JOURNAL OF PROTEOMICS 74 (2011) 2052–2059

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Verification of a biomarker discovery approach for detection of Down syndrome in amniotic fluid via multiplex selected reaction monitoring (SRM) assay

Chan-Kyung J. Cho[a,b], *Andrei P. Drabovich*[b], *Ihor Batruch*[b,c], *Eleftherios P. Diamandis*[a,b,c,*]

[a]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada*
[b]*Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada*
[c]*Department of Clinical Biochemistry, University Health Network, Toronto, ON, Canada*

ARTICLE INFO

Article history:
Received 22 March 2011
Accepted 12 May 2011
Available online 20 May 2011

Keywords:
Biomarker
Down syndrome
Prenatal screening
CEL
MMP2
MUC13
DPP4
CPA1

ABSTRACT

Prenatal screening test for Down syndrome (DS) can be improved by discovery of novel biomarkers. A multiplex selected reaction monitoring (SRM) assay was developed to test previously identified thirteen candidate proteins in amniotic fluid (AF). One unique peptide was selected for each protein based on discovery data, while three MS/MS transitions were selected based on intelligent SRM results. For one of the candidates, matrix metalloproteinase-2 (MMP2), ELISA was also performed to validate SRM results in AF and to test serum samples. Comparison of AF samples from DS versus controls via SRM assay revealed five proteins that were differentially expressed. Bile salt-activated lipase, mucin-13, carboxypeptidase A1, and dipeptidyl peptidase 4 showed a decrease in DS-affected AF, and MMP2 showed an increase, in comparison to controls ($P<0.05$). Discovery-based spectral counting ratios and SRM ratios showed a strong correlation, and MMP2 ELISA further confirmed the validity of the SRM data. Potential implications of differentially expressed proteins during fetal development are proposed. Our data also shows that SRM can provide a high-throughput and accurate platform for biomarker verification.

1. Introduction

Down syndrome, also known as Trisomy 21, is caused by complete or partial triplication of human chromosome 21 (HSA21), which is the smallest autosome with a length of 47 megabases. Down syndrome is the most common chromosomal anomaly in humans, and it also constitutes the most common congenital cause of intellectual disability in live-born infants. Currently, Down syndrome prevalence is roughly 1 in 750 live births [1]. This rate, however, underestimates the true incidence since it does not include induced and spontaneous abortions [2]. The currently available screening tests for DS perform risk calculation, mostly based on measurements of biochemical markers from maternal serum, resulting in 85–

Abbreviations: AD, Alzheimer's disease; AF, amniotic fluid; CEL, bile salt-activated lipase; CN, chromosomally normal; CPA1, carboxypeptidase 1; DPP4, dipeptidyl peptidase 4; DS, Down syndrome; iSRM, intelligent SRM; MMP2, matrix metalloproteinase-2; MUC13, mucin-13; SRM, selected reaction monitoring.

Human genes discussed in paper: APP, amyloid precursor protein; CEL, bile salt-activated lipase; CPA1, carboxypeptidase 1; DPP4, dipeptidyl peptidase 4; MMP2, matrix metalloproteinase-2; MUC13, mucin-13; TNC, Tenascin-C.

* *Corresponding author at:* Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 6th Floor, Room 6–201, Box 32, 60 Murray Street, Toronto, ON, Canada M57 3L9. Tel.: +1 416 586 8443; fax: +1 416 619 5521.
E-mail address: ediamandis@mtsinai.on.ca (E.P. Diamandis).

1874-3919/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jprot.2011.05.025

95% detection rate [3]. Due to the uncertainties associated with the current screening, invasive diagnostic procedures such as amniocentesis are frequently used, although only a small fraction (1–5%) of the tested individuals actually have an affected fetus [4,5]. To further reduce or even eliminate unnecessary invasive procedures, it is important to advance the current screening tests by discovering additional biomarkers to improve the predictive power and specificity.

Amniotic fluid (AF), among all biological fluids, has two major advantages over plasma as a reservoir of biomarkers for Down syndrome detection. First, it contains the greatest concentration of fetal and pregnancy-related proteins. AF before skin keratinization of the fetus is especially similar to fetal plasma in composition. Secondly, the complexity of AF poses less challenge for proteomic analysis compared to that of plasma or serum. Therefore, AF has been proteomically studied in depth to explore its potential as a medium for biomarker discovery [6–8]. Furthermore, several studies have attempted to compare AF proteins from chromosomally normal (CN) and DS-affected pregnancies semi-quantitatively and quantitatively [9–11]. In our previous study, a comparison of spectral counts of proteins from unaffected and affected (Down syndrome) AF proteome revealed 60 candidates. Since spectral counts endow only semi-quantitative comparisons, candidates revealed through spectral counts must be confirmed by more accurate and sensitive assays [9]. Previously, two candidates among the 60 proposed candidates that showed differential expression in AF, amyloid precursor protein and tenascin-C, were confirmed by ELISA. However, many other candidates could not be readily confirmed or verified due to lack of sensitive assays or specific antibodies.

