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Amniotic Fluid Proteome Analysis from Down Syndrome Pregnancies for Biomarker Discovery

Chan-Kyung J. Cho,[†,‡] Christopher R. Smith,[§] and Eleftherios P. Diamandis*[,†,‡,§]

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada, and Department of Clinical Biochemistry, University Health Network, Toronto, Ontario, Canada

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Down syndrome (DS) is an anomaly caused by an extra chromosome 21, and it affects 1 in 750 live births. Phenotypes include cognitive impairment, congenital defects, and increased risk for several diseases such as Alzheimer's disease and leukemia. Current DS-screening tests subject many women to invasive procedures for accurate diagnosis due to insufficient specificity. Since amniotic fluid (AF) surrounds the developing fetus, understanding the changes in AF composition in the presence of DS may provide insights into genotype−phenotype associations, and aid in discovery of novel biomarkers for better screening. On the basis of our previous study, in which we reported an extensive proteome of AF, we performed two-dimensional liquid chromatography followed by MS/MS to analyze triplicates of pooled AF of chromosomally normal and DS-affected pregnancies (10 samples per pool). A total of 542 proteins were identified from the two sets of triplicate analyses by the LTQ-Orbitrap mass spectrometer and data were compared semiquantitatively by spectral counting. Candidate biomarkers were selected based on the spectral count differences between the two conditions after normalization. Comparison between the two groups revealed 60 candidates that showed greater than 2-fold increase or decrease in concentration in the presence of DS. Among these candidates, amyloid precursor protein and tenascin-C were verified by ELISA, and both showed a 2-fold increase, on average, in DS-AF samples compared to controls. All proteins that showed significant differences between the two conditions were bioinformatically analyzed to preliminarily understand their biological implications in DS.

Keywords: Down syndrome • prenatal screening • amniotic fluid • spectral counting • APP • TNC • hCG

Introduction

Down syndrome (DS), also known as Trisomy 21, is the most common chromosomal anomaly, and it constitutes the most common congenital cause of intellectual disability in live-born infants. DS phenotype varies from individual to individual, and only few phenotypic features, such as cognitive impairment from birth and early onset Alzheimer's disease, are common in all affected patients. Individuals with DS suffer from many congenital malformations, such as heart defects. Patients are also at higher risk for a number of noncongenital diseases, such as transient or acute megakaryocytic leukemia. DS incidence ranges from 1 to 2 per 1000 live births; however, this rate underestimates the true incidence since it does not include induced and spontaneous abortions.[1] Currently, maternal serum screening tests for DS involve multiple serum markers, such as PAPP-A, β-hCG, AFP, and inhibin. Combined with ultrasonography, the integrated test can provide detection rate of 90−95% at a 5% false positive rate.[2,3] Such integrated tests, however, require procedures at both the first and second gestational trimesters, and their relatively low specificity produces many false-positive results that require further invasive procedures such as amniocentesis. Thus, this study was a first step to discover additional biomarkers to improve current screening, and to identify proteins involved in fetal development that are dysregulated due to the presence of DS, for possible therapeutic applications.

Amniotic fluid (AF) provides both physical and biochemical support for the developing fetus. Its composition is complex and includes fetal and maternal proteins, amino acids, carbohydrates, hormones, lipids, and electrolytes. Since AF is in direct contact with multiple organs of the fetus, AF contains high concentrations of proteins that are directly secreted from the fetus.[4] Not surprisingly, most of the currently used and putative biomarkers for pregnancy-related pathologies, such as premature rupture of amnion, intra-amniotic infection, and aneuploidies, have been identified in our extensive proteomic analysis of chromosomally normal AF samples by mass spectrometry.[5] More specifically, the list of current and putative biomarkers found in the AF proteome included β-hCG chain, AFP, and inhibin A, which are clinically used serum screening markers for Down syndrome, as well as many proteins that

* Corresponding author, Dr. E. P. Diamandis, Department of Pathology and Laboratory Medicine. Mount Sinai Hospital, 60 Murray Street, Toronto, Ontario, M5T 3L9, Canada Tel: 416-586-8443, 416-619-5521, Email: ediamandis@mtsinai.on.ca.
[†] University of Toronto.
[‡] Mount Sinai Hospital.
[§] University Health Network.

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Table 1. Clinical Characteristics of Controls and Subjects (Fetuses) with DS

sample	karyotype	gestation	sample	karyotype	gestation
DS1	47, XY, +21	17.5	**CN1**	46, XY	17.5
DS2	47, XX, +21	16	**CN2**	46, XX	16
DS3	47, XX, +21	19.3	**CN3**	46, XX	18.2
DS4	47, XY, +21	19.6	**CN4**	46, XX	19.2
DS5	47, XX, +21	17	**CN5**	46, XX	18
DS6	47, XY, +21	18.5	**CN6**	46, XY	18.2
DS7	47, XX, +21	16.5	**CN7**	46, XY	16
DS8	47, XY, +21	16.3	**CN8**	46, XY	16.6
DS9	47, XX, +21	18.6	**CN9**	46, XX	18.5
DS10	47, XY, +21	19.2	**CN10**	46, XX	18

have been previously reported to exhibit differential expression in DS samples.

In the pursuit of discovery of novel biomarkers for Down syndrome, two groups analyzed the AF proteome and another two groups analyzed the plasma proteome from chromosomally normal and DS pregnancies. Tsangaris et al. resolved AF proteins by two-dimensional electrophoresis (2DE), identifying 28 proteins in total from excised gels, and reported differential expression for 7 of them.[6] More recently, Wang et al. fractionated AF proteins by two-dimensional liquid chromatography (2D-LC) followed by tandem mass spectrometry (MS/MS), and tested some of the candidates by Western blots and ELISA.[7] Other groups chose to analyze maternal plasma directly, and reported candidate biomarkers based on 2DE analysis.[8,9] For the present study, AF was chosen rather than plasma, since the plasma proteome poses greater challenges due to its greater complexity, while containing significantly smaller amounts of fetal proteins than AF. Knowing that AF contains over 800 proteins, it was our aim to identify proteins with differential expression between chromosomally normal-AF (CN-AF) and Down syndrome-AF (DS-AF) by quantitatively comparing hundreds of proteins via a shotgun approach.

