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Review

Application of proteomics to prenatal screening and diagnosis for aneuploidies

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Abstract

Current screening for fetal aneuploidies relies on biochemical and ultrasound measurements, and the sensitivity and specificity needs to be improved to reduce the number of pregnant women subjected to invasive diagnostic procedures, such as amniocentesis. Proteomic technologies enable new strategies for discovering biomarkers from complex biological fluids in a high-throughput and sensitive manner. Since mass spectrometry-based techniques allow for both qualitative and quantitative analysis of a given proteome, they have been widely used to resolve and compare the proteome of maternal plasma, serum, urine, cervical-vaginal fluid, and amniotic fluid. Comparisons of proteomes of normal fluids with those from aneuploidy pregnancies have revealed a host of candidate markers that still need to be verified. In parallel with proteomics, there is interest in other emerging techniques, such as RNA-SNP analysis or quantitation of fetal DNA by shotgun sequencing. Although these genomic techniques hold much promise, discovery of additional markers via quantitative proteomic comparisons could drastically improve current conventional screening at reasonable cost. Proteomics-based biomarker discovery is applicable to detection of not just aneuploidies, but also other pregnancy-related diseases.

Keywords: aneuploidy; biomarker discovery; Down syndrome; mass spectrometry; prenatal screening; proteomics.

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Introduction

Prenatal diagnostic tests in modern medicine are offered to detect certain abnormalities of the fetus before birth and can be classified based on the nature of fetal disorders. The first group of diagnostic tests is offered primarily for couples at high risk of certain genetic disorders. These tests can detect hereditary or spontaneous genetic disorders, such as cystic fibrosis, hemophilia A, α- and β-thalassemia, Tay-Sachs disease, and fragile X syndrome. The other group of diagnostic tests is often part of routine prenatal care and can detect structural abnormalities (such as neural tube defects) or chromosomal anomalies (such as trisomies). Unfortunately, this latter group of diagnostic tests, such as amniocentesis, chorionic villus sampling, and percutaneous umbilical blood sampling, are invasive procedures that are associated with the risk of loss of pregnancy, as well as inevitable waiting time and pain (1). Consequently, much emphasis has been placed on the development and improvement of non-invasive or minimally invasive screening tests in order to decrease the number of pregnant women being subjected to such invasive procedures (2).

Prenatal screening aims to estimate a woman's risk of having an affected pregnancy on the basis of factors, such as concentration of certain analytes (biomarkers) and advanced maternal age. The first routine screening test for pregnant women was offered in the 1980s (3–5), and screening tests have since been improved by incorporation of multiple biochemical and ultrasound markers to better detect structural anomalies and trisomies (3, 4, 6–10). Screening tests play an important role in significantly increasing the diagnosis for affected pregnancies, while reducing the frequency of unnecessary invasive tests. Previously, a high-risk group was characterized by risk factors including advanced maternal age, history of Mendelian disorders, and previous exposure to certain environmental factors. However, with the advancement of screening tests, a more precise definition of the high-risk group has been obtained based on the measurements of markers. As a result detection of chromosomal abnormalities from women who were once considered as a low-risk group (≤35 years of age) has become more efficient.

Today, the largest group of pregnant women who opt for invasive tests consists of those whose screening results indicate high risk for aneuploidies. Moreover, among all pregnancy-related pathologies, aneuploidies and a few other structural abnormalities are the only disorders for which rou-

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Table 1 Current markers used for routine prenatal screening and their applications.

Abnormalities	Markers	Screening type
Down syndrome (i.e., Trisomy 21)	Pregnancy-associated plasma protein A α-Fetoprotein Human chorionic gonadotropin Unconjugated estriol Inhibin A	Maternal serum screening (MSS)
Neutral tube defects (e.g., spina bifida)	α-Fetoprotein Human chorionic gonadotropin Unconjugated estriol	MSS
Trisomy 18 (Edwards syndrome)	α-Fetoprotein Nuchal translucency	MSS Ultrasonographic screening
Trisomy 13 (Patau syndrome)	Nuchal translucency	Ultrasonographic screening

tine screening is available (Table 1). Hence, this review will focus on the prenatal screening and diagnostic tests that are available for aneuploidies, with an emphasis on Down syndrome (DS). This review will also explore the possibility of applying new paradigms and technologies to improve screening tests for these disorders as well as other pregnancy-associated abnormalities.

