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Comparative Proteomic Analysis of Human Amniotic Fluid Supernatants with Down Syndrome Using Mass Spectrometry

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Down syndrome (DS) is an abnormality of the 21st chromosome that commonly occurs in children born to older women. Thus, amniotic fluid (AF) is usually collected from such women for prenatal diagnosis. This study analyzed human AF supernatants (AFS) using a mass spectrometric (MS) approach to search for candidate biomarkers of a DS pregnancy. The AFS were collected from older pregnant women at weeks 16–18 of their gestation by amniocentesis for cytogenetic analysis. The AFS from the pregnancies carrying DS (n=4) or chromosomally normal (n=6) fetuses, as revealed by the cytogenetic analysis, were then subjected to global protein profiling based on liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS). Affinity chromatography was also applied prior to the LC–ESI–MS/MS to minimize the masking effect of highly abundant albumin and immunoglobulin and thereby increase the diversity of the identified proteins. As a result, at least 30 new AFS proteins were identified and 44 AFS proteins were found to be differentially expressed between the DS and normal cases, where 6 of the proteins were unique to the DS cases and 11 were unique to the chromosomally normal cases. In addition, in the DS cases, 19 AFS proteins were downregulated and 8 were upregulated to varying degrees. A Western blot analysis confirmed the LC–ESI–MS/MS data, indicating that the combined detection of apolipoprotein A-II (apo A-II) and alpha-fetoprotein (AFP) could be a potential tool for diagnosing DS cases.

Keywords: Proteomics, biomarker, Down syndrome, amniotic fluid, liquid chromatography–electrospray ionization–mass spectrometry.

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Down syndrome (DS) is an abnormality of the 21st chromosome in humans and is characterized by impaired cognitive ability, physical growth, facial appearance, and gonadal function in both male and females [5, 16, 32, 36, 39]. Reported to occur in about 1 in 800 live births, the incidence increases with an increase in the maternal age [15, 17, 52]. DS offspring are generally infertile or subfertile, although there are a few reports of live births from DS men [32, 39].

DS is usually diagnosed by screening the maternal serum for alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG), and dimeric inhibin A, with ~22–25% false-negative and ~7.5–8.5% false-positive rates [4]. In families with an increased chance of having a DS child, more invasive techniques, such as amniocentesis, chorionic villus sampling, or percutaneous umbilical cord blood sampling, are employed for the diagnosis. Analysis of the amniotic fluid (AF), obtained by amniocentesis, also facilitates prenatal genetic diagnosis and can reveal inflammatory conditions affecting the pregnancy. Moreover, proteomic profiling of the AF has been shown to be useful in detecting inflammation, infection, and neonatal sepsis [6, 7], along with the identification of specific biomarkers and diagnostic profiling of pathophysiologic conditions of the pregnancy, such as Rh(–) incompatibility [13], congenital diaphragmatic hernias [35], and premature rupture of the membranes [35, 42, 47].

Proteomic profiles of human AF have already been generated by several research groups using different approaches [2, 25, 31 44]. Park *et al*. [31] and Tsangaris *et al*. [44] applied two-dimensional gel electrophoresis (2DE) combined with matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS/MS) to the AF supernatant (AFS) to create a proteome map of human AFS. As a result, Tsangaris *et al*. [48] found that several proteins were differentially expressed between the AFS from pregnancies with DS fetuses and the AFS from pregnancies with chromosomally normal fetuses, and that these proteins may be used as potential markers for a prenatal diagnosis of DS. MALDI–TOF identifies proteins by matching a list of experimental peptide masses with a calculated list of all the peptide masses for each entry in a database, a method known as peptide-mass mapping or peptide-mass fingerprinting [1]. However, since this technique requires an essentially purified target protein, it is commonly used in conjunction with prior protein fractionation using 2DE. However, this imposes limitations on separating proteins with high or low molecular masses, extreme pIs (particularly basic proteins), high hydrophobic proteins, and proteins of low abundance [9, 22]. As a result, the combined 2DE and MALDI–TOF tends to identify and overrepresent proteins that are more hydrophilic, owing to the precipitation of less soluble proteins. In addition, the narrow dynamic range of a 2DE gel makes it difficult to identify less abundant and hydrophobic proteins.