Here, we utilized a bottom-up 2D fractionation strategy, involving strong cation-exchange followed by reverse-phase LC fractionation followed by MS/MS, that was developed in our previous study.[5] We confirmed and expanded the existing human AF proteome by further characterization of CN-AF samples from the second-trimester. We qualitatively and quantitatively compared proteome results generated using Mascot and X!Tandem algorithms for CN- and DS-AF. This analysis resulted in the semiquantitation of 542 proteins that were identified by at least 2 unique peptides (95% peptide probability, 99% protein probability) in triplicates of pooled AF samples. Approximately 150 proteins showed significant concentration differences after normalization, and 60 proteins were selected as candidates for further verification. Two of these candidates have been further validated by enzyme-linked immunosorbent assays (ELISA) using individual AF samples, showing 2-fold increase in AF from DS pregnancies.

Materials and Methods

Sample Preparation. AF samples (8−10 mL) were obtained by amniocentesis from second-trimester women (16−20th week of gestation), ranging from 30 to 45 years of age with no additional exclusion criteria, after written informed consent and ethics board approval. Ten samples from CN pregnancies and 10 samples from pregnancies with DS-affected fetuses were randomly collected (Table 1). AF samples were centrifuged to isolate amniocytes for cytogenetic analysis, and the cell-free supernatants were stored immediately at −80 °C until use. Ten samples were pooled together using equal total protein amounts, based on a Coomassie (Bradford) total protein assay (Pierce), resulting in 6 mg of total protein in each pool. Pooled samples were vortexed and filtered through a 0.22 μm × 25 mm syringe-driven filter unit (Millipore). All samples were depleted for IgG with a Protein A/G column (Bio-Rad) as previously described.[5] Samples were subjected to dialysis at 4 °C using a molecular mass cutoff membrane of 3.5 kDa (Spectra/Por) for 12 h in 5 L of 1 mM ammonium bicarbonate buffer, with one buffer exchange. Each pool was divided into equal triplicates, resulting in 6 samples in total. Finally, maternal serum samples were obtained from pregnant women with DS ($n = 10$) or CN fetuses ($n = 15$) for validation by immunoassays.

Trypsin Digestion. Each lyophilized AF sample was denatured using 8 M urea, reduced with 200 mM dithiothreitol (DTT) at 50 °C, and alkylated in 500 mM iodoacetamide at room temperature in the dark. Each replicate was desalted using a NAP5 column (GE Healthcare), a Coomassie assay (Pierce) was performed to quantify total protein of each replicate, and the volumes were adjusted so that each contains equal amount of total protein before digestion into peptides. The samples were then lyophilized and resuspended in trypsin-containing buffer (1:50, trypsin/protein concentration; 120 μL of 50 mM ammonium bicarbonate; 100 μL of methanol; 150 μL of H_2O) and digested overnight at 37 °C (Promega, sequencing grade modified porcine trypsin). The samples were lyophilized to dryness and resuspended in 120 μL of mobile phase A buffer (0.26 M formic acid in 10% acetonitrile (ACN)) to be injected into the HPLC system.

Strong Cation-Exchange Liquid Chromatography (SCX). The digested peptides, in 120 μL of 0.26 M formic acid in 10% ACN (mobile phase A), were directly loaded onto a PolySULFOETHYL A column (The Nest Group, Inc.) containing hydrophilic anionic polymer (poly-2-sulfethyl aspartamide) with a pore size of 200 Å and a diameter of 5 μm. Fractionation was performed using a high-performance liquid chromatography (HPLC) system (Agilent 1100) for 1 h at a flow rate of 200 ÅL/min. Ammonium formate (1 M) and 0.26 M formic acid in 10% ACN (mobile phase B) were then used in a linear gradient. A protein cation-exchange standard (Bio-Rad) was applied before each run to ensure optimal column performance. The eluent was monitored by UV absorbance at 280 nm. A total of 10 fractions were collected between 20% and 60% of mobile phase B gradient, and were lyophilized to dryness.

Online Reversed Phase Liquid Chromatography−Tandem Mass Spectrometry. Each fraction was resuspended in 80 μL of 95% water, 0.1% formic acid, 5% ACN, 0.02% trifluoroacetic acid (Buffer A) and desalted using a $ZipTiP_{C18}$ pipet tip (Millipore). The peptides were eluted in 4 μL of 90% ACN, 0.1% formic acid, 10% water, 0.02% trifluoroacetic acid (Buffer B) and 80 μL of Buffer A was added on top. Half of this volume (40 μL) was loaded on an Agilent 1100 HPLC by the autosampler onto a 2 cm C18 trap column (inner diameter 200 μm) and the peptides were eluted onto a resolving 5 cm analytical C18 column (inner diameter 75 μm). The samples were loaded at 15 μL/min for 5 min, then the 103 min gradient was run at 400 nL/min (split from 4 μL/min) starting from 0 to 40% B, followed by 4 min linear gradient to 65%, and finally to 100% B for 1 min. The peptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in an LTQ Orbitrap XL (Thermo, Inc.) coupled online to the HPLC. Data files were created by the Mascot Daemon (version 2.2) and

Extract_MSn, and the parameters were: 300 Da minimum mass; 4000 Da maximum mass; automatic precursor charge selection; 10 minimum peaks per MS/MS scan; and 1 minimum scan per group.

