Proteomic investigation of the severe preeclampsia treatment by low molecular weight heparin

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Summary

Purpose: The primary goal of this study is to investigate the mechanism of severe preeclampsia (PE) treatment by low molecular weight heparin (LMWH). *Materials and Methods:* Using two-dimensional difference in-gel electrophoresis (2D-DIGE) combined with matrix assisted laser desorption ionization-time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF) approach to identify the proteins that expressed differently in the serum samples of five patients before and after subcutaneous injection of LMWH (0.4 ml/person). *Results:* Seven protein spots were identified in 2D-DIGE that show significant change in expression level after LMWH treatment. Further analysis of seven protein spots with MALDI-TOF/TOF identified six different proteins. To confirm the proteomic data, two meaningful proteins of the six proteins, alpha-1-acid glycoprotein (AGP) and serotransferrin are subjected to immunoblotting. All of the proteins are obviously down-regulated after LMWH treatment. *Conclusions:* PE is a pregnancy-specific disease that clinically manifests as new-onset hypertension and proteinuria after 20 weeks of gestation. LMWH is an effective treatment of severe PE. The present proteomics based investigation may provide a new angle to understand the mechanism of severe PE treatment with LMWH.

Key words: Severe preeclampsia; LMWH; DIGE; MALDI-TOF/TOF; Human serum.

Introduction

Preeclampsia (PE) is a systemic syndrome that occurs in 3% ~ 5% of pregnant women worldwide [1, 2]. It is estimated that each year more than 500,000 women die of pregnancy related diseases, and 10% ~ 15% of maternal deaths are associated with PE and eclampsia; 99% of fatalities occurs in low- and middle-income countries [3]. PE is a pregnancy-specific disease characterized by development of concurrent hypertension and proteinuria, which sometimes progresses into multiple organ cluster with varying clinical features [4]. Despite numerous clinical investigations and intensive researches, pathophysiological mechanisms of this disease still remain unknown [5]. As a result, the development of preventive and therapeutic treatments falls further behind [6]. Placental perfusion insufficiency caused by micro thrombus plays a crucial role in the development of PE [7]. Preliminary nonrandomized studies suggest some benefit from adjuvant therapy with anticoagulation by means of LMWH in PE [8-12]. LMWH is an anticoagulant drugs that can prevent and reduce thrombus including placental micro thrombus. Because LMWH does not cross the placental barriers, it does not cause fetal teratogenicity [13]. The goal of this study is to identify proteins markers that may explain the mechanism of adjuvant treating severe PE with LMWH.

Proteomic techniques play an important role in the study of changes in proteomes expression of patients serum samples [14]. Identification of these proteins can thus significantly increase the availability of molecular markers for early diagnosis and therapy, leading to better understanding the mechanisms of LMWH adjuvant treatment for PE [15]. Two-dimensional electrophoresis (2D-E) is extensively used in proteomics to compare changes in protein expression [16, 17]. Although proteomic studies of the PE are performed in the past, they are mostly limited to the samples acquired from placenta rather than from serum [18-21]. In addition, none of those studies focused on the difference between untreated and LMWH treated patients of severe PE. Thus a comparative study is required to gain insights into the mechanism of PE adjuvant treatment by LMWH.

Two-dimensional differential in gel electrophoresis (2D-DIGE) was first described by Unlü *et al.* as a technique to profile proteins [22]. 2D-DIGE is a method that labels protein samples with different fluorescent dyes before 2D electrophoresis, and then separates three different protein samples at the same time in one two-dimensional gel, The application of the internal standard ensures that the results reflect the real biological differences, and in the meantime avoids influence of systematic errors [23-26]. The most important advantage of the DIGE system lies in that it integrates CyDye DIGE dye multiple labeling method into DeCyder difference 2D analysis software. DeCyder software takes the advantage of the spots co-detecting algorithm, which automate the entire process, including acquisition of fluorescence images, background elimination, quantification, normalization, and matching spots in gel. As a result, operational errors are minimized. Therefore, it is an ideal tool to obtain the information of differential proteome in samples of the patients with severe PE before and after LMWH treatment.

