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PAPER

Placenta proteome analysis from Down syndrome pregnancies for biomarker discovery

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Down syndrome is one of the most frequent chromosomal disorders, with a prevalence of approximately 1/500 to 1/800, depending on the maternal age distribution of the pregnant population. However, few reliable protein biomarkers have been used in the diagnosis of this disease. Recent progress in quantitative proteomics has offered opportunities to discover biomarkers for tracking the progression and for understanding the molecular mechanisms of Down syndrome. In the present study, placental samples were analyzed by fluorescence two-dimensional differential gel electrophoresis (2D-DIGE) and differentially expressed proteins were identified by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). In total, 101 proteins have been firmly identified representing 80 unique gene products. These proteins mainly function in cytoskeleton structure and regulation (such as vimentin and Profilin-1). Additionally, our quantitative proteomics approach has identified numerous previously reported Down syndrome markers, such as myelin protein. Here we present several Down syndrome biomarkers including galectin-1, ataxin-3 and sprouty-related EVH1 domain-containing protein 2 (SPRED2), which have not been reported elsewhere and may be associated with the progression and development of the disease. In summary, we report a comprehensive placenta-based proteomics approach for the identification of potential biomarkers for Down syndrome, in which serum amyloid P-component (APCS) and ataxin-3 have been shown to be up-regulated in the maternal peripheral plasma of Down syndrome cases. The potential of utilizing these markers for the prognosis and screening of Down syndrome warrants further investigation.

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Introduction

Down syndrome is one of the most frequent chromosomal disorders, with a prevalence of approximately 1/500 to 1/800, depending on the maternal age distribution of the pregnant population.[1] Current protein-based screening tests for Down syndrome are based on the determination of the concentrations of the plasma protein markers: pregnancy-associated plasma protein A, alpha fetoprotein (AFP), human chorionic gonadotrophin, unconjugated estriol (uE3) and inhibin A in maternal plasma. These tests can detect approximately 80–90% of Down syndrome cases, but has a 5% false positive rate.[2] By taking a large scale approach for biomarker discovery through the use of proteomics, it is anticipated that a greater panel of biomarkers will be identified to improve the screening and diagnosis of Down syndrome.

All mammalian placentas have the same fundamental function to nourish the growing fetus by establishing contact with the maternal blood circulation. Meanwhile, the placenta acts as a protective barrier to prevent molecules such as drugs, chemical

compounds or stress hormones passing over to the fetus. Moreover, the placenta expresses an intricate pattern of major histocompatibility complex molecules to prevent and immunize the mother against the foreign fetal tissue. During pregnancy the placenta also has the capacity to translocate embryonic cells, fetal proteins, nucleic acids, growth factors, hormones such as human chorionic gonadotropin and human placental lactogen[3–7] into the maternal circulation system. Thus, it is reasonable to investigate the fetal diseases *via* discovery of disease markers in maternal serum.

After the human genome was sequenced, the elucidation of protein function has become one of the most important issues in the post-genomic era and proteomics has been used as a tool to globally study the behavior of gene expression. 2-DE combined with MALDI-TOF MS is currently a key proteomic strategy in profiling thousands of protein signatures within biological samples, as well as playing a role complementary to LC/MS-based proteomic analysis.[8] Our current 2D-DIGE platform is a reliable analysis tool to quantitatively compare protein expression alteration across gels.[9,10] In this study, a sample preparation protocol and the proteomic analysis of placental proteins has been optimized. After these, placental proteins from 5 normal and 5 Down syndrome cases have been extracted and analyzed with 2D-DIGE and MALDI-TOF MS. A panel of placental proteins, differentially expressed between Down syndrome cases and normal cases, were elucidated and their expressions were also validated with placental tissues and maternal peripheral plasma.

Materials and methods

Chemicals and reagents

All primary antibodies used in this study were purchased from GeneTex (Hsinchu, Taiwan), while reagents for 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden). All the biochemicals and chemicals used in this study were of analytical grade.

Placental sample collection and purification

From Jan 2011 to Dec 2011, five normal (2 female fetuses and 3 male fetuses) and five Down syndrome (2 female fetuses and 3 male fetuses) placentas/maternal peripheral plasma in a single hospital (Mackay Memorial Hospital, Taipei, Taiwan) were enrolled in the study. Placentas and maternal peripheral plasma from pregnancies with Down syndrome fetuses and from chromosomally normal fetuses in between 16th–18th week of gestation were collected. Written informed consent was obtained from all subjects and the research protocol has been approved by the ethics committee of the Mackay Memorial Hospital. Immediately after induced labor, placentas were intensively washed in cold normal saline to eliminate any contaminating blood; five different punches from various areas of each placenta were pooled and stored in −80 °C until protein extraction.

To improve the performance of the proteomic analysis of the placental samples, the homogenized placental tissues were precipitated by adding 1 volume of 100% TCA (at −20 °C) to 4 volumes of sample and incubated for 10 min at 4 °C. The precipitated protein was then recovered by centrifugation at 13 000 rpm for 10 min, and the resulting pellet was washed three times with ice-cold acetone. Air-dried pellets were resuspended in 2-DE lysis containing 4% w/v CHAPS, 7 M urea, 2 M thiourea, 10 mM Tris-HCl, pH 8.3 and 1 mM EDTA.

Sample preparation for 2D-DIGE and gel image analysis

The placental protein extracts were dissolved in 2-DE lysis buffer. A pool of 5 normal placentas (five different punches from various areas of each placenta) and a pool of 5 Down syndrome placentas (five different punches from various areas of each placenta) were prepared individually. After protein quantification with Bradford assay, 200 μg of protein samples from the normal placenta pool and 200 μg of protein samples from the Down syndrome placenta pool were labeled with 500 pmol of Cy3 and Cy5, respectively, and equally distributed into gel 1 and gel 3. In contrast, 100 μg of protein samples from the normal placenta pool and 100 μg of protein samples from the Down syndrome placenta pool were labeled with 250 pmol of Cy5 and Cy3, respectively, and distributed into gel 2. To facilitate image matching and cross-gel statistical comparison, a pool of all samples was also prepared and labeled with Cy2 at a molar ratio of 2.5 pmol Cy2 per μg of protein as an internal standard for all gels. Thus, the triplicate samples and the internal standard could be run and quantified on three 2-DE. The detailed procedures for fluorescence dye-labeling, isoelectric focusing electrophoresis, two-dimensional electrophoresis, fluorescence image detection/analysis were described in our previous publication.[11] Spots displayed in all 9 gel images (3 images per gel) with a $\geqq 1.3$ average-fold increase or decrease in abundance and a p-value < 0.05 were selected for protein identification.

Protein staining, in-gel digestion and MALDI-TOF MS analysis

Colloidal coomassie blue G-250 staining was used to visualize CyDye-labeled protein features in 2-DE followed by excised interested post-stained gel pieces for MALDI-TOF MS identification. The detailed procedures for protein staining, in-gel digestion, MALDI-TOF MS analysis and the algorithm used for data processing were described in our previous publication.[11] The spectrometer was also calibrated with a peptide calibration standard (Bruker Daltonics) and internal calibration was performed using trypsin autolysis peaks at m/z 842.51 and m/z 2211.10. Peaks in the mass range of m/z 700–3000 were used to generate a peptide mass fingerprint that was searched against the Swiss-Prot/TrEMBL database (release on 2012_01) with 534 242 entries using Mascot software v2.3.02 (Matrix Science, London, UK). The following parameters were used for the search: *Homo sapiens*; tryptic digest with a maximum of 1 missed cleavage; carbamidomethylation of cysteine, partial protein N-terminal acetylation, partial methionine oxidation and partial modification of glutamine to pyroglutamate and a mass tolerance of 50 ppm. Identification was accepted based on significant MASCOT Mowse scores ($p < 0.05$), spectrum annotation and observed *versus* expected molecular weight and p*I* on 2-DE.

Immunoblotting analysis and ELISA analysis

Immunoblotting and ELISA analysis were used to validate the differential abundance of mass spectrometry identified proteins. The detailed experimental procedures were described in our

previous reports.[12–14] All primary antibodies used for expression validation were purchased from Genetex (Hsinchu, Taiwan).