Meanwhile, protein identification using the collision-induced dissociation (CID) spectra of peptides from electrospray ionization–tandem MS (ESI–MS/MS) coupled to liquid chromatography (LC) is more clear-cut than mass mapping, as the peak pattern in the CID spectrum also provides information about the peptide sequence [1]. Moreover, pre-fractionation of the cellular proteins prior to liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) by one-dimensional (1D) SDS–PAGE adds another dimension to increase the depth and range of the identified proteins. LC–MS/MS also allows protein identification in a high-throughput fashion, unlike the rather slow and laborious 2DE–MALDI–MS/MS methods [2], and was recently shown, by the present authors, to be very effective for new cell surface biomarker discovery in cancer cells [20] and protein profiling of embryonic samples that are available in limited amounts [12]. Furthermore, Cho *et al*. [2] and Michaels *et al*. [25] reported an increased range of identified proteins in human AFS when using LC–MS/MS combined with SDS–PAGE. Notwithstanding, the whole proteome catalog of human AF remains incomplete and thus new proteins are regularly added to existing databases [2, 25].

Accordingly, this study applied a 1D–LC–ESI–MS/MS approach to extend the existing information on the protein composition of human AFS. To increase the chances of identifying low abundant proteins, affinity chromatography was applied prior to LC–ESI–MS/MS to remove highly abundant proteins, such as albumin and immunoglobulin. In addition, a comparison of the protein profiles of the AFS from pregnancies with DS fetuses and those from pregnancies with chromosomally normal fetuses identified differentially expressed proteins that may be potential biomarkers for DS diagnosis.

Materials and Methods

Materials

The albumin and IgG removal kit was purchased from GE Healthcare (Piscataway, NJ, U.S.A.) and the Coomassie assay reagents were obtained from Pierce (Rockford, IL, U.S.A.). The sequencing-grade modified trypsin used for the in-solution digestion was purchased from Promega (Madison, WI, U.S.A.), and the acetonitrile (ACN, HPLC grade), water (HPLC grade), and formic acid (ACS regent grade) were all purchased from Aldrich (Milwaukee, WI, U.S.A.).

Sample Preparation

This study was approved by the Institutional Review Board of Kangnam CHA Hospital, Pochon CHA University. The human AF samples (10 ml) were obtained based on written informed consent from 10 women undergoing a routine amniocentesis for genetic karyotyping at gestational weeks 16–18. The samples were centrifuged at 1,800 rpm for 10 min to collect the amniocytes for cytogenetic analysis, and the AFS stored at $-70^{o}C$ until further analysis. Four samples came from pregnancies shown by a conventional cytogenetic analysis to have a fetus with DS, and six came from pregnancies with a chromosomally normal fetus. The total protein concentration of the AFS was determined using a Bradford protein assay. To minimize the masking effects caused by highly abundant proteins, affinity chromatography was applied using an albumin and IgG depletion kit to remove the albumin and IgG as per the manufacturer's instructions. Briefly, 15 µl of a sample was loaded into 700 µl of the fully suspended resin supplied with the kit, and the resin/sample were mixed for 30 min at room temperature on a rotary shaker. Following incubation, the mixture was dispensed into the upper chamber of a microspin column and centrifuged at 6,500 ×*g* for 5 min. The eluted sample was then precipitated with acetone, resolved on 4~20% gradient SDS–PAGE, and stained with Coomassie blue, as previously described [20].