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Materials and Methods

Subjects and serum sample collection

Samples were collected at the Department of Obstetrics and Gynecology, NanFang Hospital. The clinical characteristics of patients are summarized in Table 1. Blood was drawn from the peripheral vein and serum was produced by centrifugation. Serum samples in Group 1 were taken from five pregnant women with severe PE. These patients did not receive any treatment before being admitted to NanFang hospital. Samples in Group 2 were taken from the same five patients, after receiving 0.4 ml LMWH subcutaneous injection. Since the peak concentration in blood of LMWH usually appears in six hours after injection, blood samples were drawn at eight to ten hours after LMWH injection. Table 1 shows the information of five patents in age, body mass index (BMI), gestational age, systolic blood pressure (SBP) or diastolic blood pressure (DBP).

Severe PE was diagnosed as follows: blood pressure ≥ 160/110 mmHg, proteinuria ≥ 2+ (two or more episodes, two measurements ≥ six hours apart) or proteinuria ≥ two grams in 24 hours along with any of the following: platelets < 100,000/ml, elevated aspartate aminotransferase or aminotransferase levels, elevated lactic dehydrogenase levels, creatinine > 106 μmmol/l, persistent headache, central nerve dysfunction, blurred vision, or persistent epigastric discomfort. All of the subjects' blood pressure normalized 12 weeks after delivery.

All of the five participants signed verbal informed consent prior to sample collection. The Ethics Committee of NanFang Hospital approved the research protocol. The screening criteria excluded patients who had: 1) PE history; 2) chronic hypertension before the indexed pregnancy; 3) other metabolic diseases such as hyperthyroidism or hypothyroidism, diabetes or gestational diabetes, nephropathy, and polycystic ovary syndrome; 3) other diseases such as congenital heart diseases; and 4) addiction to smoking or alcohol.

Samples preparation for 2D-DIGE analysis

Serum samples (five ml/each) were extracted from the peripheral blood by centrifuging at 5,000 rpm for ten minutes at 4°C within 30 seconds of collection and then stored at -80°C. Precise kits were used to specifically remove albumin and IgG, followed by desalting and concentration with another clean-up kit. Protein concentration of supernatant is measured with the 2D Quant kit following manufacturer recommendations and resuspended in 30 mM Tris (pH=6.8), 7 M urea, 2 M thiourea, and 4% (w/v) CHAPS.

2D-DIGE and image analysis

The internal standard was labeled with a cyanine 2 dye and equal amount of protein was loaded on each gel to control the gel-gel variation. The pre-treatment sample was labeled with Cy3, while the serum sample from the patients after LMWH treatment was labeled with Cy5.

The labeled samples (50 μg of sample/400 pmol of cydye) were loaded onto the 1st dimension immobilized pH gradient (IPG) strip. The 24 cm, pH3-11, non-linear immobiline dystrips were rehydrated with three μl IPG buffer 3-11 NL and 410 ml rehydration solution. Isoelectric focusing was conducted using an instrument, according to the following profile: 30 V for 12 hours, 100 V for one hour, 500 V for one hour, 1,000 V for one hour, 3,000 V for one hour, gradient to 8000 V for three hours and then constant at 8,000 V overnight until reaching 5,000~8,000 V.

The IPG strips were conditioned for 15 minutes in equilibration buffer containing 2% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue, and 10 mg/ml dithiothreitol (DTT). The strips were then alkylated for 15 minutes in equilibration buffer containing 25 mg/ml iodoacetamide instead of the DTT and loaded onto 12.5% polyacrylamide gels, which were cast according to specifications. The second dimension was run by a system for four hours, until the bromophenol blue dye-front reached the edge of the gel.