Results

2D-DIGE and mass spectrometry analysis of the differentially expressed placental proteome between Down syndrome fetuses and chromosomally normal fetuses

In order to study the alteration of the placental proteome in Down syndrome fetuses, comparative proteomics analysis was performed between Down syndrome and normal fetuses. The 2-DE images of the samples from the two groups were minimally labeled with Cy3 and Cy5 dyes and distributed on each gel. A pool of both samples was also prepared for labeling with Cy2 as an internal standard to run on all gels to facilitate image matching across gels (Fig. 1). The placental samples arrangement for a triplicate 2D-DIGE experiment is shown in Fig. 1A. Thus, the triplicate samples resolved in different gels can be quantitatively analyzed by means of the internal standard on multiple 2-DE. After resolving protein samples with 2D-DIGE, the DeCyder image analysis software indicated that 174 protein features showed a greater than 1.3-fold change in expression level with a student *t*-test (*p*-value) less than 0.05. MALDI-TOF MS identification revealed that 101 proteins (corresponding to 80 unique proteins) were differentially expressed

A

	Cy2	Cy3	Cy5
Gel1	Pool	Normal	Down syndrome
Gel2	Pool	Down syndrome	Normal
Gel3	Pool	Normal	Down syndrome

B

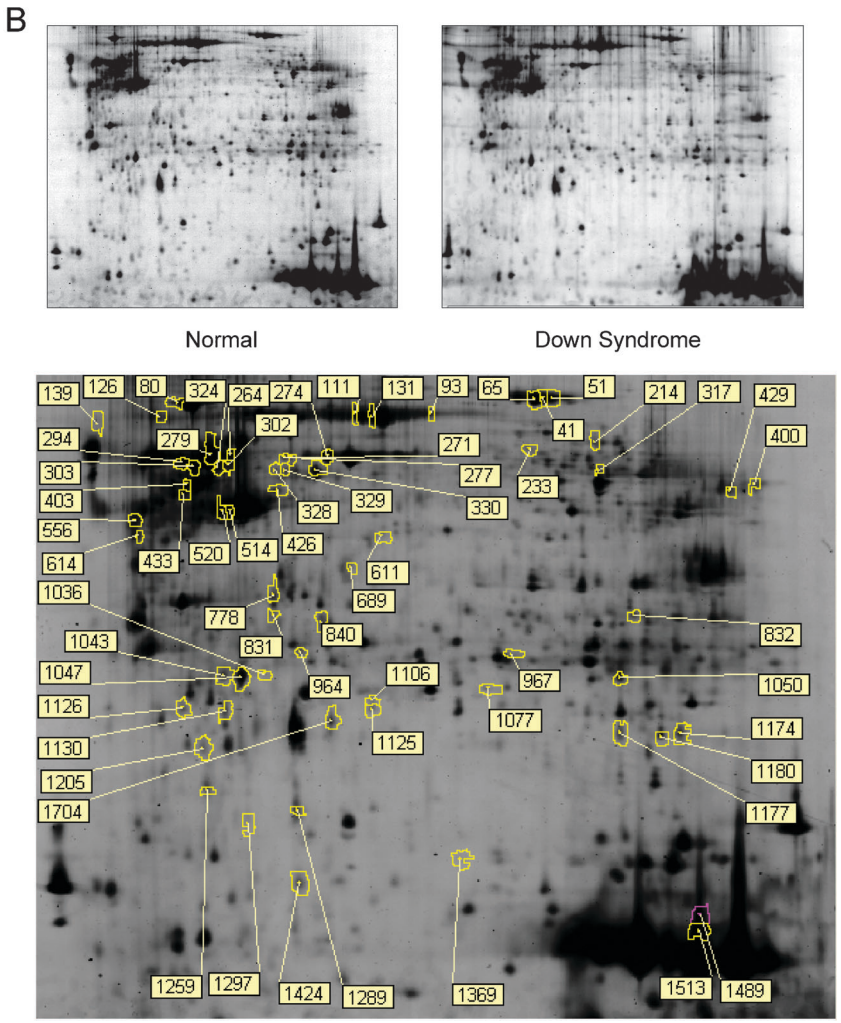

Fig. 1 2D-DIGE analysis of Down syndrome-induced differentially expressed placental proteins. (A) Placental sample arrangement for a triplicate 2D-DIGE experiment. (B) Placental samples (100 μg each) were labeled with Cy-dyes and separated using 24 cm, pH 3–10 non-linear IPG strips. 2D-DIGE images of the placental samples from Down syndrome fetus and normal fetus at appropriate excitation and emission wavelengths were shown (Upper images). The differentially expressed identified protein features are annotated with spot numbers (bottom image).

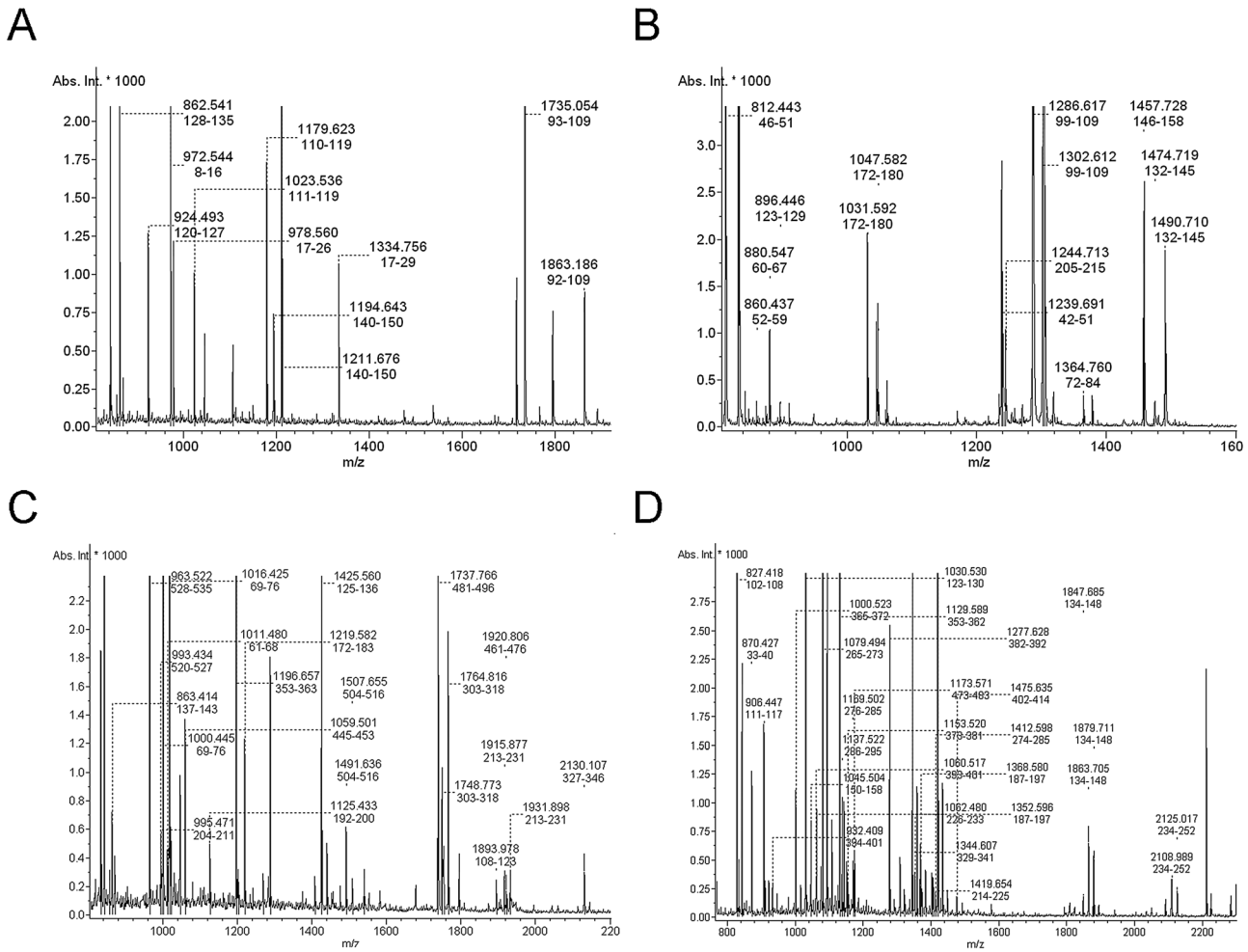

Fig. 2 Peptide mass fingerprinting of identified placental proteins (A) Peroxiredoxin-2, (B) Cyclophilin B, (C) Glutamate dehydrogenase 1 and (D) Keratin, type II cytoskeletal 8.