Enzymatic Digestion and LC–ESI–MS/MS Analysis

The gel digestion and LC–ESI–MS/MS analysis were performed as previously described with modifications [28]. Briefly, the Coomassie blue-stained protein lanes on the SDS–PAGE gel were manually cut into five sections and subjected to in-gel tryptic digestion. The tryptic digests were recovered by extraction with 50% ACN/0.1% formic acid, and the isolated peptides lyophilized and solubilized in 5% ACN/0.1% formic acid. Microcapillary reversed-phase HPLC was performed using an Agilent 1100 Series capillary LC system (Agilent Technologies, Inc., Palo Alto, CA, U.S.A.) coupled to an XCT ultra-ion-trap mass spectrometer (Agilent Technologies Inc.). The samples were applied to a C18 trapping column (0.3×5 mm, Zorvax) in line with the analysis capillary column (Zorbax 300CB-

C18, 150×0.075 mm i.d, 3.5 μm, Agilent Technologies, Inc.) connected to the mass spectrometer. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in ACN. After injecting a sample onto the column, according to the gradient program, the mobile phase was held at 5% B for 20 min, followed by a linear gradient to 70% B over 70 min, and then a linear gradient to 85% B over 45 min at a flow rate of 250 nl/min. The XCT ultra was operated in the data-dependent MS/MS mode, where a full MS scan was followed by three MS/MS scans with an electrospray voltage of 2.0 kV and normalized collision energy setting of 35%. The dynamic exclusion duration was set at 180 s.

Peptide Identification

The data files were extracted using a Spectrum Mill Data Extractor with parameters of [MH+] 600 to 40,000 and a minimum signal-to-noise (S/N) ratio of 25. Searches of the human NCBInr database were performed in both forward and reverse directions using the Spectrum Mill program (Agilent Technologies) with the following parameters: specific to trypsin with two missed cleavages; ±2.5 Da precursor-ion tolerance; and ±0.7 Da fragment-ion tolerance. The initial results were autovalidated using the following parameters for the "protein details" mode: SPI (scored percent intensity) >70% for matches with a score >7 for +1, >9 for +2, >9 for +3, >8 for +4, and SPI>90% for a score >6 for +1. The proteins were summarized for the validated peptides using the following parameters: >13 for the protein score; SPI>70% for a score >9 for a peptide; and each protein included at least 2 distinct peptide hits.

Semiquantitative Analysis

A semiquantitative analysis of the protein profile data was performed by comparing the total peptide intensity with the peptides of an identified protein. The total peptide intensity was obtained by summing up the peptide intensities of the peptide hits for the protein. The false-positive rates were calculated as described previously [34]. The data were filtered to classify the proteins that showed at least 3-fold changes compared with the normalized ratio in the pathological condition.

Western Blot Analysis

To confirm the protein expression, an immunoblot analysis of the proteins in the AFS samples was performed as described previously [45] with modifications. Briefly, equal amounts of the proteins from the AFS samples were resolved on 4–12% NuPAGE gradient SDS–PAGE, and electrotransferred to a nitrocellulose membrane (Invitrogen, San Diego, CA, U.S.A.). The blots were then incubated overnight at 4°C with specific rabbit polyclonal antibody against apolipoprotein A-II (1:5,000 dilution; Abcam, Cambridge, U.K.) or alpha-fetoprotein (1:200 dilution; Abcam, Cambridge, U.K.) after blocking with 3% nonfat milk in TBS. A horseradish peroxidase-conjugated anti-rabbit IgG secondary polyclonal antibody (Abcam, Cambridge, UK) was used at a dilution of 1:5,000 to capture the bound primary antibody. The immune complexes were detected using an ECL Western blotting detection system (GE Healthcare Amersham Bioscience) and monitored with a LAS-1000 (Fuji Film, Tokyo, Japan).

Statistical Analysis

The Student's *t*-test was used to analyze the statistical differences. A probability value of less than 0.05 ($p<0.05$) was considered significant.

