Quantitative proteomic analysis of Down syndrome in the umbilical cord blood using iTRAQ

WEIGUO SUI[1*], RUOHAN ZHANG[1,2*], JIEJING CHEN[1], HUIYAN HE[1,2], ZHENZHEN CUI[1,2], MINGLIN OU[1], WUXIAN LI[3], SUWEN QI[3], JINGLI WEN[4], XIUHUA LIN[4] and YONG DAI[1,4]

[1]Nephrology Department of Guilin 181st Hospital, Guangxi Key Laboratory of Metabolic Diseases Research, Guilin, Guangxi 541002; [2]College of Life Science, Guangxi Normal University, Guilin, Guangxi 541004; [3]Key Laboratory of Laboratory Medical Diagnostics of Education Ministry, Chongqing Medical University, Chongqing, Sichuan 400016; [4]Clinical Medical Research Center, The Second Clinical Medical College of Jinan University (Shenzhen People's Hospital), Shenzhen, Guangdong 518020, P.R. China

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Abstract. Down sydrome (DS) is a relatively frequent chromosomal disorder, which has no safe and effective method of prenatal diagnosis to date. The present study was designed to identify DS biomarkers. We quantified the changes in the umbilical cord blood protein levels between DS-affected and healthy (control) pregnant females using isobaric tags for relative and absolute quantification (iTRAQ) and Gene Ontology (GO) analysis. A total of 505 proteins were identified, and of these, five proteins showed significantly different concentrations between the DS and the control group. These proteins may thus be relevant to DS and constitute potential DS biomarkers.

Introduction

Down sydrome (DS) is a relatively frequent chromosomal disorder. It is diagnosed in 1:500 to 1:800 of pregnant females (1). DS is clinically manifested by multiple somatic anomalies, mental retardation and precocious dementia. Pathological examination of DS brains reveals poor maturation, atrophy of the dendrites and the early appearance of senile plaques, which are characteristic of Alzheimer's disease (AD). DS represents an important issue for affected families and the society, but there is no effective treatment at present. Advanced maternal age (35 years old) is an important risk factor for fetal DS (2). The most popular diagnostic strategy for DS relies in the combined detection of AFP, hCG, uE3 and inhibin A, with a detection rate of 60-75% and a 5% false positive rate (3). It is important to find a safe and effective method for diagnosis of DS. One strategy to achieve this is via identification of new biomarkers using noninvasive proteomic approaches. In a previous study, the isobaric tags for relative and absolute quantification (iTRAQ) technique was combined with matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF)/TOF select biomarkers (4). In this study, iTRAQ was combined with Gene Ontology (GO) analysis, in order to identify the proteins that are differentially expressed in DS and their predicted functions.

Proteomic approaches are a promising tool for the identification of diagnostic biomarkers of DS (5). iTRAQ was developed by Applied Biosystems, Inc. (Foster City, CA, USA) in 2004. iTRAQ-based proteomic analysis represents a major development in the rapid detection of potential biomarkers. The key advantage of the 8-plex iTRAQ system is the ability to simultaneously analyze up to 8 different biological specimens, thereby increasing the throughput while reducing experimental errors (6). iTRAQ has been recently employed in the study of certain diseases (7-9). In this study, we used iTRAQ in conjunction with multidimensional chromatography, followed by GO analysis, to detect and quantify proteome differences.

Materials and methods

Samples. Umbilical cord blood samples were obtained from April-August 2011 from pregnant females, six carrying a DS fetus (age, 27-37) and 11 carrying a healthy fetus (age, 27-37), at the Shenzhen People's Hospital (Shenzhen, China). Diagnosis of DS in these women was performed by chromosomal examination. This study was undertaken with the approval of the Institutional Ethical Board of Guilin 181st Hospital (Guilin, China), and written informed consent was provided by all subjects.