(Fig. 1B, Fig. 2 and Table 1). Most of these identified proteins are functionally involved in cytoskeleton regulation (44%), transport (17%) and gene expression (9%) (Fig. 3).

Representative examples for the evaluation by DeCyder of alteration in spot intensities using the 2D-DIGE system are displayed in Fig. 4. To visually display alterations in corresponding spot intensity proportions, selected identified spots (apolipoprotein A–I, transthyretin, serum amyloid P-component and reticulocalbin-1) are shown as 3-D images as well as the associated graph views of standardized abundances of the selected spots and spot locations.

Validation of identified proteins by immunoblotting and ELISA analysis of placental proteins and maternal peripheral plasma

To verify the abundances of proteins deduced from the results of 2D-DIGE and MALDI-TOF MS, the abundance levels of identified cytokeratin 8, ataxin-3, annexin 2, vimentin, cathepsin D, SPRED2, serum amyloid P-component, glutamate dehydrogenase and VDAC2 were investigated by immunoblotting and ELISA, respectively. As is shown in Fig. 5, the 42 kDa of ataxin-3, 53 kDa of vimentin, 36 kDa of annexin 2, 48 kDa of SPRED2, 25 kDa of serum amyloid P-component and 61 kDa of glutamate dehydrogenase were increased in the Down syndrome placentas. Additionally, the 54 kDa of cytokeratin 8, the 55 kDa of cathepsin D and 32 kDa of VDAC2 were significantly decreased in the Down syndrome placentas. These immunoblotting and ELISA results are consistent with the data from the 2D-DIGE and MALDI-TOF MS, and further suggest that these identified proteins may be employed as potential markers for the diagnosis of Down syndrome. Further study of the maternal peripheral plasma demonstrated that APCS and ataxin-3 are up-regulated in the maternal peripheral plasma of Down syndrome cases (Fig. 6). These data suggest that these proteins may be employed as potential markers for the diagnosis of Down syndrome.

Discussion

Proteomic analysis of the human diseases usually adopt a comparative strategy that is defined by the differential expression of the proteins under different disease conditions. Recent serum and amniotic fluid proteomic studies of Down syndrome cases have identified several putative Down syndrome biomarkers.[15–17] The main advantage of plasma as a starting source is that it can be obtained safely from a pregnant woman and the amount of plasma proteins is sufficient to perform numerous experiments.

Table 1 Alphabetical list of differentially expressed placental proteins between Down syndrome and normal fetuses as identified by MALDI-TOF peptide mass fingerprinting after 2D-DIGE analysis. All identified proteins listed in Table 1 have their statistical p-values < 0.05, while identified proteins with p-values < 0.01 are shaded gray

No.	Swiss-Prot No.	Protein name	pI	MW	No. Match. Peptide	Cov. (%)	Score	Down syndrome / Normal Control	T-test	Subcellular location	Functional Ontology	Match peptide Sequences
1720	P32969	60S ribosomal protein L9	9.96	21964	3/4	18	57/56	1.74	0.017	Cytoplasm	Translation control	R.VDKWWGNR.K K.GTVQQADE.-
80	P11021	78 kDa glucose-regulated protein	5.07	72402	9/33	20	71/56	-1.35	0.039	Endoplasmic reticulum	Protein folding	R.IEIESFYEGEDFSETLTR.AK.VTHAVVTVPA YFNDAQRQATK.D
520	Q96HG5	Actin, cytoplasmic 1	5.29	42052	7/31	21	75/56	-1.65	0.021	Cytoplasm	Cytoskeleton	K.AGFAGDDAPR.A K.DLYANTVLSGGTTMYPGIADR.M
514	P63261	Actin, cytoplasmic 2	5.31	42108	12/48	34	108/56	-1.31	0.02	Cytoplasm	Cytoskeleton	R.HQGVMVGMGQK.D K.QEYDESGPSIVHRK.C
293	Q86U18	Alpha-1-antitrypsin	5.34	46878	11/37	23	89/56	2.61	0.015	Secreted	Immunodefence	K.LSSWVLLMK.YK.WERPFEVK.D
1455	P02511	Alpha-crystallin B chain	6.76	20146	6/26	25	70/56	-1.38	0.083	Cytoplasm	Protein folding	MDIAIHHPWIR.R R.RPFFPFHSPSR.L
1174	Q8WXJ9	Ankyrin repeat and SOCS box protein 17	9.05	34887	6/16	13	81/56	-1.84	0.032	Cytoplasm	Protein degradation	R.IYRSLAK.I R.VLSVISVKEIK.T
857	P07355	Annexin A2	7.57	38808	10/26	27	79/56	1.54	0.049	Secreted	Calcium binding	R.QDIAFAYQR.R R.DLYDAGVKR.K
906	P08758	Annexin A5	4.94	35971	8/18	26	112/56	-1.44	0.036	Plasma membrane	Coagulation	K.YMTISGFQIEETIDR.E R.SIPAYLAETLYYAMK.G
1047	P02647	Apolipoprotein A-I	5.56	30759	15/35	46	138/56	3.02	0.063	Secreted	Lipid metabolism	K.VQPYLDDFQK.K K.ETEGLRQEMSK.D
1127	Q9H3N0	Ataxin-3	4.81	42097	5/10	19	70/56	1.39	0.03	Nucleus	Gene expression	R.VWQVTIGTR.- R.MRMAEGGVTSEDYR.T
376	P25705	ATP synthase subunit alpha, mitochondrial	9.16	59828	8/11	19	93/56	1.72	0.033	Mitochondrion	ATP synthesis	R.ILGADTSVDLEETGR.V K.GIRPAINVGLSVSR.V
1863	P25705	ATP synthase subunit alpha, mitochondrial	9.16	59828	8/22	18	83/56	1.41	0.05	Mitochondrion	ATP synthesis	R.VGLKAPGIIPR.I K.FENAFLSHVVSQHQALLGTIR.A
160	P27797	Calreticulin	4.29	48283	6/11	11	78/56	1.62	0.00072	Endoplasmic reticulum	Calcium binding	K.GQTLVVQFTVK.H K.KVHVIFNYK.G
914	P07339	Cathepsin D	6.10	45037	5/8	8	63/56	-1.56	0.026	Lysosome	Protein degradation	R.YYTVFDR.D K.QPGITFIAAK.F
894	P01243	Chorionic somatomammotropin hormone	5.34	25289	8/23	35	93/56	-1.39	0.0059	Secreted	Hormon activity	R.LFDHAMLQAHR.A K.DLEEGIQTLMGR.L
1713	P23284	Cyclophilin B	9.42	23785	14/35	48	119/56	1.57	0.05	Endoplasmic reticulum	Protein folding	K.DTNGSQFFITTVK.T K.IEVEKPFAIAK.E
887	O43543	DNA repair protein XRCC2	5.67	32620	4/8	11	57/56	-1.31	0.002	Nucleus	DNA repair	.MCSAFHR.A M.CSAFHRAESGTELLAR.L
1214	Q14236	Early lymphoid activation gene protein	10.16	18117	4/7	26	59/56	-1.59	0.044	Cytoplasm	Signal transduction	R.MFYHQK.H K.HFQLGLK.G
1078	A8MZ36	Envoplakin-like protein	5.8	34331	4/4	18	73/56	1.4	0.016	Cytoplasm	Cytoskeletal linker proteins	R.RAAAEPGGAGCR.H M.QASADQVER.D
1126	Q05315	Eosinophil lysophospholipase	6.82	16556	3/11	32	56/56	-1.53	0.0089	Cytoplasmic granule	Lipid metabolism	K.FNVSYLK.R .MSLLPVPYTEAASLSTGSTVTIK.G
933	P52907	F-actin-capping protein subunit alpha-1	5.45	33073	3/5	17	62/56	-1.48	0.05	Cytoplasm	Cytoskeleton	R.LLLNNDNLLR.E K.TIDGQQTIIACIESHQFQPK.N
1180	P30043	Flavin reductase (NADPH)	7.13	22219	6/11	23	76/56	-2.11	0.0093	Cytoplasm	Redox regulation	K.HDLGHFMLR.C K.HDLGHFMLR.C
485	Q15313	G protein-coupled receptor kinase 4	7.93	67738	5/11	14	58/56	-1.34	0.05	Cytoplasm	Signal transduction	K.DALCLVLTIMNGGDLK.F R.GRVGTVGYMAPEVVNNEK.Y
1784	P09382	Galectin-1	5.34	15048	4/10	35	62/56	2.58	0.043	Secreted	Cell growth	M.ACGLVASNLNLKPGECLR.V K.DSNNLCLHFNPR.F