RESULTS AND DISCUSSION

Protein Composition of Human AFS

A proteomic analysis of the AFS is the first step to elucidate any changes related to pathological conditions during pregnancy. Although proteomic profiles of human AFS have already been generated by several research groups using different approaches [2, 25, 26, 29, 31, 44], a collective profile of AF proteins has not yet been assembled, and little is known regarding the possible molecular interactions of proteins and their contribution to fetal development and reproductive disorders. This problem originates from the complex nature of AF that contains high-abundance proteins, such as albumin, which limit the detection of other low-abundance proteins. However, the identification of more AF proteins is desirable to explore their functions within the AF [2]. Thus, to extend the existing information on the protein composition of human AFS, this study combined the LC–ESI–MS/MS approach with 1D SDS–PAGE [20]. Affinity chromatography was also used prior to the LC–ESI–MS/MS to remove highly abundant proteins, such as albumin and immunoglobulin [51]. Furthermore, all the samples from normal pregnancies and pregnancies showing DS were respectively pooled to minimize an individual variability of protein expression. Using this approach, 88 proteins were identified that mainly included extracellular proteins, although some cellular proteins were also identified. The average false-positive rate was 1.06 % for the Spectrum–Mill-validated positive peptides. The total number of identified AFS proteins in this study was higher than that in the previous studies of Liberatori *et al*. [21], Nilsson *et al*. [29], Park *et al*. [31], and Vascotto *et al*. [46], which reported the identification of 26, 58, 37, and 26 AFS proteins, respectively. The details of the proteins identified in this study are provided in supplementary Table 1. The details of the MS-generated spectra and peptide sequences for the individual proteins are also available from the authors upon request.

Among the proteins identified in this study, 44 proteins had 0.0% false-positive rates and five were also identified by Nilsson *et al*. [29] using LC–Fourier transform–ion cyclotron resonance MS (LC/ESI–FTICR–MS) with albumin depletion. The proteins identified in the current study also included 15 out of the 37 and 26 AFS proteins identified by Park *et al*. [31] and Vascotto *et al*. [46], respectively, using 2D–MALDI-MS/MS and 11 out of the 26 AFS proteins identified by Liberatori *et al*. [21] using an immunoblot assay. The AFS proteins identified for the first time in this study are highlighted with bold type in Table 1. The increased number of AFS proteins identified in this study may be ascribed to the combined methodology and depletion of the highly abundant albumin and IgG proteins. The depletion of albumin from the AFS to identify more proteins was first used by

Nilsson *et al*. [29] in combination with LC/ESI−FTICR−MS. However, Cho *et al*. [2] suggested that the "sponge effect" of these major proteins, especially of albumin, could lead to a simultaneous loss of the proteins bound to these major proteins. Thus, the loss of some low-abundance proteins that may be valuable biomarkers cannot be ruled out. Nonetheless, the number of proteins identified in this study was lower than that reported by Tsangaris *et al*. [44], who identified 136 proteins in human AFS when using 2DE−MALDI−MS/MS. This difference probably arose because Tsangaris *et al*. [44] conducted their peptide search using a collective protein database, thereby identifying many non-human proteins from human AFS.

The identified AF proteins were also annotated into a biological process and subcellular location categories based on the Gene Ontology (GO) database (http://www.geneontology.org/) using a house-made FindGo program (available from the authors upon request). As shown in Fig.1A, the identified proteins mainly included extracellular proteins found in the extracellular space and region (58%), extracellular matrix (8%), or collagen (8%). The presence of these extracellular proteins is quite obvious. Some cellular proteins were also found in the cytoplasm (6%) or membrane (3%). These cellular proteins are likely to result from cell lysis or cell component release [42, 44, 47]. The distribution of the identified proteins in the biological process is shown in Fig. 1B. It was evident that the majority of the identified proteins were related to cellular transport (22%), immune response (14%), and metabolism/catabolism (10%), whereas others were related to homeostasis/circulation (5%), cell adhesion (5%), acute-phase response (4%), development (3%), complement activation (3%), cell proliferation (3%), stimulus response (2%), signal transduction (2%), and biosynthesis (2%). However, the biological function

Table 1. Identification of human amniotic fluid supernatant (AFS) proteins by LC−ESI−MS/MS.
A. Human AFS proteins unique to either Down syndrome (DS) or chromosomally normal (normal) cases.