DS is the most common congenital anomaly, with a prevalence of 1 in 732 in the US (11). It is caused by triplication of chromosome 21. The current screening tests for DS involve risk calculation based on measurements of biochemical markers in the maternal serum, often complemented by ultrasound for fetal nuchal translucency, to increase the overall detection rate to 90%–95% (2). Screening in the first-trimester is performed between 11 and 14 weeks' gestation, based on ultrasound, pregnancy-associated plasma protein A (PAPP-A), and the free β chain of human choriogonadotropin (hCG-β); and in the second trimester, between 15 and 20 weeks, based on α-fetoprotein (AFP), unconjugated estriol, inhibin A, and hCG-β. Despite intense efforts to improve current screening, the highest obtainable detection rate is 95% at a false positive rate of 5% (2, 12). Moreover, these markers lack specificity and are used to detect not only DS, but also Trisomy 18 and neutral tube defects (Table 1). The detection rate is even lower if a pregnant woman misses the first trimester screening or if certain screening techniques (e.g., nuchal translucency) are not offered (2, 13, 14).

Due to the uncertainties associated with the outcome of current screening, many pregnant women and physicians feel compelled to choose invasive diagnostic procedures which afford an accuracy of approximately 99%. A recent study showed that most (92%) obstetrician–gynecologists in America routinely offer these tests for women aged $\geq$35 years (15). Even for the age group of those $\leq$35 years, amniocentesis is frequently offered based on the patient request or abnormal screening results. Therefore, of all women undergoing invasive tests, only 1%–5% actually have an affected fetus (16, 17). Therefore, to further reduce, or even eliminate, unnecessary invasive procedures, screening tests for prenatal abnormalities need to be further improved in terms of predictive power and specificity.

Developments in proteomics and application to prenatal biomarker discovery

The post-genomics era has been enabled by the development of mass spectrometry (MS) and extensive bioinformatic resources. MS-based proteomics allows global analysis of the complex proteome of any biological compartment at a given condition and time. Moreover, there is much promise that MS-based diagnostics will be robust, accurate, rapid and high-throughput for a number of diseases and conditions. Therefore, proteomic analysis has become a popular platform for discovery of biomarkers for numerous pathologies.

Pregnancy progression and birth involve complex feto-maternal physiological processes that rely on intricate interactions at multiple levels. Therefore, when a major problem arises the balance among these interactions will be disturbed at more than one level. Since proteins constitute the functional units of genes, such disturbances, as well as changes in the number of gene copies and/or gene regulatory mechanisms, will be subsequently reflected at the level of protein production and expression. Given that the presence of an extra chromosome in trisomies causes disruptions in gene expression, identification of proteins that are involved in altered biochemical pathways may provide insights into molecular mechanisms of trisomy phenotypes, potential therapeutic targets, and diagnostic proteins that could be detected in maternal blood.

The majority of proteomic analyses for biomarker discovery thus far can be grouped into a few categories, depending on their objectives. In this review, two major groups are presented and discussed because they fully represent the previous proteomic investigations in the field of prenatal diagnostics. The first group of studies can be referred to as discovery phase or descriptive proteomic studies, since they aim to achieve global proteomic profiles of relevant biological samples, such as plasma, utilizing prefractionation techniques. The second group of studies can be referred to as comparative proteomic studies, since they aim to discover differentially expressed proteins that could potentially serve as biomarkers. These studies rely either on similar technologies as the discovery phase studies, or they utilize quanti-

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tative techniques that involve differential labeling of proteins or peptides for the different conditions.