Table 1 (*continued*)

No.	Swiss-Prot No.	Protein name	pI	MW	No. Match. Peptide	Cov. (%)	Score	Down syndrome / Normal Control	T-test	Subcellular location	Functional Ontology	Match peptide Sequences
317	P00367	Glutamate dehydrogenase 1	7.66	61701	22/41	38	188/56	1.31	0.016	Mitochondrion	Neurotransmition	K.YNLGLDLR.T K.GFIGPGIDVPAPDMSTGER.I
926	P04406	Glyceraldehyde-3-phosphate dehydrogenase	8.57	36201	10/36	36	109/56	-1.65	0.031	Cytoplasm	Glycolysis	K.VGVNGFGR.I K.LTGMAFR.V
778	P09466	Glycodelin	5.36	20953	4/13	20	70/56	-1.47	0.044	Secreted	Reproduction	R.AFRPLPR.H R.VLVEDDEIMQGFIR.A
1125	A6NH11	Glycolipid transfer protein domain-containing protein 2	10.19	31964	4/8	18	74/56	-1.42	0.026	Cytoplasm	Glycolipid transfer	R.FHASLKPEGDVGLSPYLAGWR.A .MGVAARPPALR.H
1177	P62826	GTP-binding nuclear protein Ran	7.01	24597	7/24	33	100/56	-1.32	0.017	Nucleus	Nucleocytoplasmic transport	K.YVATLGVEVHPLVFHTNR.G K.KYVATLGVEVHPLVFHTNR.G
1009	Q6FH47	Guanine nucleotide-binding protein subunit beta-2-like 1	7.60	35511	6/23	26	85/56	1.62	0.00068	Plasma membrane	Signal transduction	R.DETNYGIPQR.A R.VWQVTIGTR.-
1036	Q9UC36	Heat shock protein beta-1	5.98	22826	4/6	16	78/56	2.23	0.019	Cytoplasm	Protein folding	R.GPSWDPFR.D R.DWYPHSR.L
1496	P32456	Interferon-induced guanylate-binding protein 2	5.54	67680	6/10	11	68/56	1.32	0.05	Plasma membrane	Signal transduction	K.TLSGGIPVNGPR.L R.DSEREAIEVFMK.N
1662	P48735	Isocitrate dehydrogenase [NADP], mitochondrial	8.88	51333	5/6	11	68/56	1.42	0.012	Mitochondrion	TCA cycle	R.NILGGTVFR.E R.GKLDGNQDLIR.F
426	P05783	Keratin, type I cytoskeletal 18	5.34	48029	9/26	15	81/56	-1.34	0.024	Cytoplasm	Cytoskeleton	R.STFSTNYR.S R.IVLQIDNAR.L
832	P04264	Keratin, type II cytoskeletal 1	8.15	66170	16/59	22	108/56	1.67	0.0054	Cytoplasm	Cytoskeleton	R.LLRDYQELMNTK.L K.AQYEDIAQK.S
271	P08729	Keratin, type II cytoskeletal 7	5.4	51414	7/16	15	94/56	-1.31	0.0093	Cytoplasm	Cytoskeleton	R.SAYGGPVGAGIR.E K.WTLLQEQK.S
330	Q6GMY0	Keratin, type II cytoskeletal 8	5.52	53671	27/54	45	196/56	-2.61	0.0071	Cytoplasm	Cytoskeleton	R.ELQSQISDTSVVLSMDNSR.S R.ISSSSFSR.V
329	Q6GMY0	Keratin, type II cytoskeletal 8	5.52	53671	14/31	24	112/56	-1.64	0.019	Cytoplasm	Cytoskeleton	K.WSLLQQQKTAR.S R.FLEQQNK.M
328	Q6GMY0	Keratin, type II cytoskeletal 8	5.52	53671	14/34	25	122/56	-1.67	0.047	Cytoplasm	Cytoskeleton	K.LALDIEIATYRK.L R.LQAEIEGLKGQR.A
663	Q8N1A0	Keratin-like protein KRT222	5.63	34308	4/10	14	60/56	-1.51	0.015	Cytoplasm	Cytoskeleton	K.YENENVETVTK.QK.ESTEAHGTIQTEK.V
967	Q9NZS2	Killer cell lectin-like receptor subfamily F member 1	8.14	27219	5/12	23	67/56	1.5	0.016	Plasma membrane	Immunodefence	K.CCYWFSNEMK.S K.ENSCAAIK.E
1205	Q04760	Lactoylglutathione lyase	5.12	20992	6/14	23	68/56	-1.62	0.045	Cytoplasm	Gene expression	K.RFEELGVK.F K.IAWALSR.K
689	P07195	L-lactate dehydrogenase B chain	5.71	36900	4/7	11	68/56	-1.35	0.048	Cytoplasm	Glycolysis	R.VIGSGCNLDSAR.F K.IVVVTAGVR.Q
1369	P24666	Low molecular weight phosphotyrosine protein phosphatase	6.30	18487	4/11	27	71/56	2.97	0.0093	Cytoplasm	Signal transduction	K.SVLFVCLGNICR.S R.SPIAEAVFR.K
611	Q14168	MAGUK p55 subfamily member 2	6.32	64882	5/8	13	61/56	-1.44	0.029	Plasma membrane	Signal transduction	R.ELQTAMEKLR.T R.ILHGGMVAQQGLLHVGDIIKEVNGQPVGSDPR.A
788	P40926	Malate dehydrogenase	8.92	35937	6/26	19	64/56	-1.47	0.044	Mitochondrion	TCA cycle	K.AGAGSATLSMAYAGAR.FK.GYLGPEQLPDCLK.G
837	P40925	Malate dehydrogenase,MDH1	6.91	36631	7/21	18	59/56	-1.34	0.028	Cytoplasm	TCA cycle	K.ENFSCLTR.L K.GEFVTTVQQR.G
214	Q96EZ8	Microspherule protein 1	9.41	51999	4/5	13	60/56	-1.41	0.04	Nucleus	Gene expression	K.DSQGLLDSSLMASGTASR.S R.WYALLYDPVISKLACQAMR.Q
1050	Q99714	Mitochondrial RNase P protein 2	7.66	27134	3/4	17	66/56	1.59	0.011	Mitochondrion	RNA processing	K.GQTHTLEDFQR.V K.VCNFLASQVPFPSR.L

Table 1 (*continued*)

