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Gene Expression Analysis of Amniotic Fluid: New Biomarkers and Novel Antenatal Treatments

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Introduction

Over the past thirty years there has been a significant increase in the use of prenatal diagnosis to improve perinatal outcome. Frequently, at least in developed countries, this involves prenatal sonography to image fetal anatomy and detect congenital anomalies. This has led to a primarily surgical approach to fetal and neonatal treatment. While effective, this strategy has revealed little about fetal *functional* development.

Since 2005, our laboratory has explored the hypothesis that valuable fetal developmental gene expression information is present in amniotic fluid (AF). In pilot studies performed using large quantities of amniotic fluid obtained from pregnancies complicated by polyhydramnios, we showed that cell-free fetal messenger RNA (cff mRNA) could be successfully hybridized to gene expression microarrays. Comparative analyses of the arrays from different subjects demonstrated that fetal gene expression changes could be detected as a result of fetal sex, gestational age, and disease status [1]. Furthermore, we showed that cff mRNA in AF originated from the fetus and not the placenta, which suggested that AF could be used directly to analyze fetal development and well-being.

AF is widely available but generally under-utilized [2]. It is obtained primarily for fetal chromosome analysis and alpha-fetoprotein measurement to diagnose open neural tube defects. AF is an excellent source of material for research, as large quantities of supernatant are obtained for clinical diagnosis and then discarded, samples generally have correlated medical record and karyotype information available, and there is no contamination by maternal nucleic acids [2]. AF provides a way to directly query fetal development from as early as 15 weeks of gestation. We have used cff mRNA in AF to identify new biomarkers of fetal disease, develop new insights into fetal pathophysiology, and to suggest new approaches to fetal treatment.

Methods

In general, our methods can be grouped into four categories: isolation and extraction of RNA from AF, hybridization of cDNA to gene expression arrays, initial statistical analysis to identify a list of differentially-regulated probe sets and their corresponding genes, and

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functional analysis of the identified gene lists to identify the networks, diseases, and disorders associated with the differentially-regulated genes [Figure 1]. Although at first we required large quantities of AF, our newer methods require only 5–10 ml [3, 4]. Briefly, we perform RNA extraction using TRIzol/chloroform and purify it using Qiagen columns. We then synthesize cDNA, fragment it, and label it with biotin before hybridization to Affymetrix U133 Plus 2.0 arrays. Initial analysis is performed using the GeneChip Microarray Suite 5.0 (Affymetrix) and results are normalized [3, 4]. Genes that are differentially-expressed between two groups are identified via two-sided paired t tests with the multtest package in Bioconductor, using the Benjamini-Hochberg false discovery rate adjustment (FDR) adjustment for multiple testing (adjusted P value < 0.05). Functional analysis of the gene lists is performed in Database for Annotation, Visualization, and Integrated Discovery (DAVID), a publicly-available resource, and Ingenuity® Pathways Analysis (IPA) software, version 7.5, a commercially-available resource (www.ingenuity.com). Lastly, the Connectivity Map (www.BroadInstitute.org/CMap), a publicly-available reference collection of gene expression profiles from human cultured cells treated with bioactive small molecules corresponding to US Food and Drug Administration (FDA) approved drugs, is used to identify possible new therapies. The Connectivity Map received its name because it reveals connections between diseases, genes, and drugs.

Results

To test whether analysis of cff mRNA adds novel prenatal diagnostic information, we have studied AF from fetuses with the two most common human autosomal aneuploidies, trisomies 21 and 18 [3, 4] and compared the results to euploid fetuses matched for sex and gestational age. In the first study of seven living second trimester fetuses with trisomy 21 we found 414 differentially-expressed probe sets, which corresponded to 311 annotated genes. Unexpectedly, only 5 of the genes physically mapped to chromosome 21. These included *CLIC6* (a chloride intracellular channel protein), *ITGB2* (integrin beta chain beta 2), *RUNX1* (a transcription factor associated with hematopoiesis), *C21orf67*, and the hypothetical protein *LOC642582*. In the second study of five living second trimester fetuses with trisomy 18 we found 352 differentially-expressed probe sets, which corresponded to 251 annotated genes. Only seven of these genes were physically located on chromosome 18, including *ROCK1* (RHO-associated, coiled-coil containing protein kinase 1), *EMILIN2* (elastin microfibril interface 2), *ONECUT2* (a homeobox transcription factor), *ATP8B1* (an ATPase class 1 gene), *ZNF516*, *MGC11082*, and the hypothetical protein *LOC339290*. We examined the genes identified in the trisomy 21 and the trisomy 18 gene sets, and found that only six of them were differentially regulated in both aneuploidies (as compared to euploid controls). Of these, four were up-regulated in both aneuploidies (*ROCK1*, *NKX1-1* [NK1 homeobox 1], *CCDC85B* [coiled-coil domain containing 85B], *TTLI2* [tubulin tyrosine ligase-like family, member 12]) and two were down-regulated in both (*CYP2C9*, a cytochrome P450 family gene, and a solute carrier gene, *SLC25A33*). These results showed that (from the gene-expression perspective) each chromosome abnormality is essentially a distinct condition, and the differential gene regulation was not due to a generalized process associated with aneuploidy. Subsequent functional pathway analyses demonstrated that response to oxidative stress was the major problem in the fetuses with trisomy 21 [2], along with disruption of ion transport, G protein signaling, immune and stress response, circulatory system function, and sensory perception. Analysis of the trisomy 18 fetuses specifically highlighted abnormalities in pathways associated with adrenal development, as well as ion transport, immunity, DNA repair, and G protein signaling [3].