Database Searching and Bioinformatics. The resulting spectra from each fraction were searched separately against the International Protein Index human database V3.46 (72 079 entries), by two database search engines: Mascot (version 2.2, Matrix Science) and X!Tandem (v2006.06.01.2 GPM, Beavis Informatics Ltd.). The following parameters were used: (i) enzyme, trypsin; (ii) one missed cleavage allowed; (iii) fixed modification, carbamidomethylation of cysteines; (iv) variable modification, oxidation of methionine; (v) peptide tolerance, 7 ppm; (vi) MS/MS tolerance, 0.4 Da. The files were run on Scaffold v02_00_00 (Proteome Software). All DAT files (from Mascot) and all XML files (from GPM) that were searched for all fractions from a single chromatography run (HPLC or FPLC) were loaded together as one "biological sample" within Scaffold with the collation and MudPIT options. Since the aim of this project is quantitative comparison rather than thorough profiling of the AF proteome, stringent cutoffs of 95% peptide identification probability, 99% protein identification probability, and 2 or more unique peptides were used to exclude any false-positive protein identifications from the Scaffold output.

The sample reports were exported to MS Excel from Scaffold, and relevant information and annotations for each protein were searched from databases including Swiss-Prot, Human Protein Reference Database, Entrez Gene, the Plasma Proteome Database, and other related literature. The total number of spectra for each protein per condition was generated using 'normalized number of assigned spectra' option. The false-positive error rate was calculated based on the search result against a concatanated IPI Human forward/reverse database. The false-positive rate (FPR) was calculated as: FPR = no. of false peptides/(no. of true peptides + no. of false peptides).

Measurement of Two Candidates in Individual AF and Serum. The concentration of APP and Tenascin-C was measured using sandwich ELISA with a Human APP ELISA kit (US Biological, MA) and a Tenascin-C Large (FNIII-B) Assay kit (Immuno-Biological Laboratories), respectively. Individual AF samples, which were used to create the pools for MS/MS analysis ($n = 10$ per group; CN or DS), as well as an additional eight, randomly selected CN-AF samples, were measured, along with unmatched serum samples (true positives and true negatives by cytogenetic analysis). A 100-fold dilution of the samples was prepared for both AF and serum samples to measure APP, and a 400-fold dilution was prepared for both AF and serum for TNC-C assay. Both ELISAs were performed according to the instructions provided with the kit, and results were analyzed using GraphPAD Prism (GraphPAD Software, San Diego, CA). Statistical analysis was performed using one-factorial analysis of variance (ANOVA) and independent samples t test. Values of $p < 0.05$ were considered significant.

Results

Characterization of Normal and Trisomy 21-Affected Amniotic Fluid Proteomes. AF, like most of biological fluids, contains high-abundance proteins that complicate proteomic analysis by hindering identification of low-abundance proteins. Albumin and immunoglobulin families together represent over 70% of total proteins in AF, and our previous experiments[5] showed improved identification of low-abundance proteins after immunoglobulin depletion. Although various depletion columns for high-abundance proteins are available, we chose to deplete immunoglobulins only, since this method allows larger amount of starting material and lower loss of low-abundance proteins due to "sponge effects".[5] From our previous study, the 2D-LC fractionation platform had the least variability without compromising identification efficiency, among three different fractionation methods. Therefore, pooled normal and DS-AF samples were digested, and the peptides were fractionated on an SCX column in triplicate, to increase confidence for low-abundance quantification. A complete list of proteins from both CN- and DS-AF samples was compiled via Scaffold, applying stringent cutoffs for three criteria: 95% peptide probability, 99% protein probability, and 2 or more unique peptides. A total of 475 proteins were identified from DS AF, and 462 from Normal AF, representing a combined list of 542 proteins (Figure 1a; Supplemental Data 1). The total number of unique peptides and percentage sequence coverage identified from MS/MS data for these 542 proteins are listed in Supplemental Data 4.

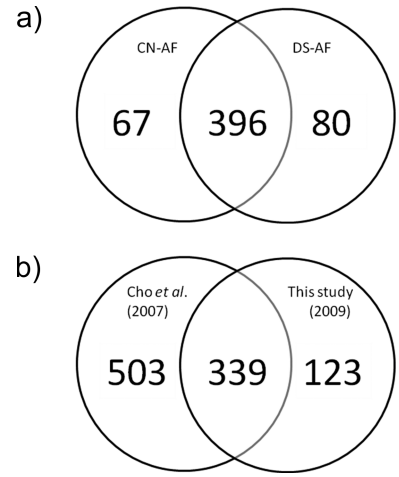

Figure 1. Venn diagrams of amniotic fluid proteome. (a) A total of 542 proteins were identified from CN-AF and DS-AF and quantified based on spectral counting, and 396 proteins were identified from both conditions. (b) This study identified 123 additional proteins from CN-AF, increasing the current number of human CN-AF proteins from 852 to 965.

Our previous study[5] used three different fractionation protocols, identifying 842 proteins from AF. Compared to our new list of Normal AF, 339 proteins were shown to overlap between the two data sets, and 123 proteins have been newly identified from CN-AF in this study, expanding the CN-AF proteome to 965 proteins (Figure 1b). When the newly identified proteins from DS-AF were added to this list, the number of newly identified proteins from human AF increases to 172, increasing the total number of AF proteins identified by our group to 1014. We searched databases to annotate these proteins for tissue expression, chromosomal origin, subcellular localization, and biological functions. Two out of the newly identified 172 proteins are located in human chromosome 21 (HSA21); ADAM metallopeptidase with thrombospondin type 1 motif (ADAMTS5) and pituitary tumor-transforming 1 interacting protein (PTTG1IP). Both were identified from DS-AF but not from CN-AF (Supplemental Data 1).