DIGE gel was visualized with excitation at 488 nm (Cy2), 532 nm (Cy3), and 633 nm (Cy5). The corresponding emission was detected at 520 nm (Cy2), 580 nm (Cy3), and 670 nm (Cy5). The imager utilized allowed sensitivity adjustment by means of tuning the voltage setting of the photomultiplier tube (PMT) that captured the fluorescence image. The starting points for the PMT is around 600 V. Data obtained from gels were quantified using specific software. The differential in-gel analysis (DIA) was used to detect differential expression. Quantitative differences were only accepted when at least a 1.5-fold change was confirmed.

Table 1. — *Demographic information of the pregnant women included in this study.*

Patient number	Maternal age (y)	Gestational weeks (w)	BMI (Kg/m²)	SBP (mmHg)	DBP (mmHg)
1	26.7	36^{+4}	28.07	168	112
2	25.8	36^{+1}	29.11	170	110
3	27.1	35^{+6}	27.93	169	114
4	26.2	36^{+2}	28.85	178	116
5	27.4	35^{+5}	27.69	171	113
*statistic	26.64±0.65	35^{+6} ±0.39	28.38±0.55	171.2±3.96	113±2.24

Data are described as mean ± standard.

In-gel digestion and protein identification

Proteins were identified by peptide mass fingerprinting (PMF) from spots that are digested from preparative gels stained with SYPRO Ruby. Protein spots were then excised from the 2D gel. The spots were in-gel digested with trypsin following Bruker's standard in-gel digestion protocol and stored at -20°C.

Samples on the MALDI target plates were then analyzed using a proteomics analyzer MALDI-TOF/TOF mass spectrometer. For MS analysis, 1,000 shots were typically accumulated. MS/MS analysis was performed using air at collision energy of 2 kV. MASCOT search engine (version 2.1) was used to search all of the tandem mass spectra. GPS software (version 3.6.2) was used to create and search files with the MASCOT search engine for protein identification. Protein identities were obtained by using the MASCOT search engine against non-redundant databases selected for human taxonomy.

Western blot analysis

Two differentially expressed proteins, alpha-1-acid glycoprotein (AGP) and serotransferrin (Tf), were further validated by Western blot using the ubiquitous protein β-Actin as the loading control.

Equal amount of total protein (20 μg) extracted from the individual serum samples was resolved in 12% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked for one hour at room temperature in TBST (20 mM Tris-HCl pH7.6, 140 mM NaCl, pH 7.5, 0.05% Tween-20) containing 5% skim milk before the mouse monoclonal primary antibodies were added. Membranes were incubated with each primary antibody at room temperature for two hours. Blots were then washed for five times, incubated with the dylight flour conjugates labeled polyclonal goat anti-mouse IgG (diluted 1:10000) for one hour at room temperature. After being washed five times with TBST, immuno-reactive complexes were visua-

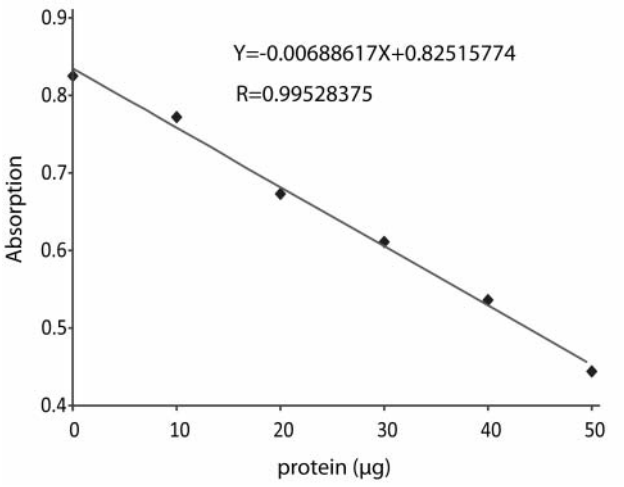

Figure 1. — Standard curve for protein quantification using 2-D Quant kit.