Unique in DS cases

Total intensity[a]		Unique Peps No[b].		Unique score[c]		Percent coverage		Accession number	Entry name
DS	Normal	DS	Normal	DS	Normal	DS	Normal		
5.61E+09	0.00E+00	5	0	71.6	0	45	0	4504349	**Beta-globin**
5.01E+09	0.00E+00	4	0	60.83	0	36	0	13650074	Hemoglobin alpha-1 globin chain
4.90E+08	0.00E+00	4	0	49.49	0	15	0	4503271	**Decorin isoform-a preproprotein**
5.42E+08	0.00E+00	3	0	40.13	0	2	0	219510	**Collagen alpha 1(V) chain precursor**
6.81E+08	0.00E+00	2	0	26.15	0	2	0	62088774	**Collagen alpha 1 chain precursor variant**
2.95E+08	0.00E+00	2	0	25.41	0	4	0	5031863	**Galectin-3 binding protein**

Unique in chromosomally normal cases

Total intensity		Unique Peps No.		Unique score		Percent coverage		Accession number	Entry name
DS	Normal	DS	Normal	DS	Normal	DS	Normal		
0.00E+00	3.44E+09	0	3	0	46.91	0	9	11321561	**Hemopexin**
0.00E+00	1.95E+09	0	4	0	45.84	0	13	2765425	**Immunoglobulin lambda heavy chain**
0.00E+00	4.57E+08	0	3	0	44.15	0	3	1418928	**Prepro-alpha1(I) collagen**
0.00E+00	4.66E+08	0	2	0	32.33	0	0	55665909	**Heparan sulfate proteoglycan 2 (perlecan)**
0.00E+00	1.24E+09	0	2	0	31.16	0	18	21669387	**Immunoglobulin kappa light-chain VLJ region**
0.00E+00	1.69E+08	0	2	0	28.36	0	21	671882	**Apolipoprotein-AII**
0.00E+00	9.22E+07	0	2	0	23.64	0	10	18088326	**Retinol-binding protein 4, plasma**
0.00E+00	5.94E+08	0	2	0	22.54	0	8	21669637	**Immunoglobulin lambda light-chain VLJ region**
0.00E+00	7.30E+08	0	2	0	22.25	0	12	119239	**Bone-marrow proteoglycan precursor (BMPG) (Proteoglycan 2)**
0.00E+00	1.08E+08	0	2	0	21.99	0	2	68533033	**C4A variant protein**
0.00E+00	1.83E+08	0	2	0	19.68	0	2	62988777	**Collagen, type III, alpha 1 preproprotein**

[a] The total peptide intensity was obtained by summing up the peptide intensities of the peptide hits for the protein.
[b] Numbers of validated peptides assigned to the corresponding proteins.
[c] For confident identification, scores should be higher than 13, as explained in the Materials and Methods section.

Table 1. B. Human AFS proteins differentially expressed between Down syndrome (DS) and chromosomally normal (normal) cases.