Quantitative Comparison of Normal versus Down Syndrome-AF Proteome. For the 542 proteins that were identified from CN- and DS-AF in this study, semiquantitative

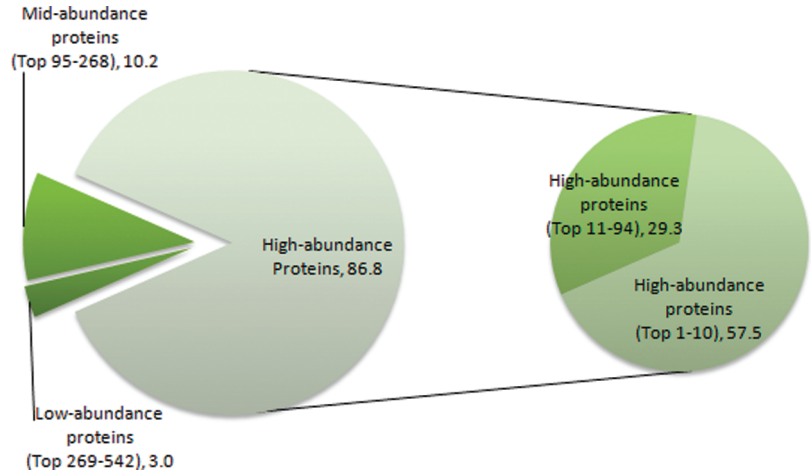

Figure 2. Classification of CN-AF proteins based on the number of spectra. The proteins identified by 20 or less total peptides (from triplicates) were considered as low-abundance; 21−99 total peptides as mid-abundance; and 100 or more peptides as high-abundance. Top 10 high-abundance proteins were separately represented to emphasize their proportion (57.5%) in AF proteome.

analysis was performed based on the ratio of the spectral counting of each protein for the two conditions using the cutoff of 2 or more unique peptides, 99% peptide probability, and 99% protein probability. The total spectral counting results were compiled by Scaffold and exported to an MS Excel file. The proteins were classified into three groups using two cutoffs of 20 and 99 (≤20, 21−99, and ≥100; triplicates combined) spectral counts, respectively, to estimate how many of the 542 proteins are of low-abundance (Figure 2). The high-abundance proteins that were identified by more than 100 spectra were subdivided based on rank, to top 10 high-abundance proteins and top 11−94 high-abundance proteins. As predicted, the vast majority of spectra (86.8%) belonged to the high-abundance proteins; 57.5% of the total number of spectra accounted for the top 10 proteins. A total of 174 of relatively mid-abundance proteins that were identified with number of spectra ranging from 21 to 99 constituted 10.2% of total spectra. The majority of the identified proteins belonged to the relatively low-abundance group. Although they represented more than half of the proteins (274), their spectra constituted only 3.0% of the total spectra.

The percentage abundance of each protein, for each condition, was calculated by dividing the number of spectra of a specific protein by the total number of spectra found in each condition. These values were then used to calculate the ratio between the two conditions and estimate the degree of differential expression. Excluding the proteins that are uniquely present in one condition (for which ratio could not be calculated), the frequency of all ratio values of the normalized spectral counting for 396 proteins was graphed (Figure 3). The plot shows that most of these proteins (327; 83%) fall within the range of 2-fold increase or decrease, and that only a small percentage of these proteins (17%) shows greater than 2-fold increase or decrease. Not represented in this histogram are the uniquely identified proteins for CN-AF (67) and DS-AF (80).

To reveal pathways that are potentially dysregulated due to the extra copy of HSA21, we generated two lists of proteins: one with proteins that are significantly decreased in DS-AF, and one with proteins that are significantly increased in DS-AF. We used Ingenuity Pathway Analysis v7.5 (Ingenuity Systems) to annotate 91 proteins (decreased in DS-AF) and 115 proteins (increased in DS-AF), respectively, on each list (Supplemental Data 2). Compared to the CN-AF proteome, over-

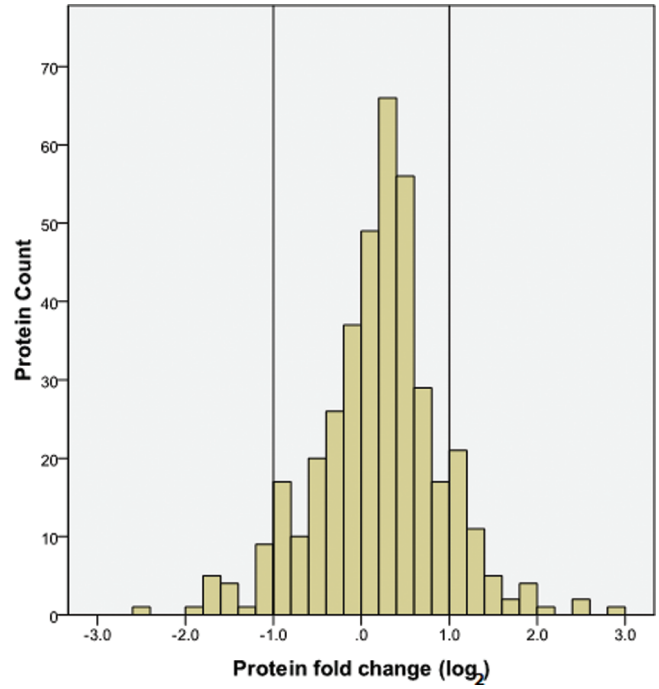

Figure 3. Histogram representing quantitative comparison of 396 proteins between CN- and DS-AF. The majority of proteins showed less than 2-fold increase or decrease as represented by the two reference lines.

represented pathways included protein degradation, hematopoiesis, organismal survival, reproductive system development and function, and nutritional disease, while underrepresented pathways were involved in ophthalmic and neurological processes (Supplemental Data 3). Finally, we looked at proteins encoded by chromosome 21 and examined the spectral counts to determine if the gene-dosage hypothesis is observed in the proteome of AF (Table 2). Out of 542 proteins that were quantified in this study, 7 proteins were encoded by chromosome 21, and all were identified with greater number of spectra from the DS-AF samples, compared to the number of spectra found in the CN-AF samples.