Correspondence to: Dr Yong Dai, Clinical Medical Research Center, The Second Clinical Medical College of Jinan University (Shenzhen People's Hospital), 1017 Dongmen North Road, Shenzhen, Guangdong 518020, P.R. China
E-mail: daiyong2222@gmail.com

*Contributed equally

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Table I. The upregulated and downregulated proteins in umbilical cord blood samples of Down syndrome fetuses.

UniProt accession no.	Protein name	Peptides	Ratio
Upregulated proteins (n=13)			
Q8N1G4	Leucine-rich repeat-containing protein 47	3	60.27
Q9UNM6	26S proteasome non-ATPase regulatory subunit 13	1	60.27
P55209	Nucleosome assembly protein 1-like 1	2	60.27
Q6P1M3	Lethal (2) giant larvae protein homolog 2	1	60.27
Q96HE7	ERO1-like protein α	2	60.27
Q9ULD0	2-oxoglutarate dehydrogenase-like, mitochondrial	10	60.27
P63313	Thymosin β-10	4	60.27
O43681	ATPase ASNA1	3	60.27
Q9UHI5	Large neutral amino acids transporter small subunit 2	2	60.27
Q9Y6X5	Ectonucleotide pyrophosphatase/phosphodiesterase family member 4	1	60.27
P02649	Apolipoprotein E	40	3.75
P00751	Complement factor B	7	1.96
P02743	Amyloid P component, serum	7	1.68
Downregulated proteins (n=6)			
Q9H1E5	Thioredoxin-related transmembrane protein 4	1	0.01
Q9UHV9	Prefoldin subunit 2	3	0.01
Q01433	AMP deaminase 2	1	0.01
Q00765	Receptor expression-enhancing protein 5	4	0.01
P43243	Matrin-3	4	0.01
P10451	Osteopontin	1	0.44

Ratio, the ratio of the ionic strength of the DS group and the ionic strength of the control group. Proteins that provided tryptic peptides with an average reporter ion ratio≥1.5 were classified as upregulated and those with an average reporter ion ratio ≤0.67 were classified as downregulated.

Sample preparation. Cord blood (10 ml) was collected from enrolled subjects in heparinized vacutainers. The samples were centrifuged at 3,000 x g for 30 min at 10˚C, the plasma was separated, and 100-μl aliquots were stored at -80˚C until further use.

Protein extraction. Total protein was extracted from the plasma samples with the ProteoMiner protein enrichment kit (Bio-Rad Laboratories, Hercules, CA, USA) as per the manufacturer's instructions. Protein concentration was measured with the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). A previous study indicated that extensive analysis of well-characterized pooled samples is more effective than analysis of individual samples (10). In this study, 40 μg of protein from each sample was pooled with protein samples of the DS or control group prior to proteomic analysis.

iTRAQ, strong cation exchange (SCX) and mass spectrometry (MS)/MS. The proteins from each pool were blocked, digested, and labeled in accordance with the protocol of the Applied Biosystems iTRAQ™ Reagents system (Thermo Fisher Scientific). The iTRAQ tags were as follows: healthy control, 114; DS, 116. The labeled peptides were combined into one sample.

Multidimensional liquid chromatography was performed to separate the tryptic peptides prior to MS. The combined samples were separated into 10 SCX fractions using a 35x0.3 mm, 300Å, 3.5-μm particle size column (Zorbax Bio-SCX; Agilent, Santa Clara, CA, USA) with a potassium formate gradient in 25% acetonitrile. The peptides in these fractions were further separated on a Tempo™ LC nanoflow and MALDI spotting system, equipped with a reversed-phase Magic C18AQ column (Applied Biosystems Life Technologies, Foster City, CA, USA). Each chromatography run yielded ~380 MALDI spots on a stainless steel MALDI target plate, by the same method as previously described (4).

MS data acquisition was conducted with a 4800 Plus MALDI TOF/TOF™ analyzer (Applied Biosystems, Inc.). Only peaks with a signal-to-noise ratio ≥40 were selected for tandem mass spectrometry. Mass spectra from 500 laser shots were acquired for each spot. The combined MS/MS data from all 10 fractions were used for a Paragon Algorithm (11) search. Human version 3.62 proteome data were downloaded from the EBI website (http://www.ebi.ac.uk/) (4).