Recent improvements in mass spectrometry-based quantitative technologies can provide alternative quantitation tools even for complex biological samples. Selective reaction monitoring (SRM) based on triple quadrupole mass spectrometry is one of the most versatile label-free quantitation techniques, since it allows for relative and absolute quantitation of multiple proteins simultaneously. SRM assays provide not only high selectivity and sensitivity, but also a more feasible and automated means to verify putative biomarkers for which traditional immunoassays are unavailable. Moreover, SRM offers a unique set of advantages. For example, SRM allows differentiation of protein and peptide isoforms as long as they present unique sequences. This advantage becomes especially important when analyzing complex biological mixtures, such as AF and serum, which contain numerous proteins that may cross-react in immunoassays without the optimal specificity. Another advantage is that SRM allows high-throughput quantitation for multiple proteins in a single experiment. For example, Whiteaker et al. monitored over 80 proteins in plasma by SRM, reporting detection of many proteins at the concentration of 0.5 fmol/μL in plasma [12]. Finally, improvements in SRM technology have been substantial for the past several years. The methodologies are rapidly becoming more refined with introduction of more powerful mass spectrometers, more sophisticated analysis tools and software, and introduction of stable isotope-labeled standards for absolute quantification.

In the present study, we developed a mass spectrometry-based SRM assay to assess the differential expression of thirteen candidate proteins in AF in order to verify our discovery data. We performed relative quantitation in 18 individual AF samples from unaffected and Down syndrome-affected pregnancies. Our multiplex SRM assay was developed based on the fragmentation information obtained through experimental outputs from our previous LC–MS/MS study, and it targeted one unique peptide for each of the thirteen candidate proteins (Table 1). This report presents the first SRM study to assess candidate biomarkers for detection of Down syndrome in 18 clinical (amniotic fluid) samples.

Table 1 – Top three transitions selected based on iSRM data for each peptide (precursor ion) and their retention time.

Gene name	Precursor ion (m/z)	Top three transitions (m/z)	Retention time (min)
CA1	485.800	459.292 572.376 758.440	12.4
TF	489.748	464.286 563.355 735.403	7.9
CPA1	594.316	573.335 888.442 987.510	5.7
CEL	638.861	880.441 993.525 1163.63	24.3
KLK3 (spiked-in internal standard)	640.848	654.391 951.523 1080.566	9.9
CTGF	706.834	652.341 799.409 912.493	13.1
COMP	743.878	630.356 701.394 886.474	8.0
FBN2	750.359	909.406 1124.496 1237.580	10.5
DPP4	755.827	869.399 1032.463 1195.526	11.5
MUC13	808.899	894.467 1007.551 1154.620	18.1
AHSG	830.885	923.465 1095.513 1224.556	11.8
IGFBP5	879.932	915.496 1099.581 1196.634	16.7
NPC2	922.437	732.370 1058.529 1315.613	7.2
MMP2	1054.512	694.315 1120.526 1235.553	19.6
ADAMTS1	1157.099	630.320 840.457 968.515	30.8

2. Materials and methods

2.1. Sample collection

Amniotic fluid (AF) samples were obtained from pregnant women at 16–20th week gestation who underwent amniocentesis at the second trimester of pregnancy. Samples were collected with written consent and ethics board approval. In our previous semi-quantitation study, we used pooled AF samples by combining equal amount of proteins from each individual sample [9]. In this study, we used the original set of AF samples which have been separately aliquoted and stored at −80 °C for verification studies. AF samples fell into two groups: chromosomally normal controls (n=9) and Down syndrome group (n=9). Samples were matched for gestational age and gender of the fetus. Each sample was stored after centrifuging at 13,000x*g* for 10 min to eliminate any cellular debris.

2.2. Sample preparation

Before analysis, the samples were thawed at 4 °C and centrifuged once more at 13,000×*g* for 10 min. Total protein for each AF sample was measured by the Bradford assay (Pierce, USA), and the volume was adjusted to extract equal amounts of total protein from the individual samples. AF proteins (30 μg) were denatured with 0.1% Rapigest (Waters, Milford, MA, USA) at 60 °C, and the disulfide bonds were reduced with 10 mM dithiothreitol (Sigma-Aldrich, St. Louise, MO, USA) before being subject to alkylation with 20 mM iodoacetamide. Samples were then digested with sequencing grade modified trypsin (Promega, Masdison, WI, USA) overnight at 37 °C. Ninety six femtomoles of heavy $^{13}C_6$, $^{15}N_2$ L-Lysine-labeled peptide (LSEPAELTDAVK*) of KLK3 protein was added as a relative internal standard peptide for the SRM runs. Rapigest was cleaved with 1% trifluoroacetic acid, and all samples were centrifuged at 4000×*g* for 10 min. Peptides were purified and extracted using 10 μL OMIX C18 tips (Varian, Lake Forest, CA, USA), and were eluted using 5 μL 65% acetonitrile solution (0.1% formic acid, 0.02% trifluoacetic acid). The final sample was diluted to 130 μL to yield three replicates of 40 μL.

2.3. Peptide selection for SRM

A total of 13 candidate proteins were selected out of the 60 candidates reported previously [9]. From this list, we further reduced the number of candidates based on their relative abundance in AF. Mid- to high-abundance proteins were preferred over low-abundance proteins, and the selection was based on the number of target protein peptides that showed high frequencies in an MS scan. Also, proteins that were quantified based on unique peptides, via spectral counting in our previous study, were preferred. Proteotypic peptide for each candidate was manually selected based on identification data acquired using an LTQ-Orbitrap instrument.

