulness of the combination of affinity chromatography, 2-DE and nanoLC-ESI-MS/MS methods for identification of serum glycoprotein profile in both quality and quantity. Comparative results indicated that 8 glycoproteins (ATAL, D3h44, TTR, AIM/CD69, C3, HpA2, BOT, IGHM) were up regulated while 3 glycoproteins (AAT, AHSG, ZAG) were down regulated in the LC serum, which are significantly informative and might be considered as a useful signature for assessing LC.

Abbreviations

LC: Lung Cancer; ConA: Concanavalin A; 2-DE: Two-dimensional Electrophoresis; ATAL: Anti TNFαAntibody light chain; D3h44: Chain L, structure of Fab D3h44; TTR: Transthyretin; AAT: Alpha1-antitrypsin; AHSG: Alpha2-HS-glycoprotein; ZAG: Zinc-alpha2-glycoprotein; HpA2: Haptoglobin Alpha2 chain.

Acknowledgment

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