Statistical and GO analysis. Proteins that provided tryptic peptides with an average reporter ion ratio ≥1.5 were classified as upregulated and those with an average reporter ion ratio ≤0.67 as downregulated. To further investigate the functions of the identified proteins, we used the online GO tool WEGO (http://wego.genomics.org.cn/), which allowed annotation of the proteins with regards to the molecular function (MF), cellular component (CC) and biological process (BP) with which they are associated.

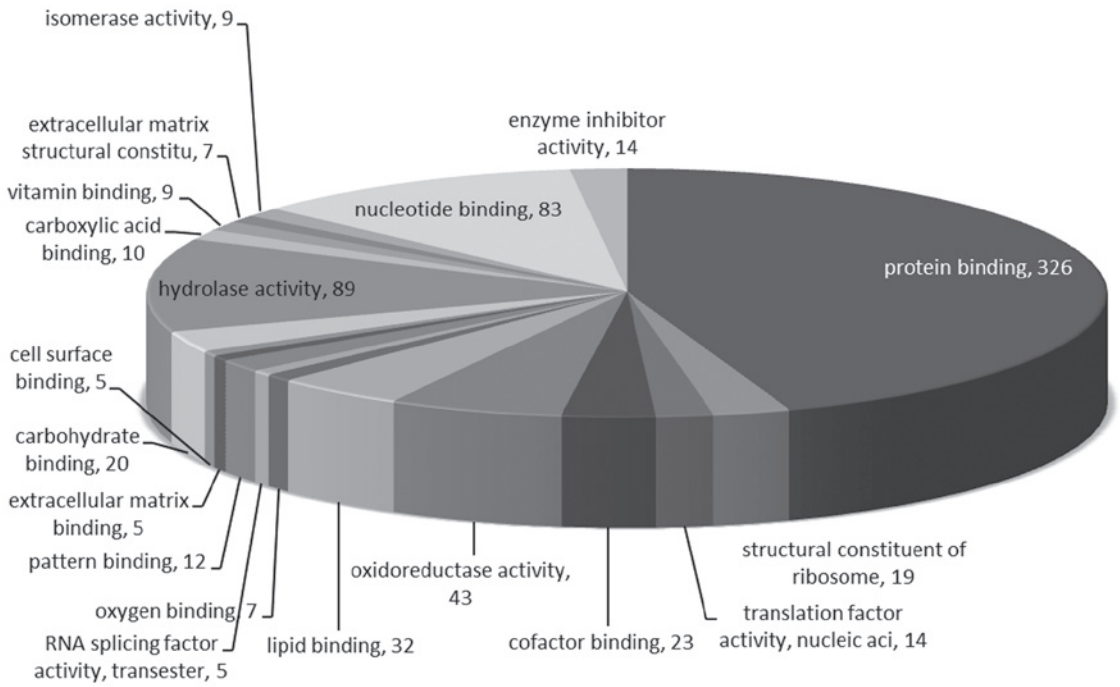

Figure 1. Classification of the 505 plasma proteins in different molecular function categories based on Gene Ontology terms.

Table II. The top 5 molecular function, cellular component and biological process Gene Ontology terms for the 505 proteins.

A, Top 5 molecular functions

Molecular function	Count	Proportion (%)
Protein binding	326	64.94
Hydrolase activity	89	17.73
Nucleotide binding	83	16.53
Oxidoreductase activity	43	8.57
Lipid binding	32	6.37

B, Top 5 cellular components

Cellular component	Count	Proportion (%)
Intracellular	405	80.68
Intracellular part	403	80.28
Intracellular organelle	318	63.35
Membrane-bounded organelle	278	55.38
Organelle part	191	38.05

C, Top 5 biological processes

Biological process	Count	Proportion (%)
Primary metabolic process	244	48.61
Cellular metabolic process	230	45.82
Establishment of localization	124	24.7
Transport	123	24.5
Response to stress	91	18.13

Results

Proteome of the umbilical cord blood. Using peptides of >1 an average reporter ion ratio ≥1.5 and an average reporter ion ratio ≤0.67 as cutoffs, 505 proteins were identified and quantified from the plasma. These proteins, from the pooled sample composed of individuals diagnosed with DS, were divided into 13 upregulated and 6 downregulated compared to healthy subjects, as shown in Table I.