Total intensity		Ratio (DS/Normal)	Unique Peps No.		Unique score		Percent coverage		Accession number	Entry name
DS	Normal		DS	Normal	DS	Normal	DS	Normal		
Downregulated proteins in DS										
3.01E+09	2.10E+09	0.70	2	2	31.48	24.74	19	19	179318	Beta-2-microglobulin
4.18E+08	2.96E+08	0.71	3	3	34.87	34.62	14	13	39725934	**Serine (or cysteine) proteinase inhibitor, clade F**
5.09E+09	4.14E+09	0.81	3	4	39.19	46.8	15	20	20070760	**Orosomucoid 1**
3.85E+09	3.37E+09	0.88	7	6	110.59	94.1	21	19	1340142	Alpha1-antichymotrypsin
1.27E+09	1.13E+09	0.89	5	4	73.25	70.24	10	9	25058739	ALB protein
Upregulated proteins in DS										
9.86E+08	1.07E+09	1.09	2	3	22.34	32.99	8	10	2521983	Alpha2-HS glycoprotein
7.80E+08	8.57E+08	1.10	2	2	31.6	26.46	15	15	55962673	**Prostaglandin D2 synthase, 21 kDa (brain)**
1.36E+09	1.24E+09	1.10	4	4	48.25	54	11	13	4502261	**Serine (or cysteine) proteinase inhibitor, clade C**
9.79E+10	1.08E+11	1.10	20	22	325.96	340.39	54	58	15080499	**Serpin peptidase inhibitor, clade A**
8.74E+09	9.74E+09	1.11	5	4	70.61	50.95	12	12	32609	**IGF-binding preprotein**
1.47E+09	1.66E+09	1.13	6	6	80.94	93.87	15	15	62089238	**Plasma protease C1 inhibitor precursor variant**
1.04E+10	1.22E+10	1.17	10	10	141.4	149.05	30	31	4505047	**Lumican precursor**
1.97E+08	2.43E+08	1.23	2	2	19.42	27.59	11	12	627578	**Prolactin precursor, placental (clone 204)**
3.78E+10	4.91E+10	1.30	19	23	286.1	345.72	24	35	4557871	Transferrin
3.87E+09	5.06E+09	1.31	8	6	117.89	102.6	22	17	46577680	Alpha-1B-glycoprotein
2.26E+09	2.97E+09	1.31	4	7	72.81	109.45	14	20	62089124	Angiotensinogen precursor variant
5.08E+08	6.88E+08	1.35	2	4	25.56	54.42	10	21	38026	Zn-alpha2-glycoprotein
1.81E+10	2.58E+10	1.43	27	37	451.23	589.6	18	25	34364820	**Fibronectin precursor**
3.96E+09	5.91E+09	1.49	3	3	46.69	48.66	11	11	4502067	Alpha-1-microglobulin/bikunin
5.78E+08	9.28E+08	1.61	3	4	35.16	49.65	4	10	4507467	**Transforming growth factor, beta-induced, 68 kDa**
3.56E+09	5.86E+09	1.65	10	13	141.48	197.4	14	18	4557485	Ceruloplasmin precursor
7.96E+09	1.42E+10	1.78	8	10	120.13	153.33	19	29	34785355	Group-specific component (vitamin D binding protein)
3.86E+09	8.23E+09	2.13	10	9	130.95	135.76	35	34	4557321	Apolipoprotein A-I preproprotein
2.99E+09	6.40E+09	2.14	3	3	49.81	51.05	22	22	4884164	**Glycodelin precursor**
6.61E+08	1.51E+09	2.28	4	17	50	192.4	3	15	116594	**Complement C3 precursor**
2.33E+09	5.55E+09	2.38	6	7	99.69	126.03	49	65	4507725	Transthyretin
4.15E+08	2.27E+09	5.47	3	8	37.56	99.09	8	18	4501989	Alpha-fetoprotein

of only 22% of the identified proteins was ascertained from the GO database, and thus it is likely they may have unique functions in human AFS that are as yet unknown.

Altered Protein Profile of Human AFS in DS Cases

As AFS proteins are produced and secreted by either the fetus or the placenta as the pregnancy progresses, their expression pattern may be a reflection of pathologies, such as Down syndrome, intra-amniotic infection, preterm delivery, pre-eclampsia, neonatal sepsis, and premature rupture of the membrane [6, 7, 13, 35, 42, 47]. The methodology used in this study allowed the assessment of 44 proteins that were differentially expressed between the pregnancies with DS fetuses and chromosomally normal fetuses (Table 1). In the DS cases, 19 of these proteins were downregulated and 8 were upregulated to varying degrees (Table 1B). Moreover, 6 proteins were only detected in the AFS from the cases with DS, whereas 11 proteins were only detected in the AFS from the cases with chromosomally normal fetuses (Table 1A). Among these proteins, 14 were already identified in AFS in previous studies; however, 30 proteins (highlighted in bold type, in Table 1) were detected for the first time in this study and may be potential biomarkers. The genes encoding many of these proteins were located in chromosomes other than chromosome 21, and their activation was possibly due to the overexpression of transcription