No.	Swiss-Prot No.	Protein name	pI	MW	No. Match. Peptide	Cov. (%)	Score	Down syndrome / Normal Control	T-test	Subcellular location	Functional Ontology	Match peptide Sequences
1130	P25189	Myelin protein P0	9.57	27709	4/7	11	60/56	-1.49	0.00049	Plasma membrane	Synaptic transmission	R.QAALQRR.L R.RLSAMEK.G
1106	P19404	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	8.22	27659	3/4	12	60/56	-1.36	0.037	Mitochondrion	Electron transfer	.MFFSAALR.A K.VAEVLQVPPMR.V
1218	O43181	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	10.30	20059	4/6	29	65/56	1.44	0.05	Mitochondrion	Electron transfer	R.LAQDQTQDTQLITVDEK.L R.IFVPARNNMQSGVNNTK.K
1244	P32119	Peroxiredoxin-2	5.66	22049	5/12	21	60/56	-2.52	0.0032	Cytoplasm	Redox regulation	R.QITVNDLPVGR.S R.QITVNDLPVGR.S
400	P32119	Peroxiredoxin-2	5.66	22049	12/28	38	157/56	-1.55	0.009	Cytoplasm	Redox regulation	R.LSEDYGVLK.T R.QITVNDLPVGR.S
1315	P30041	Peroxiredoxin-6	6.00	25133	8/24	36	9/56	1.64	0.006	Cytoplasm	Redox regulation	R.NFDEILR.V K.LPFPIIDDR.N
1173	P30041	Peroxiredoxin-6	6.00	25133	9/42	41	102/56	1.42	0.046	Cytoplasm	Redox regulation	K.LPFPIIDDR.N K.LSILYPATTGR.N
702	Q9Y3A2	Probable U3 small nucleolar RNA-associated protein 11	10.16	30485	4/6	20	64/56	-1.59	0.016	Nucleus	Gene expression	K.QEYLKALR.K K.NPDEFYYKMTR.V
1077	P07737	Profilin-1	8.44	15219	7/21	45	73/56	1.72	0.0049	Cytoplasm	Cytoskeleton organization	K.TDKTLVLLMGK.E K.CYEMASHLR.R
1489	P07737	Profilin-1	8.44	15219	7/21	47	80/56	1.36	0.047	Cytoplasm	Cytoskeleton organization	K.CYEMASHLR.R K.CYEMASHLR.R
274	P30101	Protein disulfide-isomerase A3	5.98	57146	14/44	20	80/56	-2.08	0.000031	Endoplasmic reticulum	Protein folding	K.TVAYTEQK.M K.KFLDAGHK.L
1494	P30101	Protein disulfide-isomerase A3	5.98	57146	7/20	15	97/56	1.75	0.03	Endoplasmic reticulum	Protein folding	K.TVAYTEQK.M R.GFPTIYFSPANK.K
1513	Q6ZVW7	Putative interleukin-17 receptor E-like	8.74	38516	5/10	16	65/56	-1.55	0.019	Plasma membrane	Cytokine receptor	K.RFTCEDAGAPVR.V K.FSTSWGSWVR.C
910	Q69YJ1	Putative pleckstrin homology domain-containing family M member 1P	5.93	60303	4/7	6	63/56	-1.3	0.05	Cytoplasm	Signal transduction	R.CESVGPAHSDGR.F MGLSNPFQGLMK.L
1008	P11177	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	6.20	39550	7/22	20	88/56	-1.36	0.05	Cytoplasm	Glycolysis	K.EGVECEVINMR.T K.VVSPWNSEDAK.G
302	Q14964	Ras-related protein Rab-39A	7.57	25390	5/18	31	61/56	1.61	0.015	Plasma membrane	Transport	R.FPGLRSPACDPTVGVDFFSR.L R.SITRSYYR.N
556	Q15293	Reticulocalbin-1	4.86	328866	6/17	25	67/56	1.41	0.049	Endoplasmic reticulum	Calcium-dependent activities	R.YIFDNVAK.V R.HWILPQDYDHAQAEAR.H
126	Q96QB1	Rho GTPase-activating protein 7	5.98	172196	8/11	6	73/56	1.38	0.045	Cytoplasm	Cytoskeleton organization	K.LTYMCRVDLR.G K.QLLNSAVIAQQR.R
1289	Q13103	Secreted phosphoprotein 24	8.59	24607	4/4	15	71/56	-1.51	0.032	Secreted	Bone remodeling	M.ISRMEK.M R.DYYVSTAVCRSTVK.V
710	Q8TDX7	Serine/threonine-protein kinase Nek7	8.49	34985	4/11	16	62/56	-1.33	0.03	Nucleus	Cell cycle regulation	K.VQIFDLMDAK.A K.QLNHPNVIK.Y
41	P02787	Serotransferrin	6.81	79249	16/59	20	101/56	1.44	0.071	Secreted	Transport	R.APNHAVVTR.K K.DSAHGFLK.V
51	P02787	Serotransferrin	6.81	79249	8/17	18	79/56	1.57	0.011	Secreted	Transport	K.SDNCEDTPEAGYFAIAVVK.K R.SAGWNIPIGLLYCDLPEPR.K
65	P02787	Serotransferrin	6.81	79249	11/27	16	92/56	1.6	0.012	Secreted	Transport	K.DSAHGFLK.V K.YLGEEYVK.A
68	P02787	Serotransferrin	6.81	79249	9/21	13	80/56	1.53	0.05	Secreted	Transport	K.NPDPWAK.N K.YLGEEYVK.A
93	Q9P157	Serum albumin	5.92	71371	12/39	20	80/56	2.37	0.0023	Secreted	Transport	K.VHTECCHGDLLECADDR.A K.DVFLGMFLYEYAR.R

Table 1 (*continued*)

No.	Swiss-Prot No.	Protein name	pI	MW	No. Match. Peptide	Cov. (%)	Score	Down syndrome / Normal Control	T-test	Subcellular location	Functional Ontology	Match peptide Sequences
131	Q9P157	Serum albumin	5.92	71371	10/30	18	96/56	1.87	0.0044	Secreted	Transport	K.VFDEFKPLVEEPQNLIK.Q K.EFNAETFTFHADICTLSEK.E
91	Q9P157	Serum albumin	5.92	71371	12/24	20	111/56	1.69	0.044	Secreted	Transport	K.YLYEIAR.R K.DDNPNLPR.L
111	Q9P157	Serum albumin	5.92	71371	11/29	20	86/56	1.4	0.05	Secreted	Transport	K.DVFLGMFLYEYAR.R K.SLHTLFGDK.L
964	P02743	Serum amyloid P-component	6.1	25485	4/9	20	71/56	1.42	0.031	Secreted	Transport	R.VGEYSLYIGR.H K.IVLGQEQDSYGGK.F
1259	P30626	Sorcin	5.32	21947	9/26	33	85/56	-2.53	9.8E-06	Cytoplasm	Calcium homeostasis	K.ALTTMGFR.L R.ALTDSFR.R
1280	Q7Z698	Sprouty-related, EVH1 domain-containing protein 2 / SPRED2	6.32	48125	6/11	20	72/56	1.37	0.032	Cytoplasm	Signal transduction	R.EKIWMTGYEDYR.HR.ACYHCGVMCRCCGGK.H
233	Q8N0S2	Synaptonemal complex central element protein 1	5.89	40074	6/16	20	66/56	1.43	0.0024	Nucleus	Cell cycle regulation	R.VEVLINR.I K.EICALDSSK.E
1043	A6NH17	TBC1 domain family member 3-like protein LOC729837	9.14	62905	5/5	5	66/56	2.34	0.013	Cytoplasm	Signal transduction	K.HLRASMK.K K.RSSEHIQR.I
275	P78371	T-complex protein 1 subunit beta	6.01	57794	11/33	24	86/56	1.3	0.05	Cytoplasm	Protein folding	K.LAVEAVLR.L R.AAHSEGNTTAGLDMR.E
1297	O95881	Thioredoxin domain-containing protein 12	5.24	19365	5/15	38	96/56	1.69	0.021	Endoplasmic reticulum	Redox regulation	K.GFGDHIHWR.T K.DEDFSPDGGYIPR.I
1382	Q9BV79	Trans-2-enoyl-CoA reductase, mitochondrial	8.99	40778	4/6	8	58/56	1.93	0.019	Mitochondrion	Lipid metabolism	M.WVCSTLWR.V R.GFWLSQWKK.D
1424	P02766	Transthyretin	5.52	15991	3/4	18	65/56	1.74	0.038	Secreted	Transport	R.GSPAINVAVHVFR.K K.AADDTWEPFASGK.T
279	P32241	Vasoactive intestinal polypeptide receptor 1	8.52	52462	5/17	14	70/56	-1.68	0.0037	Plasma membrane	Signal transduction	K.AASLDEQQTMFYGSVK.T K.MWDNLTCWPATPR.G
303	Q8N850	Vimentin	5.06	53676	25/63	52	170/56	2.53	0.0012	Cytoplasm	Cytoskeleton	R.FLEQQNK.IK.MALDIEIATYR.K
294	Q8N850	Vimentin	5.06	53676	20/26	37	236/56	2.18	0.0017	Cytoplasm	Cytoskeleton	R.SVSSSSYR.R R.FLEQQNK.I
324	Q8N850	Vimentin	5.06	53676	21/37	43	212/56	1.47	0.0024	Cytoplasm	Cytoskeleton	R.LLQDSVDFSLADAINTEFK.N K.MALDIEIATYR.K
433	Q8N850	Vimentin	5.06	53676	14/36	29	103/56	-1.56	0.017	Cytoplasm	Cytoskeleton	R.FANYIDKVR.F K.FADLSEAANR.N
614	Q8N850	Vimentin	5.06	53676	11/31	27	89/56	1.75	0.023	Cytoplasm	Cytoskeleton	K.NLQEAEEWYK.S R.ISLPLPNFSSLNLR.E
264	Q8N850	Vimentin	5.06	53676	15/52	26	93/56	-1.6	0.036	Cytoplasm	Cytoskeleton	K.QESTEYR.R K.QESTEYR.R
279	Q8N850	Vimentin	5.06	53676	29/53	54	288/56	-1.68	0.037	Cytoplasm	Cytoskeleton	R.EEAENTLQSFR.Q R.LQDEIQNMKEEMAR.H
403	Q8N850	Vimentin	5.06	53676	15/45	30	98/56	-1.55	0.046	Cytoplasm	Cytoskeleton	K.VESLQEEIAFLKK.L R.FANYIDK.V
990	P45880	Voltage-dependent anion-selective channel protein 2	7.49	32060	7/30	35	100/56	-1.49	0.0097	Mitochondrion	Ion transport	K.GFGFGLVK.L K.VCEDLDTSVNLAWTSGTNCTR.F
429	A6NLU5	V-set and transmembrane domain-containing protein 2B	7.88	30393	5/16	27	66/56	-1.4	0.001	Plasma membrane	Signal transduction	R.EGDDIEMPCAFR.A R.QRHGSGTGR.S
831	Q53GI3	Zinc finger protein 394	8.14	65741	8/14	11	86/56	-1.35	0.0045	Nucleus	Gene expression	K.QRSDLFK.H R.HQRIHQNK.V
287	Q53GI3	Zinc finger protein 394	8.14	65741	7/27	19	60/56	1.48	0.024	Nucleus	Gene expression	R.THTGEKPYTCLK.C MNSSLTAQRR.G
1704	Q9H5H4	Zinc finger protein 768	8.27	61445	4/8	7	56/56	2.56	0.0056	Nucleus	Gene expression	R.THSGQKPYK.C K.AFGDSSYLLR.H