Among the differentially expressed proteins in Table I and based on published studies, 5 proteins [apolipoprotein E, complement factor B (CFB), amyloid P component, serum (APCS), matrin-3 and osteopontin (OPN)] were found to be relevant to DS, with the first three being markedly upregulated in DS. The remaining proteins [leucine-rich repeat-containing protein 47, 26S proteasome non-ATPase regulatory subunit 13, nucleosome assembly protein 1-like 1, lethal (2) giant larvae protein homolog 2, endoplasmic oxidoreductin-1-like (ERO1-like) protein α, 2-oxoglutarate dehydrogenase-like, mitochondrial, thymosin β-10, ATPase ASNA1, large neutral amino acids transporter small subunit 2, ectonucleotide pyrophosphatase/phosphodiesterase family member 4, thioredoxin-related transmembrane protein 4, prefoldin subunit 2, AMP deaminase 2 and receptor expression-enhancing protein 5] showed the highest ratios (60.27 and 0.01), compared to healthy controls.

The 505 proteins were assigned to MF, CC and BP GO terms; the distribution of these proteins in the three GO term categories is shown in Figs. 1-3. The top 5 MFs in terms of relative proportion were protein binding, hydrolase activity, nucleotide binding, oxidoreductase activity and lipid binding (Table IIA). The top 5 CCs were intracellular, intracellular part, intracellular organelle, membrane-bounded organelle and organelle part (Table IIB). The top 5 BPs were primary metabolic process, cellular metabolic process, estab-

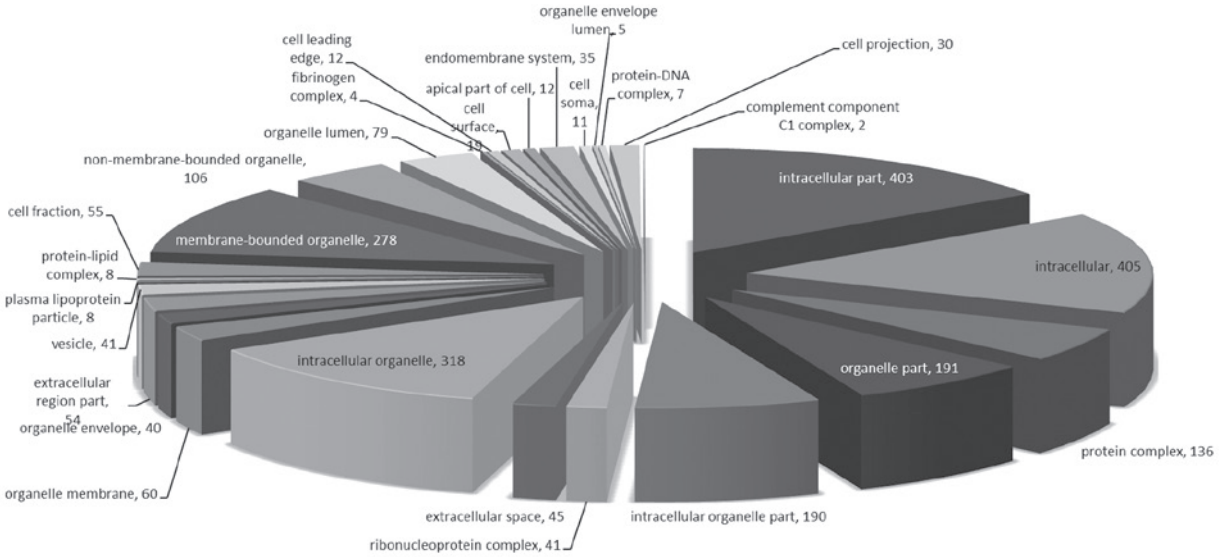

Figure 2. Classification of the 505 plasma proteins in different cellular component categories based on Gene Ontology terms.