Candidate Selection. Out of 542 proteins, we selected proteins that show significant differential expression, potentially due to DS, and developed a list of proteins that are either:

Table 2. Seven Proteins from AF That Are Encoded on HSA21

gene	protein name	spectral count in DS-AF	spectral count in CN-AF	protein spectra/ total spectra in DS-AF (%)	protein spectra/ total spectra in CN-AF (%)	ratio %DS/%CN
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1	29	10	0.076	0.035	2.2
ADAMTS5	A disintegrin and metalloproteinase with thrombospondin motifs 5	5	0	0.013	0.000	
APP	Isoform APP770 of Amyloid beta A4 protein	13	8	0.034	0.028	1.2
COL6A1	Collagen alpha-1(VI) chain	188	101	0.491	0.349	1.4
COL6A2	Isoform 2C2 of Collagen alpha-2(VI) chain	13	6	0.034	0.021	1.6
PTTG1IP	Putative uncharacterized protein PTTG1IP	3	0	0.008	0.000	
TFF3	trefoil factor 3 precursor	7	0	0.018	0.000	

(1) uniquely found in DS-AF only, by 5 or more spectra; (2) uniquely found in CN-AF by 5 or more spectra; or (3) found in both DS- and CN-AF proteome by 10 or more combined spectra, while showing 2-fold or greater increase or decrease, based on the calculated ratios. From the list of 181 proteins that show significant differential expression between the two groups, we selected 60 candidate biomarkers based on a set of filtering criteria: (1) Only proteins that were identified by 10 or more spectra were retained, since low abundance of a protein in AF often indicates less confidence in quantification and lower representation of the specific fetal protein in maternal blood, making it less attractive as a candidate. (2) Proteins that show 2-fold or more increase, or 50% or more decrease were retained, with the exception of APP. Although APP showed only 63% increase in DS-AF by raw spectral counting, we decided to include it in our list of candidates since it is encoded by HSA21. (3) Since secreted or membrane proteins have higher potential as biomarkers, we additionally removed low-abundance intracellular proteins. (4) Immunoglobulins and proteins that were identified with peptides that displayed ambiguous matches were removed. (5) Finally, proteins that show no significant difference in the median number of spectra for the replicates, despite significant difference in the sum of spectra from all triplicates, were removed, resulting in a final list of 60 proteins (Table 3).

Preliminary Examination of Two Candidates by ELISA. From the list of 60 candidates, we selected two for further verification using a more sensitive and specific quantification method (ELISA): amyloid precursor protein (APP) and tenascin-C (TNC-C). APP was one of the candidates that were included in the final list of candidates, not because of its substantial increase in DS-AF, but due to location of the gene on chromosome 21. Annotated MS/MS spectra information for representative peptides for APP and TNC-C can be found in Supplementary Figures 1 and 2. To verify the spectral counting result, an increase of 63% in number of spectra in DS-AF, APP levels were measured in DS-AF samples ($n = 10$) and CN-AF samples ($n = 18$; 10 matched for gestational age and 8 randomly selected) by ELISA. The mean concentration of APP was 630 ng/mL in the CN group, and 1170 ng/mL in the DS group (Figure 4a). The mean concentration was significantly higher in the DS group than the CN group ($p < 0.001$). The same ELISA kit was used to measure APP levels in maternal serum samples from DS ($n = 8$) and CN ($n = 36$) pregnancies, but there was no statistically significant difference between the two groups.

TNC-C was selected for two reasons: it exhibited a relatively high DS to CN spectral count ratio of 2.9, and previous studies suggested that TNC-C may have an important role in development of multiple organs,[10−13] leading us to hypothesize that TNC-C may have a potential impact on the phenotype of DS. TNC-C levels were measured in the DS-AF samples ($n = 10$) and CN-AF samples ($n = 17$; 10 matched for gestational age and 7 randomly selected) by ELISA. The mean concentration of TNC-C was 274 ng/mL in the CN group, and 759 ng/mL in the DS group (Figure 4b). The mean concentration of TNC-C was significantly higher in the DS group than the CN group ($p = 0.004$). TNC-C levels were also measured in maternal serum samples from DS ($n = 11$) and CN ($n = 24$) pregnancies, using the same ELISA kit, but no statistically significant difference was found between the two groups.

Discussion

Maternal serum screening tests for DS have evolved through multiple biomarkers, leading to the current two-stage screening system: PAPP-A and β-hCG for the first trimester, and β-hCG, AFP, uE3, and inhibin for the second trimester.[14] Since the individual markers suffer from relatively low sensitivity and specificity, the "Integrated Test" that employs all of these markers, as well as nuchal translucency by ultrasonography, was developed to achieve a detection rate of 90% or higher, at a 5% false-positive rate.[15] However, even this "Integrated Test" has significant drawbacks. Five to 10% of pregnancies with DS will pass undetected. Also, based on disease prevalence and specificity, the vast majority (95−98%) of screen-positive mothers have a normal pregnancy (i.e., they are false-positives), and must be subjected to painful and risky invasive diagnostic procedures (such as amniocentesis) for verification. Therefore, the need to discover better biomarkers, to improve prenatal DS screening, still exists.