Several criteria were applied to select candidate peptides: display of multiple spectra and clear y-ion fragmentation, peptide length of 8 to 20 amino acids, and preferably absence of modifications at cysteine, methione, and tryptophan. Additionally, any non-unique peptides were removed after the sequence search result obtained via the Basic Local Alignment Search Tool (BLAST). When more than one peptide from a single protein qualified for all the criteria, a peptide that yielded less significant overlap of time interval with other peptides was preferred. In silico digestion and fragmentation of each peptide was performed with Pinpoint software (Thermo Scientific, USA).

2.4. SRM and iSRM conditions

Tryptic peptides were loaded onto a 2 cm C_{18} column (inner diameter: 150 μm) and were eluted to a resolving 5 cm analytical C_{18} column with a 15 μm tip (inner diameter: 75 μm) for separation (New Objective). This setup was online-coupled to a triple-quadrupole mass spectrometer (TSQ Quantum Ultra, Thermo Scientific) using a nanoelectrospray ionization source (nano-ESI, Proxeon A/S). The details of liquid chromatography and MS methods can be found in our previous study [13]. Briefly, a 60-min, three-step gradient was used to load peptides onto the column via an EASY-nLC pump (Proxeon A/S). Running buffer contained 0.1% (v/v) formic acid in water, and elution buffer contained 0.1% (v/v) formic acid in acetonitrile. Peptides were analyzed by SRM methods using the following parameters: predicted collision energy values, 0.002 m/z scan width, 10 ms scan time, 0.2 resolution at the first quadrupole, 0.7 resolution at the third quadrupole, 1.5 mTorr pressure at the second quadrupole, tuned tube lens values, 7 V skimmer offset. The identity of each peptide was confirmed by performing intelligent SRM (iSRM). This method targeted peptides in 3-min scheduled acquisition windows, monitoring for two primary transitions for each peptide. Once the intensity of the two transitions overcame the threshold of 300, this triggered the program to acquire the intensity of six additional transitions for the same peptide.

2.5. Optimization of the SRM assay and data analysis

The three most intense and reproducible transitions for each peptide from iSRM results were selected to develop the final SRM method. One of the unique and abundant peptides of serotransferrin (TF), a high-abundance protein in AF, was selected to serve as an additional relative internal standard. Being one of the most abundant proteins in AF, it was hypothesized that TF abundance would not significantly differ between AF samples. Therefore, relative amounts of TF were measured to be used as an additional indication that protein digestion and the whole sample preparation protocol were similarly efficient across all individual samples. In total, the final method targeted 45 transitions of 15 peptides (13 candidates, TF and the internal standard). The method included a 60-min gradient within a 84-min method, and the detection window for transitions were scheduled with approximately 2-min intervals within the 60-min gradient. Raw output files for each sample were manually analyzed using LCquan software (version 2.5.6) to verify peak areas used for quantification (Fig. 1). Quantification was executed after normalization against an internal standard peptide (KLK3*) to offset technical errors.