In either case, in-depth analysis of proteomes of biological fluids almost always involves prefractionation methods in order to detect low-abundance proteins, because no amplification method (analogous to polymerase chain reaction for nucleic acids) exists for proteins. Prefractionation can be done by many different techniques, but the two most frequently used are gel electrophoresis (GE) and liquid chromatography (LC). For proteomics purposes, three different kinds of GE are often employed: one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional GE (2DGE), and difference in-gel electrophoresis (DiGE). 2DGE is the most commonly used for its power to separate proteins in two dimensions, according to their isoelectric point in the first dimension and to their molecular weight in the second dimension. 2DGE can be advantageous due to easy visualization of large numbers of proteins. Therefore, it has been frequently used for studying aneuploidy samples and controls. The disadvantages of 2DGE include a narrow dynamic range (10^2–10^4) compared to the actual dynamic range observed in the plasma proteome (10^{10}), and bias against low-abundance, hydrophobic, low- or high-molecular weight proteins (18). LC can be used multi-dimensionally, and two-dimensional LC (2DLC) is gaining in popularity due to a number of advantages, including its resolving power. In this method, proteins are often digested first, and the resulting peptides are separated by chromatography based on a different property, such as charge, followed by another chromatographic separation based on another property, such as hydrophobicity. This powerful technique demonstrates higher reproducibility and rigorous fractionation, but requires more time for development compared to 2DGE. 2D-LC coupled to tandem mass spectrometry (MS/MS) is now commonly known as multi-dimensional protein identification technique (MudPIT).

Proteomic analysis of pregnancy-related biological fluids

To develop a non-invasive screening or diagnostic test, attention should be paid to samples that can be obtained with minimally invasive procedures. This includes samples, such as maternal blood, maternal urine or cervical-vaginal fluid. International efforts to improve current screening for DS have resulted in proteomic studies of several related biological fluids.

Human blood (serum and plasma) has been frequently analyzed to identify differentially expressed proteins for pregnancy-related conditions. For proteomics experiments, plasma is often preferred over serum due to its advantage as a starting material: plasma can be obtained by bypassing the process of clotting which activates proteases that can degrade proteins, creating a bias in the proteome of interest. Human plasma has held promise as an effective medium for diagnosis and monitoring of human diseases, and has been the primary clinical specimen for a long time. Hence, the plasma proteome has been analyzed extensively for a number of conditions, since comparisons between pathological and normal conditions, in theory, is expected to reveal potential biomarkers for a variety of diseases. Unfortunately, studies to date have shown that the plasma proteome is probably the most complex and challenging among all human-derived proteomes due to two main reasons. First, the blood proteome contains sets of the subproteome which originates from other human tissues. Moreover, the plasma proteome boasts a huge dynamic range of individual protein concentrations (10^{10}), and a limited number (approximately 10) of high-abundance proteins, such as albumin and transferrin, which account for 90% of the total protein content (19). As a result, much effort has been invested to develop methods to reduce the complexity of plasma proteome through depletion of high-abundance proteins and/or utilizing effective fractionation technologies. Both strategies allow improved identification of low-abundance proteins; for example, the use of a multiple fractionation method has shown to reveal a set of proteins that may be normally masked by high-abundance proteins under different conditions.

To date, a very limited number of studies analyzed maternal blood in search for biomarkers of DS. The first study that analyzed maternal blood proteomically to identify biomarkers of DS combined both top-down and bottom-up approaches for a total of 56 first- and second-trimester maternal serum samples from DS and controls, matched according to gestational age. Nagalla et al. utilized fluorescence 2DGE, 2DLC-chromatofocusing, 2D-liquid chromatographic, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) approaches to characterize maternal serum proteins, resulting in a list of 25 proteins that were identified as differentially abundant in DS serum; the majority of which were mid- to high-abundance glycoproteins (20). They reported nine proteins as candidate markers based on the results from multiple (two or three) approaches.

In 2008, Kolialexi et al. reported the identification of differentially expressed proteins in maternal plasma from DS-affected pregnancies (21). Their approach involved 2DGE and MALDI-TOF-MS to analyze maternal plasma from the second-trimester for eight DS-affected and 12 normal pregnancies. Comparison of approximately 900 protein spots per gel revealed nine proteins that showed differential density: transthyretin, ceruloplasmin, afamin, α1-microglobulin, apolipoprotein E, amyloid P component, histidine-rich glycoprotein, α1-antitrypsin, and clusterin. Two of these candidates, amyloid P component and apolipoprotein E, were verified by Western blot using four DS and four normal plasma samples.