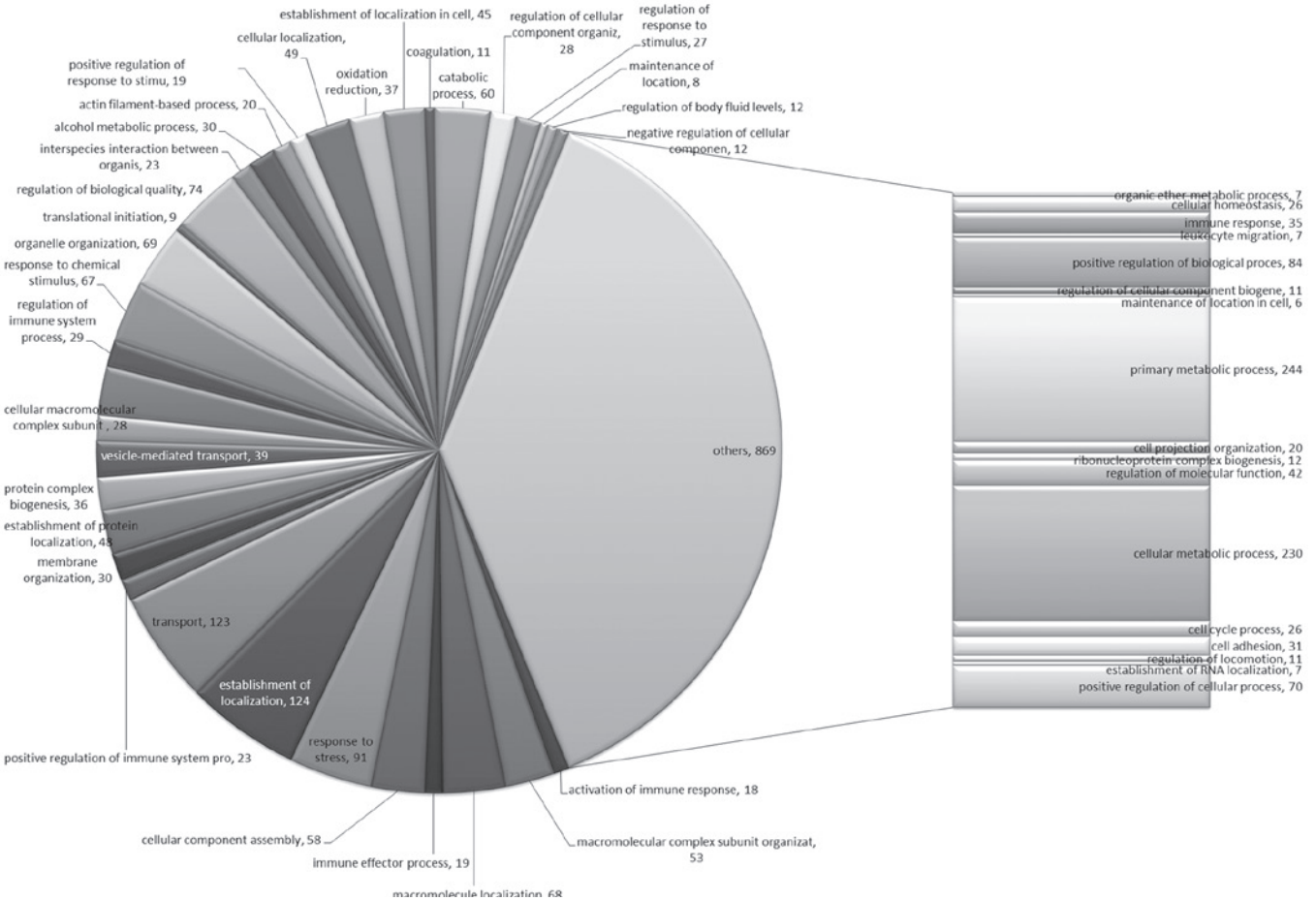

Figure 3. Classification of the 505 plasma proteins in different biological process categories based on Gene Ontology terms.