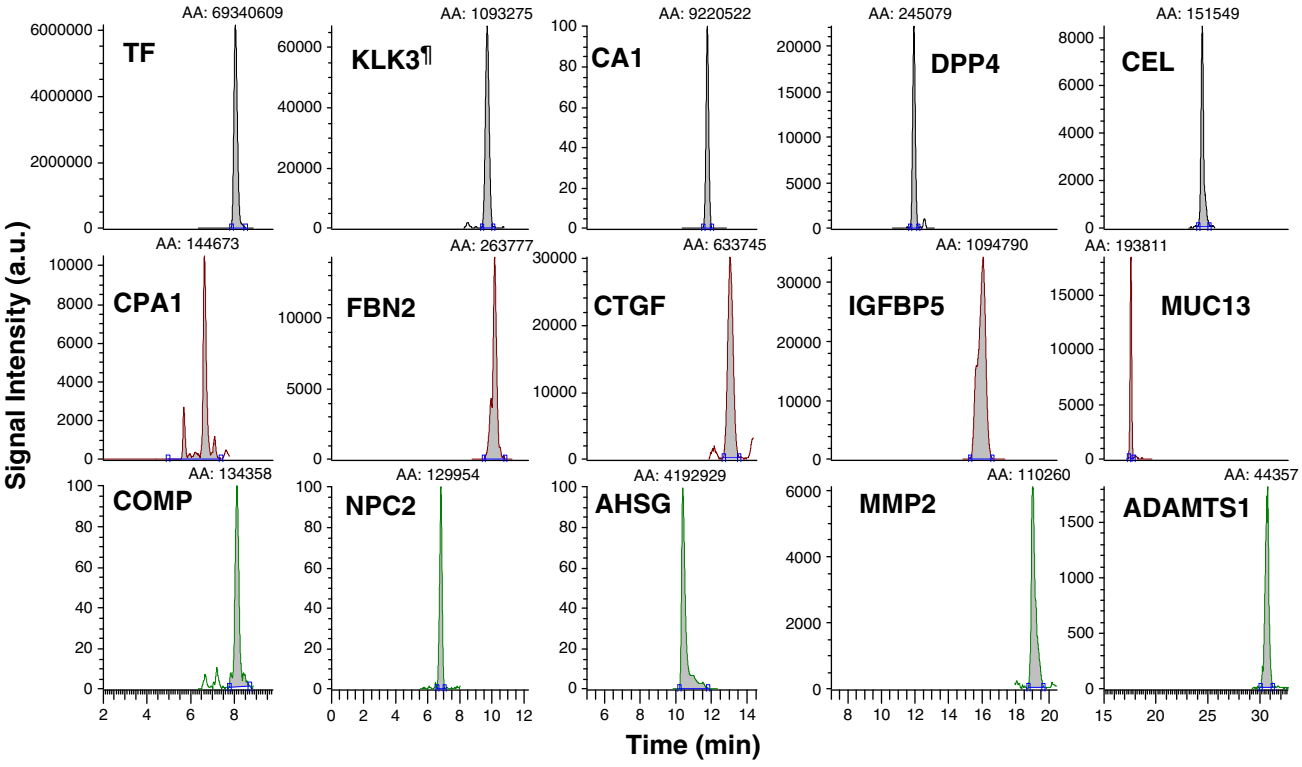

Fig. 1 – Quantification of thirteen candidates and serotransferrin in individual AF samples for comparison between DS versus control groups. Normalized area of the peaks were calculated by dividing peak intensity of each peptide by that of internal standard (heavy peptide LSEPAELTDAVK of KLK3) in each sample. Low variation of TF in individual samples could serve as an indirect indicative that sample preparation protocol was consistent for all individual samples assayed.

2.6. *Quantification of MMP2 by enzyme-linked immunosorbent assay (ELISA)*

The concentration of MMP2 was measured using sandwich ELISA with a Human MMP-2 Quantikine Kit (R&D Systems). A total of 18 AF (n=9 each for DS and CN-AF) and 71 maternal sera (n=22 for DS; n=49 for CN) samples were measured. A 10-fold dilution was prepared for both AF and serum samples, and ELISA was performed according to the instructions provided with the kit. The results were analyzed using SPSS and GraphPAD Prism (GraphPAD Software). All statistical analyses were performed via independent samples t-test using SPSS software (version 17.0). P-values of <0.05 were considered significant.

3. Results and discussion

In our previous report, 60 candidate proteins that were differentially expressed in DS-affected AF, compared to the controls, were identified by semi-quantitative spectral counting [9]. From the list of 60 candidate proteins, the target proteins were selected based on their relative abundance, estimated by the frequency of MS1 spectra. Low-abundance proteins were omitted since they are less likely to be accurately quantified with SRM assay in the unfractionated or non-enriched digests of AF. As a result, we developed an SRM assay targeting a total of 13 candidate proteins in addition to two internal standards, for quantification and comparison between the DS and unaffected groups (Table 1). In order to achieve reliable quantification via SRM assays, it is essential to precisely execute sample preparation and to assess the reproducibility of the assay. Thus, three independent SRM runs per each sample were performed to monitor the reproducibility.

In order to select SRM transitions, we used two complementary approaches. First, the previous LC–MS/MS data obtained from LTQ-Orbitrap was manually analyzed using Scaffold software to obtain experimental spectral information including the transitions for the peptides of interest. All selected precursor ions had charge state of 2 to 3. We selected against the peptide ions that display modifications, if such modifications constitute greater than 5% but less than 95% of the observed spectra, to ensure accurate quantification. Up to eight experimentally observed transitions were compared to the discovery MS/MS spectrum for evaluation. Secondly, we used Pinpoint software (Thermo Scientific, USA) to refine the list of transitions. Pinpoint generates the most abundant precursor and product ions for comparison, as well as automated optimal collision energies for SRM transitions. The MS/MS spectra of the precursor ions were examined to ensure selection of the fragment ions with the highest intensities. Fragment ions that displayed the highest and second-highest MS/MS intensities from experimental data were selected as primary ions for iSRM method.

The peak area for each of the three transitions of the target proteins was extracted from the spectrum of each sample via

LCquan. Each peak area was manually inspected on LCquan for consistent peak integration to obtain accurate AUC. The area under the curve (AUC) for each peptide ion was then normalized to the AUC of the KLK3 standard peptide to offset technical variations between the SRM runs. Thirteen candidates and TF were then assessed by comparing the mean values in DS and control groups. First, we used relative abundances for TF to evaluate the global performance of our SRM assay. Technical variation was estimated based on CVs for triplicate injections of the same sample. Technical variation was found to be 25% or less (8% on average) for all 18 samples. Biological variation was estimated based on CVs of relative abundances of 9 samples within groups of normal and DS samples. Such variations were found to be 56% and 48% on average, respectively (excluding carbonic anhydrase 1 which had a few exceptional outliers). Finally, the ratio of mean values for TF was found to be 1.1, supporting our initial assumption about similar abundances of TF in individual AF samples. Based on these observations, we believe that our sample preparation protocol and SRM assay were sufficiently reproducible to enable comparison of relative abundances for chosen candidate biomarkers.