The limitation of these two studies is that most of the findings and candidate biomarkers are, in fact, high-abundance proteins since the majority of low-abundance proteins are difficult to quantify due to ion suppression effects. Quantitative methods, such as isobaric tag for relative and absolute quantitation (iTRAQ), may render easier quantification of low-abundance proteins (22). Kolla et al. used four-plex iTRAQ labeling to compare six maternal plasma samples from the first-trimester of DS-affected pregnancies against six normal controls (23). A total of 50 proteins were reported to be differentially expressed out of 235 proteins that were identified. This study reported proteins with a variety of

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functions and expression levels, and further verification of the 50 listed proteins may reveal potential biomarkers to improve current screening.

Despite the differences in strategies and the nature and the number of samples used, three aforementioned studies demonstrate how difficult it is to perform reliable qualitative and quantitative analysis of the human blood proteome. For instance, the coverage of the plasma proteome by these studies represents only a fraction of the total plasma proteome. A non-redundant list of 1175 plasma proteins was compiled as early as 2004, but <200 proteins were common across different studies. To date, over 7518 proteins and isoforms have been identified from 3778 unique genes, but this number reflects a collective effort to achieve comprehensive plasma proteome coverage and not the coverage capacity of an individual study. Since only a small fraction of the total plasma proteome was represented in the comparative studies thus far, it would be difficult to conclude that the reported differentially expressed proteins reflect the true differences between AF-affected and normal pregnancies.

Contrary to plasma, urine and cervical-vaginal fluid have received relatively little attention for their potential as a reservoir of biomarkers for pregnancy-specific diseases. Urine proteins originate from a number of sources, such as plasma, vasculature, and the urogenital system. The issues involved with use of urine for proteomics studies include prevention of microbial growth, having to remove cells that may lyse, and concentration (19). Thus far, the urine proteome has been studied by a number of groups using 2DE or 2D-LC followed by MS/MS for both normal and disease conditions, and the most exhaustive study revealed as many as 2362 proteins (24–27). Urine holds much promise for non-invasive diagnosis of different diseases. However, due to its complexity, the urine proteome has been primarily studied in search for potential markers for renal diseases (28).

Cervical-vaginal fluid (CVF) is a complex biological fluid that protects and lubricates the uterine, cervical and vaginal area of the female reproductive tract. CVF contains proteins predominantly synthesized by the endocervix and vaginal cells (29). However, during pregnancy the CVF proteome changes due to leakage of AF into CVF caused by disruption and secretion of the chorionic-decidual interface. Since CVF can be obtained non-invasively, unlike AF, CVF may be an important diagnostic resource for pregnancy or vaginal pathologies. Dasari et al. and Pereira et al. were among the first groups to realize this potential; they identified 205 proteins from CVF (30, 31). Dasari et al. compared the CVF proteome with AF to show that some proteins are indeed common between the two fluids (30). Shaw et al. utilized two different fractionation approaches, SDS-PAGE and strong cation exchange chromatography, followed by LC-MS/MS, and identified 685 proteins (32). Another study by Di Quinzio et al. applied 2D-SDS-PAGE to detect 15 proteins common to five pregnant women at term (33). To date, the CVF proteome has been studied to discover markers for preterm labor and intra-amniotic infections (34). Therefore, further investigation will be necessary to explore the possibility of using urine or CVF for diagnosis or screening of aneuploidies.

A major limitation common to urine, serum, and cervical-vaginal fluid is that these fluids are not pregnancy-specific. Pre-existing (i.e., non pregnancy-related) proteins inevitably complicate the discovery of proteins that will closely reflect the well-being of the fetus. Since identification of proteins specific to pregnancy or the fetus will help better understand the physiology of pregnancy and fetal development, amniotic fluid (AF) has been a popular medium of proteomics analysis. AF is a complex mixture of proteins, amino acids, carbohydrates, hormones, lipids, and electrolytes that originate from fetal tissues and organs, amnion epithelial cells, maternal circulation, and the placenta. Not surprisingly, AF has great potential to reveal biomarkers that are specific for fetal diseases or complications of pregnancy.