lishment of localization, transport and positive regulation of BP (Table IIC). The full list of MF, CC and BP associated with apolipoprotein E, CFB, APCS, matrin-3 and OPN is shown in Table III. MF, CC and BP terms for the proteins which showed the highest fold difference are presented in Table IV.

Discussion

Quantification of the proteins via iTRAQ analysis has been suggested to be a suitable strategy for the identification of biomarkers, since iTRAQ allows the comparison of protein

Table III. Molecular function, cellular component, and biological process Gene Ontology terms associated with apolipoprotein E, complement factor B, amyloid P component, serum, matrin-3 and osteopontin.

UnipProt no.	Protein name	Molecular function	Cellular component	Biological process
P02649	Apolipoprotein E	Protein binding, lipid binding pattern binding, carbohydrate binding	Intracellular part, intracellular, extracellular space, intracellular organelle, extracellular region part, plasma lipoprotein particle, protein-lipid complex, membrane-bounded organelle, cell surface, cell soma, cell projection	Macromolecular complex subunit organization, macromolecule localization, cellular component assembly, response to stress, establishment of localization, transport, membrane organization, vesicle-mediated transport, response to chemical stimulus, organelle organization, regulation of biological quality, alcohol metabolic process, cellular localization, establishment of localization in cell, catabolic process, regulation of cellular component organization, maintenance of location, organic ether metabolic process, cellular homeostasis, positive regulation of biological process, maintenance of location in cell, primary metabolic process, regulation of molecular function, cellular metabolic process, regulation of locomotion, positive regulation of cellular process
P00751	Complement factor B	Protein binding, hydrolase activity	Intracellular part, intracellular, protein complex	Activation of immune response, immune effector process, response to stress, positive regulation of immune system process, response to external stimulus, regulation of immune system process, response to chemical stimulus, positive regulation of response to stimulus, regulation of response to stimulus, immune response, positive regulation of biological process, primary metabolic process
P02743	Amyloid P component, serum	Protein binding, carbohydrate binding	Protein complex, extracellular space, extracellular region part	Macromolecular complex subunit organization, cellular component assembly, response to stress, protein complex biogenesis, cellular macromolecular complex subunit, response to chemical stimulus, primary metabolic process, cellular metabolic process
P43243	Matrin-3	Protein binding, nucleotide binding	Intracellular part, intracellular, organelle part, intracellular organelle part, intracellular organelle, organelle membrane, organelle envelope, membrane-bounded organelle, organelle lumen, endomembrane system	
P10451	Osteopontin	Protein binding, extracellular matrix binding	Intracellular part, intracellular, extracellular space, extracellular region part, vesicle, apical part of cell, cell projection	Response to stress, response to external stimulus, response to chemical stimulus, regulation of cellular component organization, regulation of response to stimulus, negative regulation of cellular component, positive regulation of biological process, cell adhesion, positive regulation of cellular process

Table IV. Molecular function, cellular component, and biological process Gene Ontology terms associated with proteins that showed the highest fold difference.