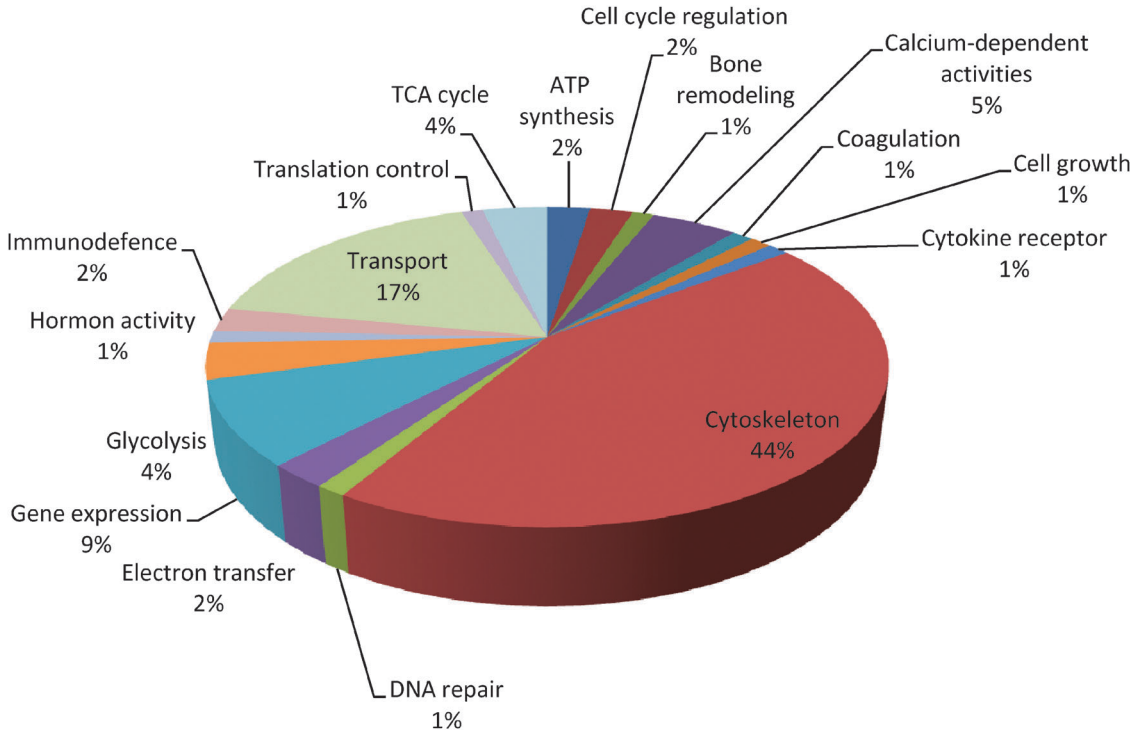

Fig. 3 Functional classification of differentially expressed placental proteins between Down syndrome fetus and normal fetus identified by 2D-DIGE/MALDI-TOF MS.

However, the dominant problem with plasma is its complexity and the large dynamic range of individual protein concentrations in plasma. The highly abundant proteins are likely to mask low-abundancy proteins and complicate their detection. In addition, amniotic fluid provides important information for the developing fetus. Its composition is complex and contains fetal and maternal proteins, amino acids, carbohydrates, hormones and lipids, which are directly secreted from the fetus. Thus it is a source to discover disease-specific biomarkers. However, the drawbacks of amniotic fluid analysis are mainly due to its invasive property. In present study, the placenta has been chosen to investigate Down syndrome biomarkers because it has the same genetic background as the fetus and has the capacity to secrete important biomolecules such as hormones, fetal proteins and growth factors into the maternal circulation system.[3,18–21] Thus, it is rational to investigate fetal disease biomarkers in placentas followed by monitoring the fetal diseases *via* discovery of disease markers in the maternal circulation system, such as serum.

During pregnancy there is a continuing accumulation of glycogen as well as a large amount of lipids in the placentas.[22] Lipid and glycogen are well known to disturb IEF-separation resulting in few spots resolved on 2D-PAGE. Thus, we used trichloroacetic acid/acetone to precipitate, desalt and enrich the placental proteins to make them well resolved by 2D-PAGE. Our results demonstrated that this strategy was not suitable for good preparation and separation of the placental proteins. 2D-DIGE/MALDI-TOF analysis revealed 101 altered expressions of placental proteins corresponding to 80 unique proteins (Table 1). The majority of altered proteins belong to two major functional groups, cytoskeleton structure and regulation and transport (Fig. 3). Of these, alpha-1-antitrypsin,[23] alpha-crystallin,[24] spolipoprotein A–I,[25] cathepsin D,[26] heat shock protein beta-1,[27] malate dehydrogenase,[28] myelin protein,[29] peroxiredoxin-2,[30] peroxiredoxin-6,[31] serum amyloid P-component,[32] T-complex protein 1,[33] transthyretin,[34] vimentin[35] and VDAC2[36] have been reported as Down syndrome markers in previous studies. In contrast, Ankyrin repeat and SOCS box protein 17, annexin A2, annexin A5, ataxin-3, calreticulin, chorionic somatomammotropin hormone, cyclophilin B, DNA repair protein XRCC2, envoplakin-like protein, eosinophil lysophospholipase, flavin reductase, G protein-coupled receptor kinase 4, galectin-1, interferon-induced guanylate-binding protein 2, cytoskeletal 8/18, keratin-like protein KRT222, killer cell lectin-like receptor, lactoylglutathione lyase, LMW phosphotyrosine protein phosphatase, MAGUK p55, microspherule protein 1, mitochondrial RNase P protein 2, profilin-1, protein disulfide-isomerase, reticulocalbin-1, protein kinase Nek7, sorcin, SPRED2 and vasoactive intestinal polypeptide receptor 1 have not been previously reported as Down syndrome markers to the best of our knowledge. Further investigation indicated that the combination of these identified proteins have not yet been described as Down syndrome markers. Accordingly, the combination of these identified proteins might be further evaluated as Down syndrome specific markers.