Since 1997, proteomic profiling of human AF has been performed by several groups utilizing different approaches. Liberatori et al. identified 31 proteins by 2D-GE followed by N-terminal sequencing to identify proteins (35). Nilsson et al. were the first group to use MS-based proteomics as well as depletion of albumin to reduce the complexity (36). They identified 58 proteins in albumin-depleted AF from the 15th to 18th weeks of gestation using LC-Fourier transformion cyclotron resonance MS. Park et al. reported 37 proteins from AF supernatant using 2DGE followed by MALDI-TOF MS (37). Tsangaris et al. reported 136 proteins by 2DE followed by MALDI-MS/MS (38). In 2007, more in-depth AF analyses were reported by different groups. Queloz et al. compared the proteome of AF from different weeks of gestation to identify the proteins that are expressed in greater quantity during the first-trimester (39). Michaels et al. also compared profiles of AF proteome from the first-, second-, and third-trimester to identify proteins that show differential expression between the first- and second-trimesters (40). They identified 69 AF proteins from albumin-depleted AF using Off-Gel™ electrophoresis/LC-MS/MS. Cho et al. generated the most extensive proteome profiling of normal AF from the second-trimester, reporting 1026 unique gene products from 842 genes (41). In this study, most of the clinically used and putative biomarkers for pregnancy-associated diseases were identified. For example, DS biomarkers, such as hCG-β, AFP, and inhibin A were identified, and some of these biomarkers and putative markers were identified for the first time by MS in AF.

In the pursuit of discovery of novel biomarkers for DS and other aneuploidies, several comparative proteomic profiling studies of AF samples, obtained from pregnancies with aneuploid fetuses, have been performed. Wang et al. used a variety of surface arrays followed by pattern recognition algorithms to analyze 20 AF samples from normal and aneuploid fetuses (42). They reported a distinct peak at 2.65–7 kd which could distinguish aneuploidy samples from controls. Oh et al. characterized AF from DS-pregnancies using proteomic techniques and reported several potentially disrupted metabolic pathways, such as carbohydrate and amino acid handling, and purine and intermediary metabolism (43). Tsangaris et al. compared AF from DS- and unaffected pregnancies using 2DE and MS to identify a total of 28 proteins (44). They reported seven proteins as potential markers:

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α1-microglobulin, α1 type I collagen, α1 type III collagen, heparan sulfate proteoglycan 2, α1 type V collagen, insulin-like growth factor binding protein, and Pre-mRNA-splicing factor SRP75. The same group also studied AF from pregnancies with Turner syndrome and reported seven biomarkers: serotransferrin, lumican, plasma retinol-binding protein and apolipoprotein A-I (APOA1), all of which showed an increase in Turner syndrome. In addition, they found that kininogen, prothrombin, and apolipoprotein A-IV showed a decrease. Mange et al. used a combination of ProteinChip technology and rule-based analysis to screen for aneuploidies using AF samples. They showed a predictive value of 90% (45). Wang et al. analyzed AF proteins from pregnancies with DS and Trisomy 18 by 2D-LC followed by MS/MS, and tested some of the candidates by Western blot and ELISA (46). A group of differentially expressed proteins were APOA1, serpin peptidase inhibitor, clade A, prealbumin, and transferrin for DS, and APOA1, AP-3mu, placental protein-14 and antitripsin for Trisomy 18.

Given that human AF contains over a thousand proteins and isoforms, the previous studies identified only a relatively small fraction of the entire proteome, resulting in candidates that are mostly highly abundant proteins. For example, proteins, such as transferrin, antitrypsin, and apolipoproteins have been reported to be in the top 15 most abundant proteins in AF. To assemble a list of candidate proteins that encompasses mid- to low-abundance proteins, in addition to the high-abundance ones, more extensive quantitative comparisons between unaffected and DS-affected AF proteomes has been conducted by Cho et al. (47). In this study, spectral counting-based shotgun comparison was reported for 542 proteins that were identified and quantified based on two or more unique proteins. A total of 60 candidates have been identified, and two of these candidates, amyloid precursor protein and tenascin-C, have been verified by ELISA in 20 individual AF samples. Seven proteins from chromosome 21 were identified, and all showed increased expression in DS-AF compared to controls, supporting the long-standing ''gene-dosage hypothesis''.

AF has two major advantages over plasma as a reservoir of biomarkers for aneuploidies. First, there exists a significantly larger amount of fetal and pregnancy-related proteins in AF. Second, the complexity of AF proteome poses less challenge for proteomic analysis than that of plasma. However, the disadvantage of using AF is that once identified, candidate proteins must be verified in maternal blood before they can be used for non-invasive diagnostic test. Proteins that originate from the fetus or placenta will inevitably be diluted in maternal plasma, and possibly be masked by higher-abundance maternal proteins. Consequently, there is a chance that some of these low-abundance candidates will be difficult to quantify in the maternal circulation.