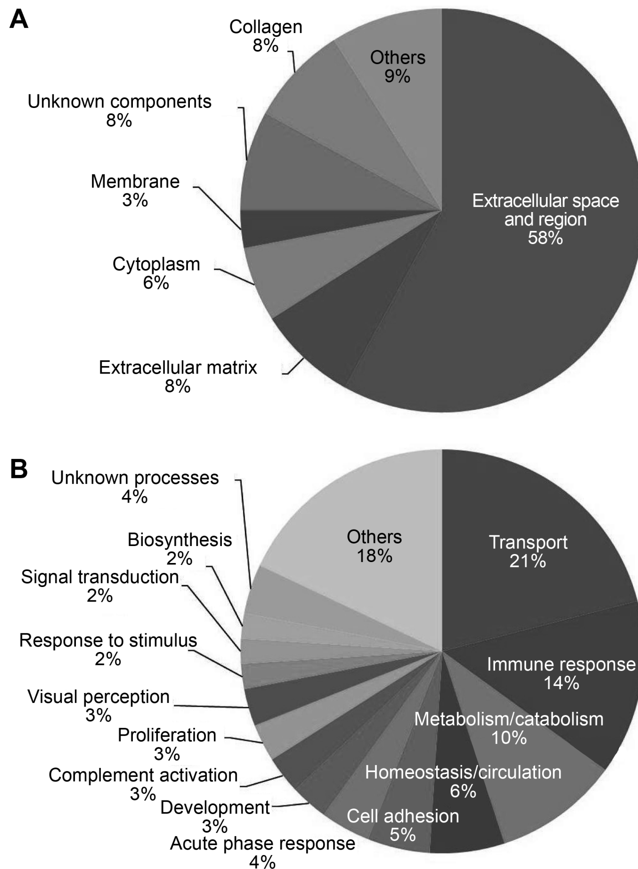

Fig. 1. Gene ontology (GO) annotation of human amniotic fluid supernatant (AFS) proteins.

The identified proteins were classified based on the subcellular location (**A**) and biological process (**B**). The extracellular space and region refer to the outermost structure of a cell, and the extracellular matrix refers to any substances produced by the cells and excreted into the extracellular space serving as a scaffolding to maintain the tissues together.

factors located in chromosome 21 or some indirect mechanism [3, 10, 44].

The present study also identified many proteins that are putative or current biomarkers for DS diagnosis: AFP, alpha-1-microglobulin, collagen $\alpha1$, heparin sulfate proteoglycan, and insulin-like growth factor binding protein (IBP) [43]. Consistent with published reports, an increased expression of AFP and collagen $\alpha1$ and a decreased expression of IBP were observed in the AFS from the pregnancies with DS fetuses. However, no upregulation of heparin sulfate proteoglycan was found in the AFS from the DS samples, whereas Tsangaris *et al*. [43] reported an increased expression of this protein in a fragmented form. The IBP acts as a regulator of fetal growth by modulating the effects of insulin-like growth factors I and II (IGF I and II), which have an important role in growth, development, metabolism, and apoptosis. IBP is highly expressed in early pregnancy, increases rapidly during gestation, and then declines postnatally. However, elevated maternal serum levels of IBP have been previously reported in pregnancies complicated by fetal growth failure, fetuses carrying trisomy 18, and premature rupture of the membrane [18, 19, 27, 37, 49]. Thus, IBP is a potential candidate for diagnosing a DS pregnancy through AFS analysis, although it would still need to be differentially diagnosed for other reproductive disorders or non-pregnancy.

Apart from published reports on up- or downregulated proteins in the AFS from DS cases, the current study detected at least 30 new AFS proteins that were differentially expressed between the two groups (Table 1). Among the newly identified proteins in the AFS from the DS cases, several proteins are important to the physiology of a pregnancy, such as decorin, hemopexin, and proteoglycan-2 proform. Decorin is a 130–150 kDa proteoglycan, which has a 46 kDa protein core and approximately 90 kDa dermatan sulfate chain [28]. This proteoglycan contains a cysteine-rich N-terminal segment followed by multiple leucine-rich repeats in the core proteins [14, 33, 50] that bind extracellular matrix components, such as collagens, fillamin A, and TGF-beta [11, 38]. It has already been shown that the high-affinity interaction between decorin and surfactant protein D in amniotic fluid samples may be related to inflammatory responses in pregnant women [28]. Meanwhile, hemopexin is a plasma beta-glycoprotein that specifically binds to heme and plays an important role in heme transport and catabolism [40, 41]. This glycoprotein has been proposed to be an important carrier protein that maintains metal-ion homeostasis [23]. In this study, the expression level of proteoglycan-2 proform was observed to be abundant in the AFS from the normal pregnancies. In the serum samples collected from the pregnant women, the proform existed as a complex with several proteins, such as pregnancy-associated plasma protein-A, AGT, and C3dg [8, 30].