Of the 13 candidates, 5 proteins exhibited a statistically significant downregulation in individual DS samples ($P<0.05$) (Fig. 2). Bile salt-activated lipase (CEL), mucin-13 (MUC13), dipeptidyl peptidase 4 (DPP4), and carboxypeptidase A1 (CPA1) showed a decrease of 4.5-, 3.7-, 2.4- and 2.3-fold, respectively. Matrix metalloproteinase-2 (MMP2) showed a statistically significant increase in DS by 1.5-fold (Table 2). The rest of the candidates (insulin-like growth factor-binding protein5, a disintegrin and metalloproteinase with thrombospondin motifs 1, epididymal secretory protein E1, alpha-2-HS-glycoprotein, fibrillin-2, cartilage oligometric matrix protein, connective tissue growth factor, carbonic anhydrase 1) did not show statistically significant increase or decrease in DS-AF compared to the controls (Supplementary Fig. 1). The mean concentration of MMP2 in AF was 221 ng/mL in the DS group, and 164 ng/mL in the CN group ($P=0.018$). The DS/CN ratio from ELISA result was 1.35, which is close to the observed SRM ratio of 1.47 (Fig. 2). Unlike in AF, MMP2 levels in serum samples showed no significant difference between CN (183 ng/mL) and DS (203 ng/mL) groups.

The Pearson's correlation coefficient that represents correlations between spectral counting ratios and SRM ratios for the candidates was 0.904 ($P=0.01$), supporting our initial rationale of candidate selection based on spectral counts from pooled samples. For example, carbonic anhydrase 1 and insulin-like growth factor-binding protein 5 initially showed a marked difference in both the spectral count and preliminary SRM results that were acquired from pooled samples. Our subsequent SRM data for individual samples revealed few AF samples with prominently high concentrations of carbonic anhydrase 1 and insulin-like growth factor-binding 5. These samples most probably affected the final concentration of both proteins in the pooled samples, which were used for our initial selection of candidates based on spectral counting comparison. Thus, analysis of individual samples via SRM assay allowed for elimination of such artifacts.

Our collective results from the previous and present study indicate that comparison of two conditions via spectral counting, if used thoughtfully, can be used as an effective strategy to select the initial list of candidates. In general, spectral counting is defined as the total number of spectra identified for a protein, and it has been widely used as a practical and rapid measure of relative protein abundance [14]. For quantitative proteomics, the more complicated sample preparation steps are, the more sources of variability are inevitably introduced. Therefore, spectral counting provides semi-quantitative information acquired together with protein identification. Due to its inherent limitations, however, spectral counting should be considered as an initial sieve to identify potential candidates out of thousands of proteins in

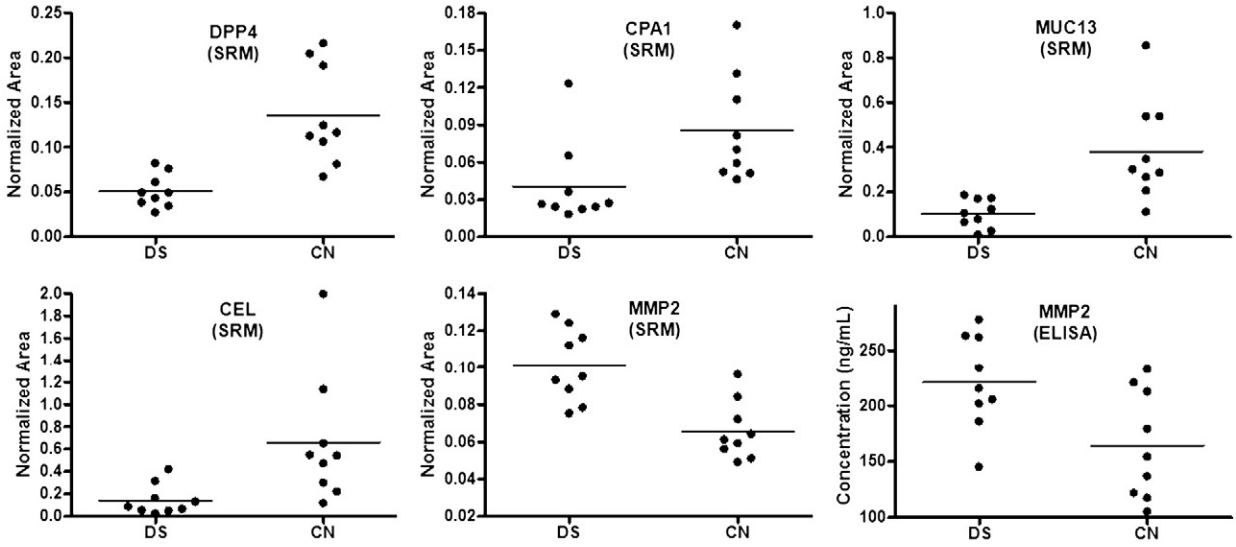

Fig. 2 – Measurement of DPP4, CPA1, MUC13, CEL, and MMP2 in amniotic fluid samples from Down syndrome-affected (DS) and chromosomally normal (CN) pregnancies. Note the decreases in DPP4, CPA1, MUC13, and CEL, and the increase in MMP2 in amniotic fluid of Down syndrome fetuses ($P<0.05$). MMP2 was also measured by ELISA. (DS = Down syndrome; CN = chromosomally normal controls).