Comparison of proteomics vs. non-proteomics approaches for DS screening

Fetal components found in maternal blood include not only proteins from the fetus and placenta, but also fetal cells and nucleic acids that are either cell-free or from nucleated red cells (48), lymphocytes (49), and trophoblasts (50). Fetal cells, among these constituents, were the first to be discovered. The first type of fetal cells to be observed were trophoblasts, discovered in 1893. Afterwards, fetal lymphocytes and nucleated erythrocytes were discovered in maternal blood. Despite many investigations to utilize fetal cells for non-invasive diagnosis, fetal cells pose great challenges as they are rarely found, and they are difficult to be isolated or separated from maternal cells.

The presence of fetal cell-free DNA in maternal plasma was first identified in 1997. Over 80% of these circulating fetal DNA fragments are short ($\leq$200 bp) (51), and evidence indicates that the placenta is the major source. These DNA fragments constitute a small fraction (3%–6%; increasing with the gestational age) of the total cell-free DNA content present in maternal serum (52). Therefore, a highly sensitive analytical method must be employed to detect this subpopulation among a heterogeneous mixture of DNA with dominating maternal DNA. Free fetal mRNA is known to result from different sources, such as the syncytiotrophoblast, the hematopoietic system and the placenta. It has been detected as early as the 4th week of gestation, demonstrating its potential as diagnostic material. Therefore, given the difficulties associated with fetal cells, fetal cell-free nucleic acids (i.e., DNA and RNA) have received increasing attention for the development of non-invasive diagnostic tests.

Fetal DNA has been used successfully to determine fetal rhesus blood group antigen D (RHD) status, and to diagnose fetal transmission of genetic disorders, such as X-linked disorders (53–56). Identification of fetal gender is another application of cell-free DNA in maternal blood, with a sensitivity of 96% (344/359) and specificity of 100% (317/317) (57). In addition, the post-genomics technological advancements in the field of transcriptomics enabled analyzing fetal nucleic acids to prove the ''gene-dosage hypothesis.'' As predicted by this hypothesis, which states that an extra copy of a chromosome would result in a 1.5-fold increase in the mRNA of the genes located in the chromosome, analysis of AF from DS-affected pregnancies by quantitative real-time PCR showed a 1.5-fold increase in cell-free fetal DNA from chromosome 21 (58).

Unfortunately, use of fetal DNA for diagnosis of aneuploidy has been more challenging due to a number of limitations. First, the amount of fetal DNA is very limited in the maternal circulation, especially during the first-trimester when screening should ideally be performed. One study reported only 3.4% free DNA in the late first-trimester to mid second-trimester, and such a low amount makes the analysis more difficult (52). Second, most fetal DNA measurement methods rely on quantifying Y-specific sequences, and therefore can only be applied to male fetuses. Third, distinguishing fetal DNA fragments from chromosomes of clinical interest (i.e., chromosome 13, 18, and 21) apart from maternal DNA has been exceedingly difficult. Recently, several developments have been made to address these issues. One approach involved enrichment of fetal DNA or suppressing the maternal DNA background based on the mean

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size difference between the two groups. For example, Lo et al. reported that use of formaldehyde increased the proportion of fetal DNA up to 25% (59, 60). Still, these improvements are not perfect, and the methodologies are known to be difficult to reproduce, labor intensive, and undesirable due to the toxicity of formaldehyde (61).

Another approach to detect aneuploidies using fetal nucleic acids involves measuring the ratio of alleles for a single-nucleotide polymorphism (SNP) in cell-free mRNA in maternal blood (62, 63). Cell-free mRNA is believed to originate from the hematopoietic system (64) and the placental components, such as syncytiotrophoblasts (65), and is often protected from degradation due to enclosure by subcellular vesicles (66). The premise of the RNA-SNP approach is as follows: if a specific gene within a chromosome of interest is heterozygous, then the ratio between the two alleles would be 1:1 in a euploid fetus, whereas the ratio will be 2:1 or 1:2 in a fetus with triplication of this chromosome. Therefore, one critical condition that must be met for this approach is that selection of informative SNPs must be done carefully so that they are specific not only for the fetus, but also for the chromosome of interest. Placenta-specific 4 (PLAC4) mRNA is transcribed from chromosome 21 in the placenta (67) and has been shown to be a specific marker for the fetus in maternal circulation (63). Unfortunately, since not all fetuses are heterozygous for PLAC4, PLAC4 RNA-SNP allelic ratios show a sensitivity of 90% and specificity of 96.5% for detecting DS (63).