Ataxin-3, one of the proteins identified in this study, shows an up-regulation in Down syndrome placentas. This protein is encoded by the *ATXN3* gene on chromosome 14 in humans. Ataxin-3 has been found to co-localize with Down syndrome critical region 1 (DSCR1) which is widely expressed in many neurodegenerative diseases and causes microtubule-dependent aggresome-like inclusion body formation.[37] Hence, we propose ataxin-3 might play roles in neurodegeneration (including Down syndrome) and modulate the aggregation of abnormal peptides in the pathogenesis of the diseases. The increase level

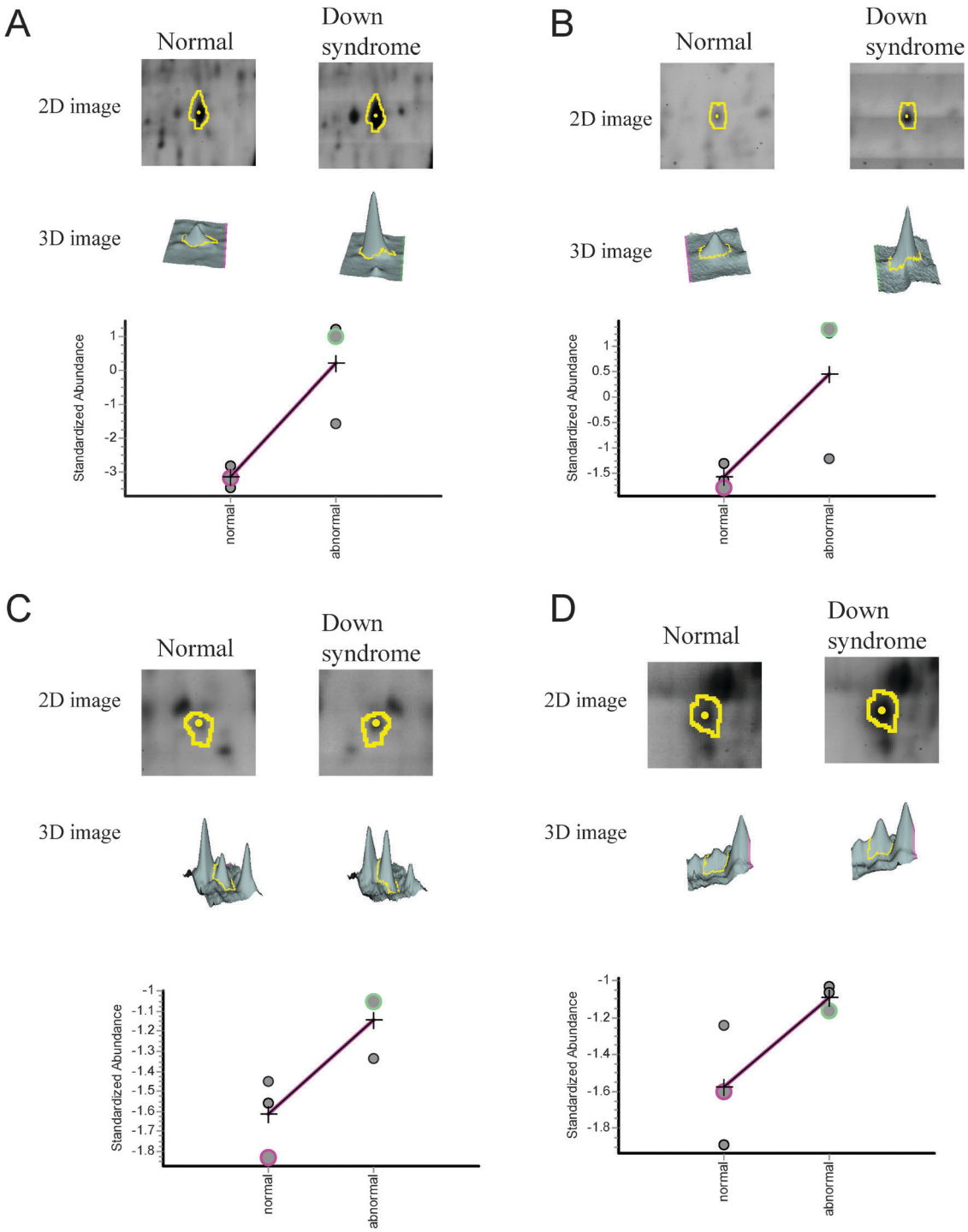

Fig. 4 Differential abundance of placental proteins ((A) apolipoprotein A–I, (B) transthyretin, (C) serum amyloid P-component and (D) reticulocalbin-1) between Down syndrome fetus and normal fetus.

of ataxin-3 might be further evaluated as a marker for Down syndrome or other neurodegenerative diseases in clinical study.

Sprouty-related EVH1 domain-containing protein 2 (SPRED2) containing a cysteine-rich domain has been shown to inhibit the Ras/ERK signaling pathway. A recent study indicated that SPRED2 directly associated with DYRK1A kinase, which is strongly linked to Down syndrome in humans since DYRK1A is localized in the Down syndrome critical region of chromosome 21. SPRED2 inhibits the activity of DYRK1A through the phosphorylation of its substrates, Tau and STAT3. This inhibition occurs *via* a protein–protein interaction of the CRD repeats of the SPRED2 with the kinase domain of DYRK1A.[38] The up-regulation of SPRED2 in proteomic, immunoblotting and ELISA analysis suggests the contribution of SPRED2 in Down syndrome and should be further evaluated as a marker for Down syndrome.