Recently, some alternative aneuploidy detection methods have been explored by several groups, based on fetal DNA, RNA, or cells that are found in the maternal circulation. For example, Lo et al. estimated the ratio between alleles of a single-nucleotide polymorphism (SNP) in PLAC4 mRNA from chromosome 21, and Dhallan et al. measured the ratio of unique fetal alleles based on SNPs.[16,17] Another new approach, shotgun sequencing of fetal DNA from maternal blood, also showed potential for a novel detection method with higher sensitivity than the Integrated Test.[18] Although these new methods hold promise, their utility is currently limited by factors such as cost, absence of certain polymorphisms in subpopulations, detection rate that is not superior to the Integrated Test, or insufficient fetal material to obtain consistently reliable results. Hence, discovery of novel biomarkers that can improve the sensitivity and specificity of the current integrated test could be an ideal solution for a cheap, accurate, fast, and noninvasive screening test.

Table 3. Top 60 Candidate Biomarkers for DS Based on Spectral Counting

gene	protein name	spectral count in CN-AF	spectral count in DS-AF	% in total spectra in CN-AF	% in total spectra in DS-AF	fold change
ABI3BP	Isoform 2 of Target of Nesh-SH3	4	11	0.014	0.029	2.1
ABP1	Isoform 1 of Amiloride-sensitive amine oxidase	3	15	0.010	0.039	3.8
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1	10	29	0.035	0.076	2.2
ANTXR1	Isoform 1 of Anthrax toxin receptor 1	8	5	0.028	0.013	−2.0
APP	Isoform APP770 of Amyloid beta A4 protein (Fragment)	8	13	0.034	0.028	−1.3
ATP6AP1	V-type proton ATPase subunit S1	3	8	0.010	0.021	2.0
C1RL	Complement C1r subcomponent-like protein	0	10	0.000	0.026	<−10.0
C8B	Complement component C8 beta chain	3	9	0.010	0.023	2.3
CA1	Carbonic anhydrase 1	7	36	0.024	0.094	3.9
CEL	carboxyl ester lipase precursor	30	0	0.104	0.000	<−30.0
CGB	Choriogonadotropin subunit beta	17	45	0.059	0.117	2.0
COL4A2	Collagen alpha-2(IV) chain	7	3	0.024	0.008	−3.3
COL5A2	Collagen alpha-2(V) chain	9	26	0.031	0.068	2.2
COMP	Cartilage oligomeric matrix protein	16	51	0.055	0.133	2.4
CPA1	Carboxypeptidase A1	14	9	0.048	0.023	−2.0
CRK	Isoform Crk-II of Proto-oncogene C-crk	7	4	0.024	0.010	−2.5
CRNN	Cornulin	3	9	0.010	0.023	2.3
CSPG4	Chondroitin sulfate proteoglycan 4	2	14	0.007	0.037	5.3
CST6	Cystatin-M	6	22	0.021	0.057	2.8
CTGF	Isoform 1 of Connective tissue growth factor	3	9	0.010	0.023	2.3
DPP4	Dipeptidyl peptidase 4	20	13	0.069	0.034	−2.0
FBN2	fibrillin 2 precursor	3	14	0.010	0.037	3.5
FLNA	Isoform 2 of Filamin-A	3	13	0.010	0.034	3.3
FOLH1	Isoform PSMA-1 of Glutamate carboxypeptidase 2	11	0	0.038	0.000	<−11.0
GC	vitamin D-binding protein precursor	10	34	0.035	0.089	2.6
GGT1	Isoform 1 of Gamma-glutamyltranspeptidase 1	17	11	0.059	0.029	−2.0
HAPLN1	Hyaluronan and proteoglycan link protein 1	16	8	0.055	0.021	−2.5
IGFBP5	Insulin-like growth factor-binding protein 5	10	0	0.035	0.000	<−10.0
LAMA5	Laminin subunit alpha-5	3	10	0.010	0.026	2.5
LDHB	L-lactate dehydrogenase B chain	4	13	0.014	0.034	2.5
MME	Neprilysin	10	4	0.035	0.010	−3.3
MMP2	72 kDa type IV collagenase	18	59	0.062	0.154	2.5
MUC13	Mucin-13	24	9	0.083	0.023	−3.3
MUC5AC	Mucin-5AC (Fragment)	63	39	0.217	0.102	−2.0
NID1	Isoform 1 of Nidogen-1	19	54	0.066	0.141	2.1
NPC2	cDNA FLJ59142, highly similar to Epididymal secretory protein E1	10	29	0.035	0.076	2.2
NRP1	Muscle type neuropilin 1	4	12	0.014	0.031	2.3
NT5E	5′-nucleotidase	11	7	0.038	0.018	−2.0
PDIA6	Isoform 2 of Protein disulfide-isomerase A6	3	10	0.010	0.026	2.5
PLOD1	cDNA FLJ59393, highly similar to Procollagen-lysine,2-oxoglutarate5-dioxygenase 1	7	21	0.024	0.055	2.3
PLUNC	Protein Plunc	9	30	0.031	0.078	2.5
PSG2	Pregnancy-specific beta-1-glycoprotein 2	3	11	0.010	0.029	2.8
PSG9	Pregnancy-specific beta-1-glycoprotein 9	7	20	0.024	0.052	2.2
SCGB3A2	Secretoglobin family 3A member 2	4	12	0.014	0.031	2.3
SERPINA5	Plasma serine protease inhibitor	0	14	0.000	0.037	>14.0
SERPINC1	SERPINC1 protein	9	28	0.031	0.073	2.4
SERPINE1	Plasminogen activator inhibitor 1	3	11	0.010	0.029	2.8
SFN	Isoform 1 of 14-3-3 protein sigma	3	10	0.010	0.026	2.5
SI	Sucrase-isomaltase, intestinal	130	60	0.449	0.157	−3.3
SPINK5	Serine protease inhibitor Kazal-type 5	5	17	0.017	0.044	2.6
SPRR1B	Cornifin-B	4	12	0.014	0.031	2.3
SPRR3	Small proline-rich protein 3	6	18	0.021	0.047	2.3
TAGLN2	Transgelin-2	2	11	0.007	0.029	4.2
TF	11 kDa protein	14	6	0.048	0.016	−3.3
TNC	Isoform 1 of Tenascin	13	49	0.045	0.128	2.8
TUBA1B	Tubulin alpha-1B chain	14	9	0.048	0.023	−2.0
TXN	Thioredoxin	2	9	0.007	0.023	3.4
VCAM1	Isoform 1 of Vascular cell adhesion protein 1	6	18	0.021	0.047	2.3
VCAN	Isoform V1 of Versican core protein	4	13	0.014	0.034	2.5
VCL	Isoform 1 of Vinculin	2	19	0.007	0.050	7.2