The RNA-SNP approach requires that the amount of fetal mRNA be sufficient in maternal serum to ensure reliable measurement of allelic ratios. Recently, a ''digital RNA-SNP'' approach based on digital PCR has been developed to meet this requirement, allowing precise counting of the number of each allele. Integration of measuring total PLAC4 RNA concentration and detecting RNA-SNPs ratio increased the diagnostic sensitivity and specificity (68).

Recently, a new technology, shotgun sequencing of fetal DNA from maternal blood, also showed potential for detection of aneuploidies, including DS, Trisomy 18, and Trisomy 13 (69). Briefly, Fan et al. sequenced directly cell-free DNA and obtained sequence tags followed by mapping to the chromosome of origin to achieve digital quantification of DNA. Similarly, Chiu et al. used massively parallel sequencing-by-ligation of maternal plasma DNA and calculated genomic representations of sequenced reads from chromosomes to detect Trisomy 13, 18 and 21 (70). Sequencing DNA to detect aneuploidies is currently costly, and both studies were based on a very limited number of samples ($n_{total} = 15$ for both), which were somewhat biased by gender and gestational age. Further improvements in the use of massively parallel sequencing will certainly benefit the field of diagnostics for pregnancy-associated diseases as well as other fields, such as developmental biology.

Improvements in either the maternal screening tests or the diagnostic methods for DS will benefit society in many ways. For the new technologies are emerging for the use of fetal DNA for non-invasive prenatal diagnosis, it is important to verify their predictive power and specificity using a large number of samples before they can be introduced into clinical practice. It is therefore clear that the inexpensive and fast multi-parametric screening test for DS will continue to be used for a while, especially if discovery of additional biomarker improves the predictive power of the current screening.

Conclusions

Proteomic studies based on MS and bioinformatic databases offer a powerful platform for biomarker discovery, and there exists increased hope that identification of novel protein biomarkers will revolutionize diagnostics for many diseases. Currently, validation of candidate biomarkers stands as the major bottleneck in the field of biomarker-related proteomics (71), and this trend is conspicuous throughout the studies presented in this paper. One of the ways to alleviate this problem would be to identify candidates based on highly stringent criteria. Furthermore, it would be prudent to confirm the presence of established markers from the list of candidates in a qualitative study, and the up/down-regulation of the established markers in a quantitatively study.

There are a few other caveats to note in order to achieve the desired outcomes through proteomic experiments, especially when dealing with complex samples. First, biological samples must be collected carefully and processed to minimize human errors and technical variability. Second, optimization of prefractionation methodologies must be performed to obtain increased depth of proteome coverage. Third, the balance between the extent of protein identification and the false detection rate must be stringently modulated (e.g., by applying high probability of protein or require two or more unique peptides per protein identified) so that fewer insignificant ''hits'' are reported. Finally, due to the inherent variability among biological samples, it is recommended that replicates be processed to confirm the list of differentially expressed proteins. One major limitation of current proteomic approaches is that the performance of candidate markers discovered via proteomic approaches is not reproducible between studies or laboratories. This reproducibility issue is pronounced primarily for candidates that were identified by either a ''discovery phase'' profiling or by using semi-quantitative platforms.

Much promise lies in proteomic approaches to discover additional biomarkers with improved predictive power for the detection of DS. Such novel markers will either improve the current multi-parametric screening or replace the current diagnostic methods with one that is non-invasive. Moreover, the newly found biomarkers will likely be associated with biochemical pathways that could lead to prospective studies to further understand the mechanisms associated with DS. Findings from biological fluids, such as AF, CVF, urine, and plasma will also extend our knowledge on other aneuploidies and pregnancy-related conditions, and we may be able to apply similar paradigms to develop diagnostic methods for these pathologies in the future.

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