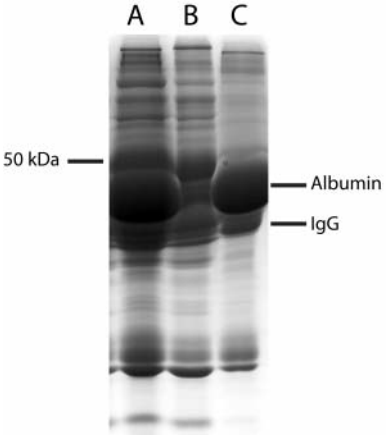

Figure 2. — Albumin and IgG were effectively removed from the serum samples. A: total protein of one serum sample. B: albumin/IgG-depleted serum fraction. C: the eluted fraction from albumin removal column.

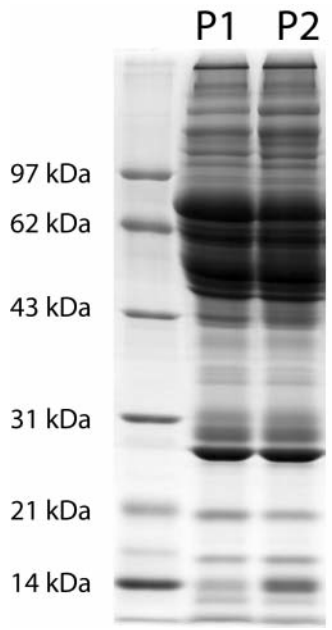

Figure 3. — SDS-PAGE image of proteins after the removal of albumin and IgG.
Left lane: marker, P1: combined sample of group 1, P2: combined sample of group 2.

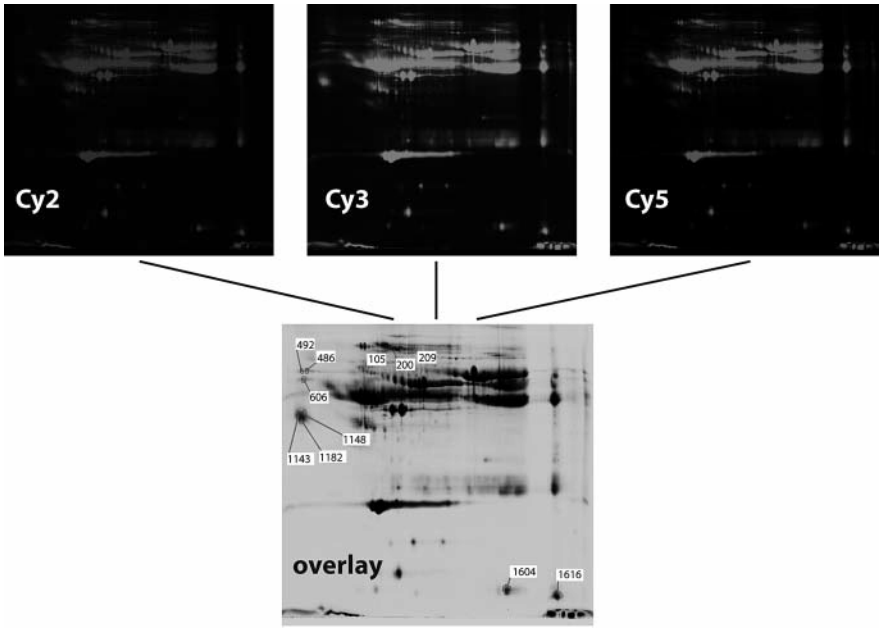

Figure 4. — Representative 2D-DIGE image of serum protein from severe PE patients before and after LMWH treatment. The image shows the distribution of the 11 differentially expressed protein spots after analyzing by specific software. Circled and numbered spots in the images were identified as differentially expressed protein spots. Cy2 (blue) image: protein from internal standard samples, Cy3 (green) image: proteins from patients without any treatment. Cy5 (red) image: proteins from patients after LMWH treatment.