Table 2 – Quantification of candidate biomarkers for Down syndrome detection in amniotic fluid.

Gene name	Protein name	Peptide	Parent ion (m/z)	SC[1] ratio (DS/CN)	SRM ratio[2] (DS/CN)	P-value[3]
CEL	Bile salt-activated lipase	LGLLGDSVDIFK	638.86	0.00	0.22	0.029
MUC13	Mucin-13	SSSSNFLNYDLTLR	808.90	0.36	0.27	0.006
DPP4	Dipeptidyl peptidase 4	WEYYDSVYTER	755.83	0.67	0.42	0.001
CPA1	Carboxypeptidase A1	ISVADEAQVQK	594.32	0.64	0.44	0.027
MMP2	Matrix metalloproteinase-2	IIGYTPDLDPETVDDAFAR	1054.51	2.90	1.50	0.002
IGFBP5	Insulin-like growth factor-binding protein 5	ALSMC*PPSPLGC*ELVK	879.93	0.82	0.77	>0.05
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1	GAFYLLGEAYFIQPLPAASER	1157.10	0.29	0.91	>0.05
NPC2	Epididymal secretory protein E1	EVNVSPC*PTQPC*QLSK	922.44	3.00	1.00	>0.05
AHSG	Alpha-2-HS-glycoprotein	EHAVEGDC*DFQLLK	830.89	1.62	1.14	>0.05
FBN2	Fibrillin-2	YVISQGNDDSVFR	750.36	1.79	1.11	>0.05
COMP	Cartilage oligomeric matrix protein	ELQETNAALQDVR	743.88	2.89	1.18	>0.05
CTGF	Connective tissue growth factor	DGAPC*IFGGTVYR	706.83	2.00	1.33	>0.05
CA1	Carbonic anhydrase 1	VLDALQAIK	485.80	5.14	2.44	>0.05
TF	Serotransferrin	DGAGDVAFVK	489.75	1.31	0.91	>0.05
KLK3[4]	Prostate-specific antigen	LSEPAELTDAVK[HeavyK]	640.85			>0.05

[1]Spectral counts from pooled samples reported previously [9].
[2]Ratio between means of each group (DS/CN; SRM values were derived by analysis of individual samples; n=9 per group).
[3]P-values for difference in mean amount (normalized peak area) of each candidate from CN- and DS-AF by SRM.
[4]KLK3 heavy peptide injected as an internal standard.
*These cysteins have S-carbamidomethyl modification.

complex mixtures. Ideally, the next step should involve a high-throughput quantitative technique to assess the quantification of dozens of potential candidates identified via spectral counting, since the list of candidates discovered via spectral counting may include some false candidates.

SRM, also known as multiple reaction monitoring (MRM), has long been utilized to quantitate small molecules such as hormones in biological samples. For the past decade, SRM has been explored and optimized for proteomic quantitation with the improvements of triple quadrupole mass spectrometers. Once optimized, SRM can offer an antibody-independent specific assay, and its multiplexing capability will yield cost-efficient means to analyze many target proteins simultaneously. Thus, coupling spectral counting with SRM would provide an effective and fast pipeline for biomarker discovery. Sensitivity of SRM assay, however, may be compromised due to the complexity of sample and the nature of candidate proteins. For example, unlike ELISA or other immunoassays, detection of low-abundance proteins (100 ng/mL or less) via SRM in the unfractionated digest of complex sample will not show consistency, for either qualitative or quantitative study, due to the current limitations of mass spectrometry. Efforts to improve sensitivity of SRM have focused on enrichment of target proteins by techniques such as depletion of high-abundance proteins and SISCAPA [15]. In this study, we aimed to confirm some of the candidates that were previously proposed based on the semi-quantitative spectral counting data. To our knowledge, this is the first study to quantify proteins in amniotic fluid using SRM assays.

MMP2 levels were assessed in both individual AF and maternal serum samples, since a sensitive ELISA kit was commercially available for MMP2. Unlike in AF, MMP2 levels in serum samples showed no significant difference between CN and DS groups. This is likely due to abundance of MMP2 in serum, irrespective of pregnancy. MMP2 is a ubiquitously secreted or membraneous metalloproteinase found in fibroblasts, and it is involved in multiple processes such as angiogenesis, tissue repair, and inflammation, atherosclerotic plaque rupture, and valve pathology [16–18]. Our finding of increased (1.5-fold) levels of MMP2 in AF of DS fetuses may provide clues for explaining as to why most DS patients possess cardiovascular defects but have reduced risk for solid tumors and atherosclerotic plaque formation. Moreover, increased MMP2 activity has been linked to increased degradation of secreted amyloid-β [18], a key player of Alzheimer's disease (AD) [19]. Recently, multiple MMPs were measured in blood and cerebrospinal fluid in Alzheimer's patients, and MMP-2 showed significant decrease in cerebrospinal fluid but not in blood of AD patients [20]. Therefore, increases in amyloid precursor protein [9] together with increases in MMP2 during fetal development may be involved in the eventual pathogenesis of early-onset AD, which is prevalent among DS patients.