Among the various AFS proteins, AFP has probably been the most actively investigated, as a change in its concentration in the maternal serum or AFS correlates with several abnormalities of the fetus, including Down syndrome [4, 43, 48]. This study also found that the expression of AFP was significantly decreased in the AFS from the DS cases. The decreased expression of AFP in the DS cases was confirmed by Western blotting (Fig. 2). In addition, the expression of apolipoprotein A-II (apo A-II) was also evaluated and found to be downregulated in the AFS from the pregnancies with DS fetuses. Increased apolipoprotein in the AFS influences the triacylglycerol levels by controlling the lipoprotein lipase activity, and a high concentration in the plasma is related to hyperlipidemia during pregnancy [24]. Indeed, the Western blotting revealed that the expression level of apo A-II was significantly downregulated in the AFS from the DS cases, further confirming the LC–ESI–

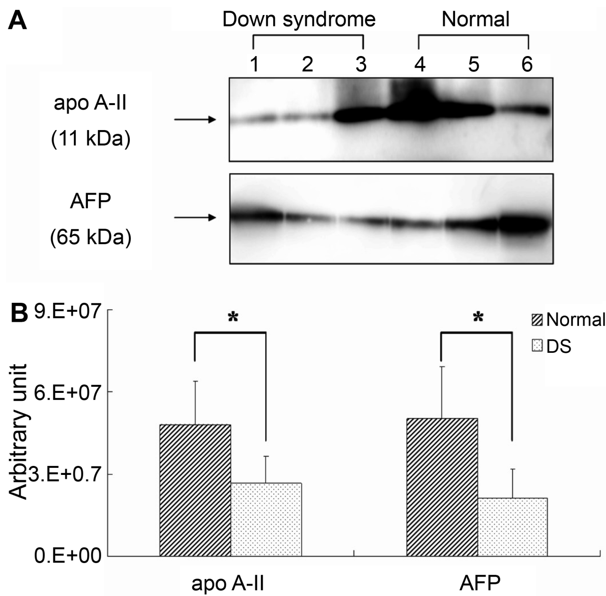

Fig. 2. Determination of differential expression for apo A-II and AFP (alpha-fetoprotein) proteins by Western blotting.

The Western blotting analysis was performed on equal amounts of human amniotic fluid supernatants (AFS) from pregnancies with Down syndrome fetuses (dotted bar) or chromosomally normal fetuses (crossed bar). (**A**) Western blot; (**B**) Quantification of protein content of apo A-II and AFP using densitometry. Values are expressed as mean ± SEM. The asterisk (*) denotes statistical significance ($p<0.05$).

MS/MS data (Fig. 2B). While variations in the decrease were noted, the expressions of AFP and apo A-II were generally decreased in the AFS from the DS cases (Fig. 2A). Therefore, this result suggests that a battery of tests would be more ideal for diagnosing DS by an AFS analysis, rather than a single-protein analysis. Thus, the combined detection of apo A-II and AFP proteins could work as a combined biomarker for diagnosing DS from AFS.

In conclusion, when using a modified proteomic approach based on a LC–ESI–MS/MS platform, new AFS proteins were identified that could contribute to a better understanding of the molecular mechanism of AF proteins. The present results also reconfirmed that the global protein profile is altered in the AFS from DS cases. Several proteins that were differentially expressed in the AFS from DS cases may be potential biomarkers. In particular, the present results suggest that the combined detection of apo A-II and AFP could be a possible tool for diagnosing DS cases. However, further studies are needed to investigate the clinical utility of these identified protein biomarkers through systematic analysis of the maternal serum.

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