Table 2. — *Differentially expressed proteins identified in PE from two groups by DIGE and MALDI-TOF/TOF.*

Spots Position	Protein description	Accession No.	Protein Score	C.I.%	Protein MW (kDa)	Protein PI	Pep. count
200&209	Alpha-2-macroglobulin	Sp|P01023|A2MG_HUMAN	155	100	164.6004	6	8
486	Serotransferrin	Sp|P02787|TRFE_HUMAN	56	99.99	79.2805	6.81	2
606	Serum Albumin	Sp|P02768|ALBU_HUMAN	52	100	71.3172	5.92	3
1182	Alpha-1-acid glycoprotein	Sp|P02763|A1AG1_HUMAN	130	100	23.7248	4.93	5
1604	Hemoglobin Subunit beta	Sp|P68871|HBB_HUMAN	168	100	16.1023	6.75	7
1616	Hemoglobin Subunit Alpha	Sp|P69905|HBA_HUMAN	224	100	15.305	8.72	5

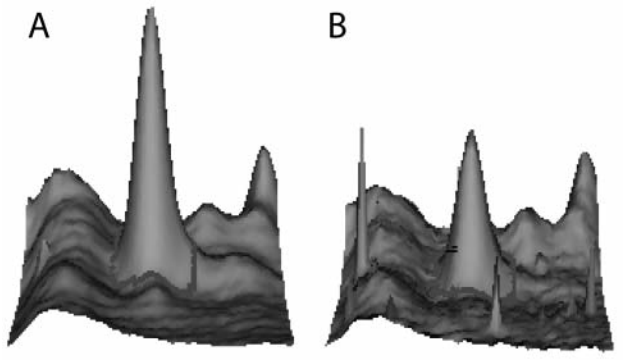

Figure 5. — 3D images of differentially expressed protein. After LMWH treatment, the protein was down-regulated. The peak of the protein is lower than before.

ized using ECL reagents and imaged with X-ray films that was later scanned. A semi-quantitative analysis, which was basing on OD was performed by a specific software.

Results

Subject characterization

Five pregnant women complicated with severe PE are recruited in this study. After received cesarean section in their trimester to terminate the pregnancy, their blood pressures dropped to the normal range. The characteristics of these pregnant women are listed in Table 1, which shows the information regarding their maternal age, gestational weeks, BMI, SBP or DBP.

Serum sample preparation

Accurate quantification of proteins is necessary for DIGE. Figure 1 shows the standard curve generated by the 2-D quant kit. To increase the resolution, albumin and IgG, two major plasma proteins of the human were removed (Figure 2) prior to electrophoresis, and no significant difference in SDS-PAGE was observed between the samples of the two sample groups (Figure 3).

Protein identification

The identification of proteins differentially expressed in Group 1 and Group 2 was performed by DIGE. Figure 4 shows the spots of differentially expressed proteins between serum samples. Seven spots were detected using the image analysis software (Figure 5). Spots with a fold change equal or above 1.5 (in absolution value) were exclusively considered as differentially expressed spots. Spots shown in Fig. 4 were selected for further identification using a MALDI-TOF/TOF mass spectrometer. As a result, six different proteins were identified differentially expressed between Group 1 and Group 2, among which alpha-1-acid glycoprotein (AGP) and serotransferrin (Tf) may have physiological significance. All six proteins were down-regulated after LMWH subcutaneous injection (Table 2).