Nuchal translucency test, which measures the thickness of the skin fold behind the nape of the neck of a fetus, is often used for first-trimester screening of DS. In DS fetuses, such thickening may be attributed to lymphatic engorgement, although the molecular mechanism of this symptom is unknown [21]. DDP4 is a cell surface glycoprotein receptor mainly expressed in lymphatic vessels [22], and studies have shown that DDP4 may be involved in the T-cell activation and lymphatic development [22,23]. Hence, decreased DPP4 expression in fetus may be related to lymphatic maldevelopment in DS-affected individuals.

CEL is a secreted lipolytic protein that hydrolyzes cholesteryl esters, triacylglycerols, and phospholipids, and it is mainly produced from the mammary gland and pancreas. Its dysregulated expression in macrophages has been linked to promotion of atherosclerosis, indicating its importance in lipid regulation [24]. Interestingly, hypercholesterolemia and

other types of lipid dysregulation have been reported in DS fetuses, and hypercholesterolemia has been proposed as a risk factor for dementia in AD [25,26]. Therefore, it may be that the impact of reduced CEL expression on its immediate pathways leads to lipid dysregulation, contributing to the eventual dementia in DS-affected individuals.

MUC13 is a transmembrane protein that is highly expressed in epithelial tissues, particularly along gastrointestinal tracts [27]. Little is known about the functions of MUC13 other that its involvement in barrier functions of epithelium. Abnormal expression of MUC13 has been linked to gastric, colon, and ovarian cancer, as well as ulcerative colitis [28–30]. Further investigation on the functions and expression of MUC13 during fetal development will allow us to understand its potential involvement in DS pathogenesis. Finally, CPA1 is a secreted protein that catalyzes the release of a C-terminal amino acid, but little is known about its biological function.

Traditional techniques for biomarker discovery include Western blotting, ELISA, or immunohistochemistry, and they all require suitable antibodies which often require ample time and cost for development. SRM analysis, therefore, allows us to effectively determine which biomarkers are worth pursuing by developing appropriate antibodies. Our data confirm that SRM can provide a high-throughput and accurate platform for biomarker verification, when immunoassays are unavailable. With continuous improvements, SRM may soon become a standard method for biomarker verification, replacing some of the immunoassays. The next step to this study would involve development of ELISA and performing clinical validation using a larger number of samples, both AF and serums.

In this study, we verified previous discovery data for 13 candidate markers of DS in AF which were previously identified from a semi-quantitative study, and showed for the first time that five different proteins, which are not previously known biomarkers or related proteins of DS, are differentially expressed in AF. We also proposed their potential involvement in DS phenotypes and pathology. As indicated by the case of MMP2, even if a biomarker may be informative in AF, its performance in maternal serum may be compromised, especially when the marker is not specific to pregnancy.

Supplementary materials related to this article can be foundonline at doi:10.1016/j.jprot.2011.05.025.

Conflict of interest statement

There are no conflicts of interest regarding the publication of this article.

Employment or leadership

None declared.

Consultant or advisory role

None declared.

Stock ownership

None declared.

Honoraria

None declared.

Role of funding source

This work was supported by a University-Industry Grant from the Natural Sciences and Engineering Research Council in Canada and Proteomic Methods, Inc., Toronto, Ontario, Canada.

Acknowledgements

We thank Dr. Elizabeth Winsor for providing amniotic fluid samples. Chan-Kyung Cho is a recipient of the Canadian Institutes of Health Research (CIHR) Frederick Banting and Charles Best Canada Graduate Scholarship. Dr. A. P. Drabovich is supported by the Canadian Institutes of Health Research (CIHR) post-doctoral fellowship.

REFERENCES

[1] Canfield MA, Honein MA, Yuskiv N, Xing J, Mai CT, Collins JS, et al. National estimates and race/ethnic-specific variation of selected birth defects in the united states, 1999–2001. Birth Defects Res A Clin Mol Teratol 2006;76:747–56.
[2] Antonarakis SE, Lyle R, Dermitzakis ET, Reymond A, Deutsch S. Chromosome 21 and down syndrome: from genomics to pathophysiology. Nat Rev Genet 2004;5:725–38.
[3] Malone FD, Canick JA, Ball RH, Nyberg DA, Comstock CH, Bukowski R, et al. First-trimester or second-trimester screening, or both, for Down's syndrome. N Engl J Med 2005;353:2001–11.
[4] Haddow JE, Palomaki GE, Knight GJ, Williams J, Pulkkinen A, Canick JA, et al. Prenatal screening for Down's syndrome with use of maternal serum markers. N Engl J Med 1992;327:588–93.
[5] Halliday JL, Watson LF, Lumley J, Danks DM, Sheffield LJ. New estimates of down syndrome risks at chorionic villus sampling, amniocentesis, and livebirth in women of advanced maternal age from a uniquely defined population. Prenat Diagn 1995;15:455–65.
[6] Cho CK, Shan SJ, Winsor EJ, Diamandis EP. Proteomics analysis of human amniotic fluid. Mol Cell Proteomics 2007;6:1406–15.
[7] Park SJ, Yoon WG, Song JS, Jung HS, Kim CJ, Oh SY, et al. Proteome analysis of human amnion and amniotic fluid by two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Proteomics 2006;6:349–63.
[8] Gravett MG, Novy MJ, Rosenfeld RG, Reddy AP, Jacob T, Turner M, et al. Diagnosis of intra-amniotic infection by proteomic profiling and identification of novel biomarkers. JAMA 2004;292:462–9.
[9] Cho CK, Smith CR, Diamandis EP. Amniotic fluid proteome analysis from down syndrome pregnancies for biomarker discovery. J Proteome Res 2010;9:3574–82.