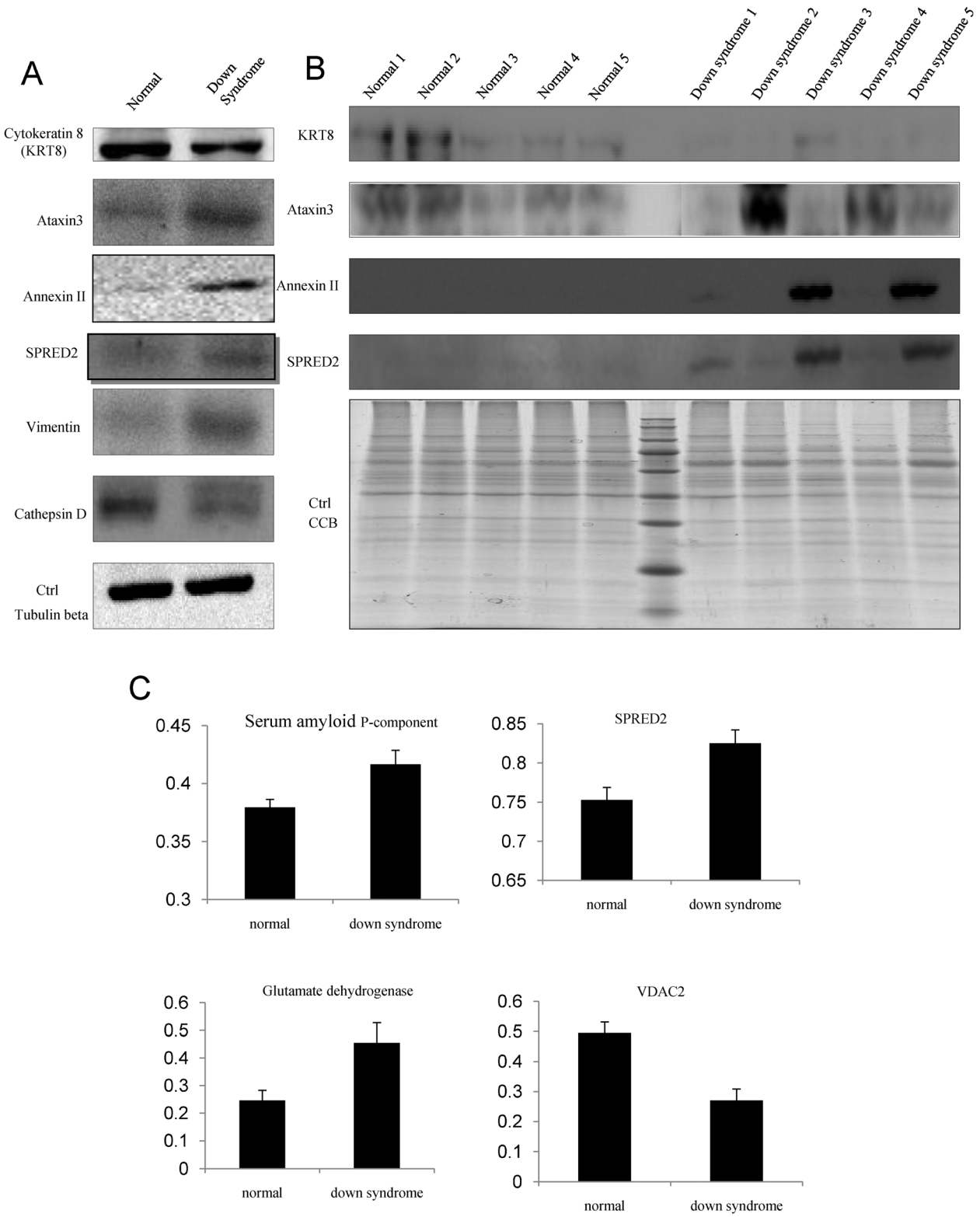

Fig. 5 Representative immunoblotting and ELISA analysis of cytokeratin 8, ataxin-3, annexin 2, vimentin, cathepsin D, SPRED2, serum amyloid P-component, glutamate dehydrogenase and VDAC2 for selected differentially expressed proteins identified by placenta proteomic analysis in between Down syndrome fetus and normal fetus. (A) 20 μg of the pooled placental proteins were loaded and resolved by SDS-PAGE followed by immunoblotted with cytokeratin 8, ataxin-3, annexin 2, SPRED2, vimentin and cathepsin D and blotted with tubulin beta as an internal loading control. (B) 20 μg of the individual placental proteins were loaded and resolved by SDS-PAGE followed by immunoblotted with cytokeratin 8, ataxin-3, annexin 2 and SPRED2. The individual placental proteins were resolved by corresponding SDS-PAGE followed by staining with colloidal coomassie blue G-250 as an internal loading control. (C) The levels of serum amyloid P-component, SPRED2, glutamate dehydrogenase and VDAC2 were confirmed by ELISA.

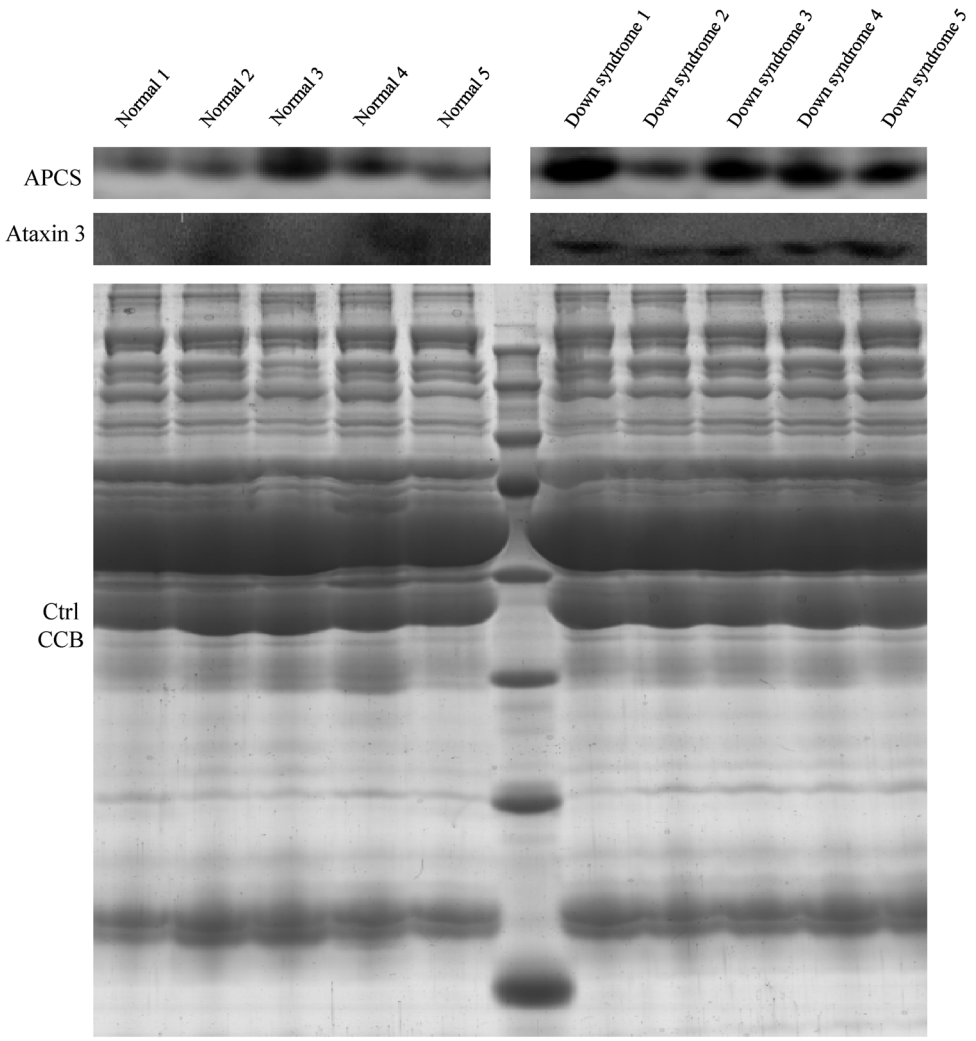

Fig. 6 Representative immunoblotting analysis of identified proteins APCS and ataxin-3 in maternal peripheral plasma. 5 blood samples were obtained from normal pregnancies and 5 blood samples were obtained from Down syndrome affected pregnancies in the 16–18 week gestation period. 20 μg of plasma were loaded and resolved by SDS-PAGE followed by immunoblotted with APCS and ataxin-3 or stained with colloidal coomassie blue G-250 as an internal loading control.

Down syndrome is linked to developmental disorders of the central nervous system that lead to Alzheimer-type neurodegeneration and mental retardation. Some of the neurodegenerative symptoms such as β-amyloid deposition and abnormal dendritic arborization are almost identical between Alzheimer's disease and Down syndrome.[39–41] In the current study, galectin-1, a member of carbohydrate-binding proteins with an affinity for β-galactosides, is over-expressed in our proteomic analysis and has been reported to induce neuronal process degeneration including Alzheimer's neurodegeneration.[42] Hence, although there is no straightforward evidence demonstrating the relationship between galectin-1 and Down syndrome, we propose galectin-1 might involve Alzheimer-type neurodegeneration in Down syndrome. Further large-scale studies are required to demonstrate the potential of galectin-1 as a marker for Down syndrome.

Our 2D-DIGE experiment is based on fluorescence-based quantitation which can detect sub-nanograms of dye-labeled proteins; however, our post-staining experiment is based on modified colloidal coomassie blue staining with a sensitivity around 10–50 nanograms.[43] For this reason, numerous differentially expressed dye-labeled low-abundancy placental proteins can be captured by the fluorescence scanner but failed to be imaged with colloidal coomassie blue staining. This is the reason why only 60% of 174 differentially expressed features on 2-DE can be picked for MALDI-TOF analysis with only 95 placental proteins identified.

Notably, we have reported a proteomics-based approach to obtain differentially expressed proteins between male and female aminocytes.[44] The results demonstrated that 28 unique proteins were differentially expressed between male and female amniocytes from three independent batches of amniotic fluid, in which 4 identified proteins, including 78 kDa glucose-regulated protein, actin, cytoplasmic 1, cathepsin D and vimentin, were identified in the current Down syndrome study. Hence, it is essential to discriminate the differential expression of these proteins owing to gender-induced changes or Down syndrome-induced alterations prior to confirming these proteins as Down syndrome associated disease signatures.

In conclusion, the quantitative placental proteomics analysis provided a valuable approach for Down syndrome research.

Our quantitative proteomic approach has identified numerous previously reported disease markers of Down syndrome. Additionally, we have presented several putative Down syndrome biomarkers which may be associated with the progression and development of the disease and has a potential to serve as a useful tool for monitoring the course of the disease in convenient sources such as serum. The potential of utilizing these markers for screening and treating Down syndrome warrants further investigation.

Abbreviations

2-DE	two-dimensional gel electrophoresis
Ab	antibody
CCB	colloidal coomassie blue
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)
ddH_2O	double deionized water
DIGE	differential gel electrophoresis
DTT	dithiothreitol
MALDI-TOF MS	matrix assisted laser desorption ionization-time of flight mass spectrometry
TFA	trifluoroacetic acid

Declaration of competing interests

The authors confirm that there are no conflicts of interest.

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