UniProt no.	Protein name	Molecular function	Cellular component	Biological process
Upregulated proteins				
Q8N1G4	Leucine-rich repeat-containing protein 47	Protein binding		Primary metabolic process, cellular metabolic process
Q9UNM6	26S proteasome non-ATPase regulatory subunit 13	Protein binding	Intracellular part, intracellular, protein complex	Catabolic process, positive regulation of biological process, primary metabolic process, regulation of molecular function, cellular metabolic process, cell cycle process, positive regulation of cellular process
P55209	Nucleosome assembly protein 1-like 1	Protein binding	Intracellular part, intracellular, protein complex, organelle part, intracellular organelle part, intracellular organelle, vesicle	Macromolecular complex subunit organization, cellular component assembly, cellular macromolecular complex subunit, organelle organization, positive regulation of biological process, primary metabolic process, cellular metabolic process, establishment of localization in cell
Q6P1M3	Lethal (2) giant larvae protein homolog 2		Intracellular part, intracellular	Establishment of localization, transport, vesicle-mediated transport, cellular localization, establishment of localization in cell
Q96HE7	ERO1-like protein α	Protein binding, cofactor binding, oxidoreductase activity, nucleotide binding	Intracellular part, intracellular, organelle part, intracellular organelle part, intracellular organelle, organelle membrane, cell fraction, membrane-bounded organelle, endomembrane system	Response to stress, establishment of localization, transport, response to chemical stimulus, oxidation reduction, primary metabolic process, cellular metabolic process
Q9ULD0	2-oxoglutarate dehydrogenase-like, mitochondrial	Cofactor binding, oxidoreductase activity, vitamin binding	Intracellular part, intracellular, organelle part, intracellular organelle part, intracellular organelle, membrane-bounded organelle, organelle lumen	Alcohol metabolic process, oxidation reduction, catabolic process, primary metabolic process, cellular metabolic process
P63313	Thymosin β-10	Protein binding	Intracellular part, intracellular, intracellular organelle, non-membrane-bounded organelle	Macromolecule localization, organelle organization, regulation of biological quality, actin filament-based process, regulation of cellular component organization, maintenance of location, negative regulation of cellular component, regulation of cellular component biogene
O43681	ATPase ASNA1	Hydrolase activity, nucleotide binding	Intracellular part, intracellular, organelle part, intracellular organelle part, intracellular organelle, cell fraction, membrane-bounded organelle, non-membrane-bounded organelle, organelle lumen	Establishment of localization, transport, response to chemical stimulus, regulation of biological quality, cellular homeostasis
Q9UHI5	Large neutral amino acids transporter small subunit 2	Protein binding	Intracellular part, intracellular	Establishment of localization, transport, response to chemical stimulus, regulation of biological quality, primary metabolic process, cellular metabolic process

Table IV. Continued.

UniProt no.	Protein name	Molecular function	Cellular component	Biological process
Q9Y6X5	Ectonucleotide pyrophosphatase/ phosphodiesterase family member 4	Hydrolase activity		
Downregulated proteins				
Q9H1E5	Thioredoxin-related transmembrane protein 4		Intracellular part, intracellular, intracellular organelle, membrane-bounded organelle	Establishment of localization, transport, regulation of biological quality, oxidation reduction, cellular homeostasis, cellular metabolic process
Q9UHV9	Prefoldin subunit 2	Protein binding	Intracellular part, intracellular, protein complex	Primary metabolic process, cellular metabolic process
Q01433	AMP deaminase 2	Hydrolase activity		Primary metabolic process, cellular metabolic process
Q00765	Receptor expression-enhancing protein 5	Protein binding		

abundance among samples by measuring the peak intensities of reporter ions released from the iTRAQ-tagged peptides. In this study, we adopted iTRAQ technology and GO analysis to quantitatively analyse the proteome of plasma from the umbilical cord blood of DS fetus-carrying mothers, in order to identify useful biomarkers for DS. As a result, 505 proteins were identified, and were classified to MF, CC and BP terms by GO analysis. Among the up- and downregulated proteins in DS (Table I), we focused on 5 (apolipoprotein E, CFB, APCS, matrin-3, and OPN) to verify whether these may constitute novel DS biomarkers.

A putative risk factor for AD in the general population, the E4 allele of the apolipoprotein E gene, has highlighted the role of genetic influences in AD. It has long been recognized that DS is associated with early and severe development of AD neuropathological lesions (12). APCS and the Ig λ chain C region were also found in the sera of patients with DS in another study (8). We found that the concentration of apolipoprotein E in the plasma of umbilical cord blood was 3.75 times (x) higher in the DS compared to the control group The MF, CC and BP GO terms associated with apolipoprotein E are shown in Table III.