[10] Perluigi M, di Domenico F, Fiorini A, Cocciolo A, Giorgi A, Foppoli C, et al. Oxidative stress occurs early in down syndrome pregnancy: a redox proteomics analysis of amniotic fluid. Proteomics Clin Appl 2011;5:167–78.
[11] Cheng PJ, Wang TH, Huang SY, Kao CC, Lu JH, Hsiao CH. Differential proteomics analysis of amniotic fluid in pregnancies of increased nuchal translucency with normal karyotype. Prenat Diagn 2011;31:274–81.
[12] Whiteaker JR, Zhao L, Abbatiello SE, Burgess M, Kuhn E, Lin C, et al. Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. Mol Cell Proteomics 2011;10(4): M110.005645 [Electronic publication, 2011 Jan 18].
[13] Drabovich AP, Diamandis EP. Combinatorial peptide libraries facilitate development of multiple reaction monitoring assays for low-abundance proteins. J Proteome Res 2010;9:1236–45.
[14] Cho CK, Diamandis EP. Application of proteomics to prenatal screening and diagnosis for aneuploidies. Clin Chem Lab Med 2011;49:33–41.
[15] Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (siscapa). J Proteome Res 2004;3:235–44.
[16] Soini Y, Satta J, Maatta M, Autio-Harmainen H. Expression of mmp2, mmp9, mt1-mmp, timp1, and timp2 mrna in valvular lesions of the heart. J Pathol 2001;194:225–31.
[17] Brown DL, Hibbs MS, Kearney M, Loushin C, Isner JM. Identification of 92-kd gelatinase in human coronary atherosclerotic lesions. Association of active enzyme synthesis with unstable angina. Circulation 1995;91:2125–31.
[18] Yang Z, Strickland DK, Bornstein P. Extracellular matrix metalloproteinase 2 levels are regulated by the low density lipoprotein-related scavenger receptor and thrombospondin 2. J Biol Chem 2001;276:8403–8.
Roher AE, Kasunic TC, Woods AS, Cotter RJ, Ball MJ, Fridman R. Proteolysis of a beta peptide from Alzheimer disease brain by gelatinase a. Biochem Biophys Res Commun 2001;205: 1755–61.
[19] Yankner BA, Lu T. Amyloid beta-protein toxicity and the pathogenesis of alzheimer disease. J Biol Chem 2009;284: 4755–9.
[20] Horstmann S, Budig L, Gardner H, Koziol J, Deuschle M, Schilling C, Wagner S. Matrix metalloproteinases in peripheral blood and cerebrospinal fluid in patients with Alzheimer's disease. Int Psychogeriatr 2010;22:966–72.
[21] Bellini C, Rutigliani M, Boccardo FM, Bonioli E, Campisi C, Grillo F, et al. Nuchal translucency and lymphatic system maldevelopment. J Perinat Med 2009;37:673–6.
[22] Shin JW, Jurisic G, Detmar M. Lymphatic-specific expression of dipeptidyl peptidase iv and its dual role in lymphatic endothelial function. Exp Cell Res 2008;314: 3048–56.
[23] Ikushima H, Munakata Y, Ishii T, Iwata S, Terashima M, Tanaka H, et al. Internalization of cd26 by mannose 6-phosphate/insulin-like growth factor ii receptor contributes to T cell activation. Proc Natl Acad Sci U S A 2000;97:8439–44.
[24] Kodvawala A, Ghering AB, Davidson WS, Hui DY. Carboxyl ester lipase expression in macrophages increases cholesteryl ester accumulation and promotes atherosclerosis. J Biol Chem 2005;280:38592–8.
[25] Bocconi L, Nava S, Fogliani R, Nicolini U. Trisomy 21 is associated with hypercholesterolemia during intrauterine life. Am J Obstet Gynecol 1997;176:540–3.
[26] Prasher VP, Airuehia E, Patel A, Haque MS. Total serum cholesterol levels and Alzheimer's dementia in patients with down syndrome. Int J Geriatr Psychiatry 2008;23:937–42.
[27] Williams SJ, Wreschner DH, Tran M, Eyre HJ, Sutherland GR, McGuckin MA. Muc13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. J Biol Chem 2001;276:18327–36.
[28] Chauhan SC, Vannatta K, Ebeling MC, Vinayek N, Watanabe A, Pandey KK, et al. Expression and functions of transmembrane mucin muc13 in ovarian cancer. Cancer Res 2009;69:765–74.
[29] Walsh MD, Young JP, Leggett BA, Williams SH, Jass JR, McGuckin MA. The muc13 cell surface mucin is highly expressed by human colorectal carcinomas. Hum Pathol 2007;38:883–92.
[30] Moehle C, Ackermann N, Langmann T, Aslanidis C, Kel A, Kel-Margoulis O, et al. Aberrant intestinal expression and allelic variants of mucin genes associated with inflammatory bowel disease. J Mol Med 2006;84:1055–66.