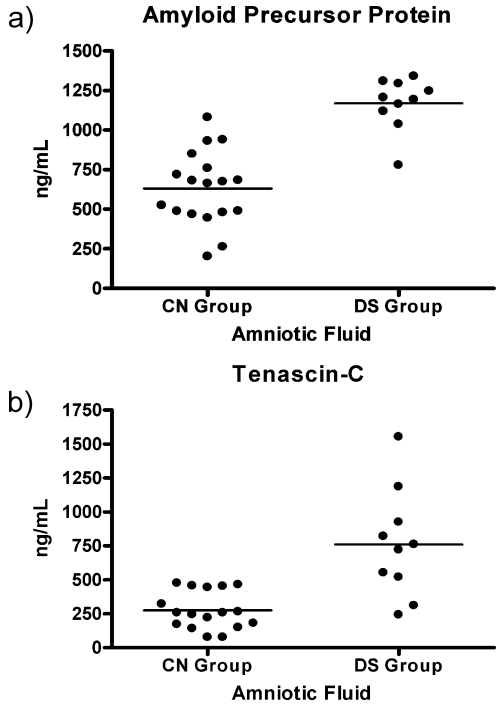

Figure 4. Verification of two candidates in amniotic fluid by ELISA. (a) APP; (b) TNC-C. CN = chromosomally normal and DS = Down syndrome pregnancies.

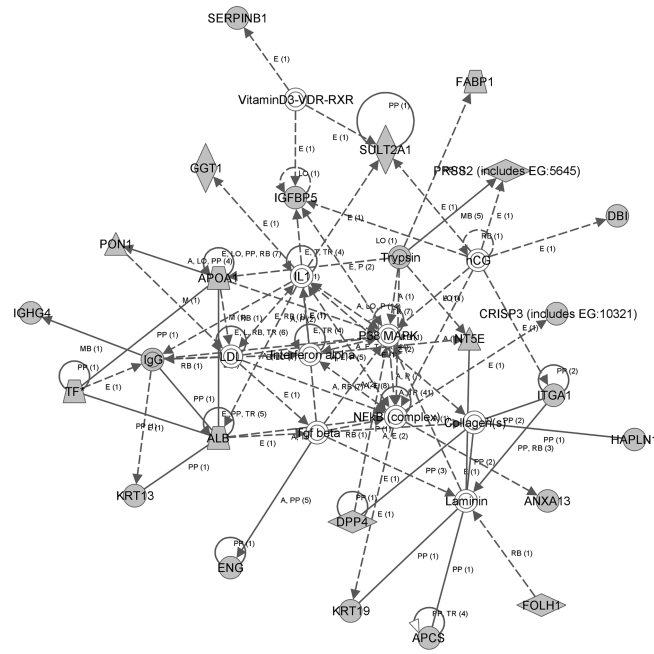

Figure 5. Top pathway from the list of proteins that show significantly decreased expression in DS-AF. This pathway is involved in organ morphology, reproductive system development and function, and lipid metabolism.

Recent improvements in mass spectrometry for proteomic analysis allow for a wide detection range of proteins in complex biological samples. High-throughput MS/MS analysis can be utilized for identification and quantification of proteins, and there are a number of strategies developed for large-scale quantification.[19−21] Spectral counting has been applied in this study for the initial high-throughput quantification, rendering dozens of candidates for further verification. Although spectral counting is only semiquantitative and less accurate than labeling methods, the major advantage of this method is that it is not limited by labeling efficiency, allowing high-throughput quantification based on the total number of spectra.[22] To minimize the variability introduced by sample preparation steps, we chose SCX chromatography for peptide separation out of three different fractionation protocols that were previously established.[5] In this study, we quantified and compared 542 proteins from CN-AF and DS-AF in triplicates, to identify candidate markers for improved DS screening.

Spectral counts for a specific protein in complex mixtures such as biological fluids can vary significantly even if the concentration of the protein of interest and the total amount of proteins are controlled. Such variability becomes more problematic if the concentration of the protein of interest is relatively low. To offset this problem, a normalization step has been performed to take account of the variability in the total number of spectra identified in each run. Additionally, we examined the triplicate results to select candidates that consistently show differences between CN- and DS-AF. The application of stringent cutoffs and careful normalization, therefore, facilitated our aim to achieve a high-confidence semiquantitation of AF proteins, to discover candidate biomarkers. We first created a list of proteins that are either uniquely expressed in CN-AF or DS-AF, or show greater than 2-fold difference between the two conditions. These proteins were further analyzed using Ingenuity Pathway Analysis software to identify canonical and functional pathways that may be affected by the differentially expressed proteins. The top pathway from the list of decreased proteins in DS-AF, for example, was associated with organ morphology and reproductive system development and function. This pathway has 35 proteins in its network, 23 of which showed significantly less spectral counts in DS-AF in this study, and interestingly, it also contains hCG, which is another aberrantly expressed protein in the presence of DS (Figure 5). The top 11 pathways that represent proteins that show decreased expression and top 10 pathways for increased proteins were compiled to propose some of the dysregulated pathways in DS (Supplemental Data 2).