The trisomy 21 gene list was entered into the Connectivity Map database, and the following molecules were suggested to “reverse” the fetal phenotype: celastrol, a potent antioxidant,

dimethyloxalylglycine (DMOG), and scriptaid (a histone deacetylase inhibitor). Experiments are ongoing in cultured amniocytes from fetuses with trisomy 21 to determine if administration of antioxidants improves the integrity of nuclear DNA in an assay called the “comet” test. If we have *in vitro* proof of therapeutic effect, we will move on to mouse models.

Discussion

In this brief report I have tried to highlight some of our laboratory’s major findings that were derived by performing transcriptomic analyses of cff mRNA in second trimester AF. The first conclusion is that in each fetal aneuploidy, there are multiple dysregulated genes that are not located on the trisomic chromosome. Thus, aneuploidy has a *significant downstream effect on gene expression*. Additionally, each chromosome abnormality appears to have a unique pattern of differentially-regulated genes that is common amongst fetuses with the same aneuploidy, but differs from euploid fetuses at the same gestational age. These aneuploidy-associated abnormal gene expression patterns are apparent by the second trimester of pregnancy, and are easily visualized in heat map analyses [3, 4]. Second, these types of unbiased, discovery-driven approaches lead to the identification of new genes involved in fetal abnormalities. For example, *ROCK1* is up-regulated in both trisomies 18 and 21 and physically maps to chromosome 18. *ROCK1* is up-regulated at the onset of the epithelial-mesenchymal transition phase in early cardiac development. It plays an important role in endocardial differentiation and migration [4]. Prior to our study, *ROCK1* was not known to have a role in aneuploidy, but our results suggest that it should be further investigated to understand the mechanisms underlying abnormal cardiac development in trisomies 18 and 21. In another condition, twin to twin transfusion syndrome (TTTS), we identified a specific water transport gene, *aquaporin1*, that was increased 18 fold in affected fetuses [1]. This gene may have a role in the polyhydramnios that develops in TTTS.

Functional analyses of the differentially-regulated genes in each condition led to new insights into the pathophysiology of each aneuploidy. For example, oxidative stress is the predominant problem in trisomy 21. This suggests that antenatal treatment of the mother with antioxidants may improve neurocognition in Down syndrome, which is a testable hypothesis in mouse models. If this approach works, it could be used in other well-characterized fetal anomalies that are not associated with an abnormal karyotype, ushering in an era of fetal personalized medicine.

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References

1. Larrabee PB, Johnson KL, Lai C, Ordovas J, Cowan JM, Tantravahi U, Bianchi DW. Global gene expression analysis of the living human fetus using cell-free messenger RNA in amniotic fluid. *JAMA*. 2005; 293:836–842. [PubMed: 15713773]
2. Hui, L.; Bianchi, DW. Cell-free fetal nucleic acids in amniotic fluid. *Hum Reprod*. 2010. Update, http://www.oxfordjournals.org/our_journals/humupd/about.html, Epub Oct. 5
3. Slomin DK, Koide K, Johnson KL, Tantravahi U, Cowan JM, Jarrah Z, Bianchi DW. Functional genomic analysis of amniotic fluid cell-free mRNA suggests that oxidative stress is significant in Down syndrome fetuses. *Proc Natl Acad Sci USA*. 2009; 106:9425–9429. [PubMed: 19474297]
4. Koide, K.; Slonim, DK.; Johnson, KL.; Tantravahi, U.; Cowan, JM.; Bianchi, DW. Transcriptomic analysis of cell-free RNA suggests a specific molecular phenotype in trisomy 18. *Hum Genet*. 2010. <http://www.springer.com/biomed/human+genetics/journal/439>, E pub Dec 9

Figure 1. Overall approach to the analysis of cell-free fetal messenger RNA in the supernatant of amniotic fluid.