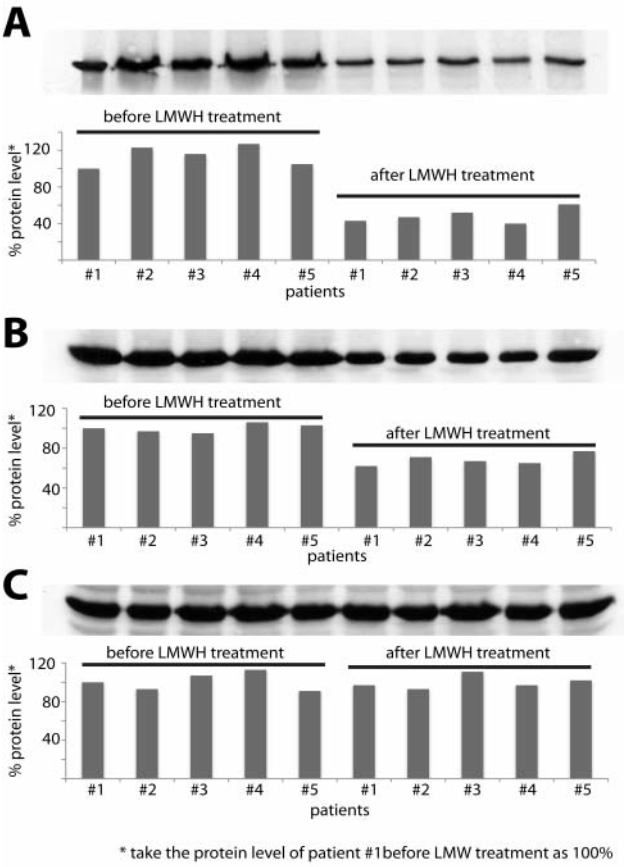

Figure 6. — Western-blot analysis of alpha-1-acid glycoprotein (AGP) and serotransferrin (Tf). Combined serum samples of group 1 and group 2 were loaded on SDS-PAGE, transferred onto Hybond-P membranes and detected by antibodies. (A) alpha-1-acid glycoprotein (AGP) , (B) serotransferrin (Tf), (C) loading control.

Protein validation

Proteins showing significantly different expression after LMWH are likely to have biological impact. To further validate the DIGE results, two proteins, AGP and Tf were selected for Western blots base upon their biological functions, high fold changes and the availability of commercial antibodies. At 42 kDa and 80 kDa, antibodies identified the bands for AGP and Tf, respectively (Figure 6). Both AGP and Tf were detected as strong bands in the samples from Group 1, while weak signals were observed in the samples from Group 2, agreeing with the DIGE results (Figure 6). In conclusion, the Western blots confirmed the down-regulation of AGP and Tf after LMWH treatment.

Discussion

In this study the authors have demonstrated a possible molecular mechanism for adjuvant treating PE with LMWH. The proteins identified in this report could also serve as potential biomarkers for the diagnosis and treatment of PE.

As a systematic syndrome primarily manifested by hypertension and proteinuria and mainly seen in the second half of pregnancy, PE affects approximately 3% to 5% of pregnancies worldwide. [27] PE was the leading cause of maternal mortality, preterm birth, and consequent neonatal morbidity and mortality. In developing countries where access to safe, emergency delivery is inadequate, PE claims the lives of more than 60,000 women every year. [28] Exciting progress has been made in pathophysiology of PE recently. However the exact cause of this disease remains unknown. For this reason, it is difficult to develop treatments or preventive strategy.

In this study, the authors have identified serum proteins that significantly down-regulated by LMWH treatment. The techniques of 2D-DIGE and MALDI-TOF/TOF allow proteomic studies and make it possible to unveil new pathophysiological mechanisms and to identify new biomarkers and/or potential therapeutic targets.

Totally, six different proteins were found. They are alpha-1-acid glycoprotein, serotransferrin, serum albumin, alpha-1-acid glycoprotein, hemoglobin submit alpha, and hemoglobin submit beta. As known, there are some physiological proteins in human bodies and these kinds of proteins participate in almost every physiological activity but not in special functions. After analysis, the authors believe the possible functions proteins are AGP and Tf. Both of them are discussed below.