We developed a list of 60 candidate markers after applying a set of selection criteria (Table 3). Since hCG is one of the best individual screening markers for DS,[23,24] the presence of β-hCG in our candidate list confirms our hypothesis that spectral counting is an effective method to select candidates by relative quantification. When a number of spectra for β-hCG between CN-AF and DS-AF were compared, the ratio concurred with the ratio between β-hCG concentrations previously reported in literature.[25] Our results showed 17 spectra for hCG from the CN-AF, and 45 from DS-AF. After normalization, it was shown that β-hCG is increased by 2-fold in DS-AF. Previous studies have shown that β-hCG levels are elevated by 1.5-fold in maternal serum and 2-fold in AF when the fetus is affected by DS.[25,26] Since we aimed to discover novel biomarkers that have equivalent or higher detection rate compared to the best marker (β-hCG), this finding adds confidence to our cutoff of 2-fold or greater difference for the candidate selection process.

From the list of 60 candidates, we attempted to preliminarily validate two candidates (APP and TNC-C) by ELISA, to investigate their potential as biomarkers and to assess the quality of quantification by spectral counts. APP is a cell surface receptor and transmembrane protein that is cleaved by a number of secretases to produce different peptides. The gene is located in 21q21.2, and it is expressed in brain, pancreas,

eye, lung, and many other tissues. This protein is of particular interest because APP ending at Aβ42 is deposited early in senile plaques, an invariant feature of Alzheimer's disease (AD).[27] It has been hypothesized that APP mutations can cause early onset familiar Alzheimer's disease,[28−30] and since the majority of individuals with DS develop AD,[31] APP was chosen as one of the candidates for validation, despite the <2-fold increase in spectral counts in DS-AF. The second protein we selected was Tenascin-C, which belongs to a family of tenascins, oligomeric glycoproteins in the extracellular matrix. Tenascin-C has an adhesion modulatory role for cell attachment and cell−matrix interactions.[32] It has been proposed that Tenascin-C has a role in gross organismal development as well as subtle regulation of morphology and physiology.[10,11,32,33]

On the basis of spectral counting, it was expected that TNC-C would show 2-fold or greater increase in DS-AF samples, and APP to show less than 2-fold increase in DS-AF samples. The results showed that APP is almost consistently increased by 2-fold in DS-AF, whereas there is a range of elevation for TNC-C in DS-AF. Our experimental design utilizes pooling of samples, reducing the impact of biological variability. This could be an advantage and a disadvantage. For example, it is difficult to assess the strength of a candidate biomarker until individual samples are measured by an accurate method such as ELISA. In this study, APP levels showed relatively insignificant biological variability, whereas TNC-C showed more variability for the same set of samples, ranging from no significant increase to greater than 6-fold increase in individual DS-AF samples compared to the mean value for CN-AF samples. Therefore, it is important to note that more accurate quantitative assays should be performed using individual samples after spectral counting is used for initial selection of candidate markers.

Analysis of CN-AF and DS-AF spectral counting revealed interesting findings that may be important for understanding the molecular mechanisms of DS, especially when combined with genomic data. A recent study of CN-AF and DS-AF at the cell-free mRNA level using microarrays revealed 414 genes that showed significantly different expression levels.[34] When we compared this list of genes with our top 60 candidates, two genes were found in common: FLNA and VCAN. Our spectral counting result showed 3.3-fold increase for filamin-A (FLNA) and 2.5-fold increase for versican core protein (VCAN), while 2.54-fold increase for FLNA mRNA and 2.56-fold increase for VCAN were observed by Slonim et al.[34] Next, we analyzed all of the HSA21-encoded proteins from our list and discovered that all were identified with greater number of spectra from the DS-AF, compared to the normal AF samples (Table 2). After normalization, the ratios for these proteins range from 1.2:1 to >7:1, indicating that they are likely significantly more abundant in DS-AF. The well-known gene dosage hypothesis of DS suggests that the phenotypes of DS are caused by a 1.5-fold increase in gene expression of genes located in HSA21 due to the presence of an extra copy.[35] This hypothesis has been tested by microarray experiments using both human samples and mouse models of DS, and the results of these transcriptome studies indicate that transcripts from over 50% of HSA21-encoded genes are indeed up-regulated by 1.5-fold or higher.[36−38] We believe that our spectral counting data for the seven proteins implicate elevated expression of these HSA21-encoded proteins, and that this finding, for the first time to our knowledge, supports the gene-dosage hypothesis for multiple genes on HSA21 at the protein level.

In this study, we used 2D-LC followed by MS/MS for in-depth comparative analysis of amniotic fluid, based on spectral counting. This is the first study to incorporate high-throughput LC-based fractionation for quantitative proteomic analysis of AF, resulting in the most extensive list of candidate markers that include many low-abundance proteins. Furthermore, proteins that showed differential expression due to DS can provide clues for understanding the pathogenesis and genotype−phenotype relationships of DS. Although APP and TNC-C did not show potential as serum biomarkers, due to their abundance in serum, the ELISA results confirmed the relative quantification data, indicating that high-throughput semiquantitative analysis based on spectral counting is an effective method to analyze complex samples. More candidates should be verified both in AF and serum, using large numbers of clinical samples, to find novel biomarkers for improved screening. Proteomic analysis of AF using this approach may be a promising method for identifying biomarkers for other pregnancy-associated pathologies.

Abbreviations: ACN, acetonitrile; AF, amniotic fluid; AFP, alpha-fetoprotein; APP, amyloid precursor protein; CN-AF, chromosomally normal (control) AF; DTT, dithiothreitol; T21-AF, AF from Trisomy 21-affected pregnancies; FPR, false positive rate; HPLC, high-performance liquid chromatography; HSA21, human chromosome 21; Igs, immunoglobulins; IPI, International Protein Identifier; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PAPP-A, pregnancy associated plasma protein-A; SCX, strong cation exchange; TNC-C, tenascin-C.

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Supporting Information Available: Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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