CFB plays an important role in the alternative complement pathway in AD (13), which shares a number of similar features with DS. Yu *et al* (14) found that the concentration of CFB is significantly increased in the serum of mothers with fetuses affected by DS. CFB may be associated with the brain damage that occurs in DS. In certain studies, CFB was associated with brain diseases, and a relationship between DS and the complement system was revealed (13,15-17). In the present study, we found that the concentration of CFB in the plasma of umbilical cord blood was 1.95x higher in the DS compared to the control group. The MFs associated with CFB were protein binding and hydrolase activity. The CCs associated with CFB were intracellular part, intracellular and protein complex. The BPs associated with CFB included activation of immune response, immune effector process, and response to stress (full list in Table III).

The APCS glycoprotein is encoded by a single gene locating on the human chromosome 1, has 204 residues, and is secreted (18). APCS immunoreactivity is commonly observed in DS, AD, and amyloid disorders with primarily cerebrovascular compromise (19,20). In the present study, we found that the concentration of APCS in the plasma of umbilical cord blood was 1.68x higher in the DS compared to the control group. The MFs, CCs and BPs associated with APCS are listed in Table III. The MFs were protein and carbohydrate binding, the CCs were protein complex, extracellular space and extracellular region part, and the BPs included macromolecular complex subunit organization, CC assembly, response to stress, and protein complex biogenesis (Table III).

OPN is a secreted glycoprotein with an arginine-glycine-aspartic acid (RGD) sequence, and able to bind a number of receptors, including the integrins and certain variant forms of CD44. OPN plays an important role in diverse fibro-inflammatory diseases. OPN is expressed in hepatocytes and macrophage-like cells in DS individuals showing perinatal liver fibrosis. OPN was thus proposed to be involved in the pathogenesis of perisinusoidal liver fibrosis, frequently observed in neonates with DS (21). We found that the concen-

tration of OPN in the plasma of umbilical cord blood was 2.27x lower in the DS compared to the control group. The MFs, CCs and BPs associated with OPN are displayed in Table III. The MFs were protein and extracellular matrix binding, the CCs included intracellular part, intracellular, and extracellular space, while the BPs included response to stress, external stimulus, and chemical stimulus (Table III).

Matrin-3 is an inner nuclear matrix protein of 125 kDa, which is highly conserved in mammals (22). Matrin-3 contains a bipartite nuclear localization signal, two zinc finger domains, and two canonical RNA recognition motifs (23). Matrin-3 is encoded by the *MATR3* gene, which is expressed in skeletal muscle (24). Autosomal and the active X chromosome territories were found to express matrin-3 (25). Matrin-3 was also found to be a target of the ataxia telangiectasia mutated (ATM) protein kinase (26). Degradation of matrin-3 may be a key cellular event, which induces a shift from apoptotic to necrotic death (27). Matrin-3 expression was significantly decreased in the fetal DS brain (28). The manifold decreased spot unambiguously assigned to matrin-3 in previous studies may reflect or induce aberrant transcription reported to occur in DS (28,29). In this study, we found that the concentration of matrin-3 in the plasma of umbilical cord blood was 100x lower in the DS compared to the control group. We did not found a BP term associated with matrin-3. The MFs associated with matrin-3 were protein and nucleotide binding, and the CCs included intracellular part, intracellular, and organelle part (Table III).

In conclusion, the iTRAQ technology, a relatively new strategy for proteomic analysis, was used here to study the changes in protein expression associated with DS. This approach identified 5 significantly differentially expressed proteins (apolipoprotein E, CFB, APCS, matrin-3 and OPN), which were previously reported to relate to DS. The MFs, CCs and BPs associated with these proteins indicate that they may be suitable DS biomarkers. However, further investigation of the functions of these proteins, as well as of the proteins with high fold changes in DS identified herein is required.

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