AGP (alpha-1-acid glycoprotein)

AGP was originally described in 1950 [29-31] and later characterized as a very unusual protein with a very low pI (in the range from 2.8 to 3.8) and a very high carbohydrate content (45%) [32]. Human AGP is the product of a cluster of three adjacent genes: AGP-A, AGP-B and AGP-C, which covers 70 kb on chromosome 9 [33, 34]. AGP is considered as a natural anti-inflammatory and immuno-modulatory agent because of its anti-neutrophil and anti-complement activity [35]. AGP was reported to act *in vitro* and *in vivo* as an immuno-modulating molecule. *In vitro*, AGP inhibits polymorph nuclear neutrophil activation [36], increases the secretion of an IL-1 inhibitor by murine macrophages and most probably the IL-1 receptor antagonist [37, 38], and modulates LPS-induced cytokine secretion by monocytes macrophages [39].

First, it is extensively realized that the inflammatory factor and disimmunity are responsible for many cases of PE. After treating with LMWH, the level of AGP was reduced. A possible explanation is that LMWH inhibits inflammatory factors, helps alleviate the syndrome of severe PE, especially in the acute phase, which in turn reduces the level of AGP. Second, human serum albumin, lipoprotein, and AGP are most important drug binding proteins in plasma that can have important pharmacokinetic implications [40]. The variations in AGP levels during inflammatory processes can considerably change the level of free drug without affecting its total plasmatic concentration. Therefore, the free concentration of the drug in plasma will reflect more accurately the intensity of the pharmacological effect. In the present experiment, AGP was down-regulated, probably because AGP bound with LWMH and facilitated the transportation of LMWH to other organs. This may result in a reduced concentration of AGP in plasma.

In summary, AGP is an acute phase protein. Its plasma levels can be used as a diagnostic and prognostic indicator during clinical therapy. The large variation observed in the binding ratios of basic drugs in plasma during several physiological and pathological states could be correlated with the large variations in the plasma level of AGP.

Tf (Serotransferrin)

Tf is a ubiquitous protein with a central role in iron transport and metabolism. During gestation, both the mothers and fetus require large amounts of iron that comes mainly form Tf [41]. It transports iron from sites of absorption and degradation to those of storage and utilization [42]. Tf is a bilobal 80 kDa iron binding glycoprotein. The single-chain polypeptide folds into two homologous lobes (N- and C-lobes) connected by a short peptide linker. Each lobe is further divided into two subdomains that come together to form the metal binding cleft [43].

There is some evidence showing that Tf has many other biological functions in addition to facilitating iron transport and metabolism. These functions may have profound effects on mammalian cell growth and productivity. The multiple functions of Tf can be exploited to develop many novel applications [44-50]. Significant progresses have been made towards expanding Tf applications in biotechnology and medicine. The effort is well-received for its potential in new therapeutic strategy for disease modification or treatment as well as a novel carrier system for targeted drug delivery [51].

Targeted drug delivery is another intensively studies area nowadays in pharmaceutical and biotechnological research and development. It is broadly defined as the selective delivery of a drug to specific biological site (tissue or cell). Among a large variety of drug delivery systems, ligand-receptor-mediated delivery systems have received considerable attention. From this perspective, Tf or its receptor may be useful as a targeting ligand to achieve targeted delivery of therapeutic agents in the treatment of PE [52]. In the present proteomic results, the down-regulation of Tf in LMWH treated samples implies that it may function in the transportation or targeted drug delivery of the LMWH.

As a proof of concept, this study is deemed to be an early effort on understanding the role of LMWH treatment for PE. Two proteins were identified by 2D-DIGE and MALDI-TOF/TOF and further verified by Western blots. Further studies focusing on the functional properties of these proteins may lead to the identification of protein biomarkers for early diagnosis and to the development of new diagnostic methods.

Acknowledgements

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