PRELIMINARY COMMUNICATION

Global Gene Expression Analysis of the Living Human Fetus Using Cell-Free Messenger RNA in Amniotic Fluid

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Context No molecular biological tests are available to monitor the ongoing development of human fetuses in vivo.

Objective To determine whether cell-free fetal messenger RNA (mRNA) in amniotic fluid can be detected using oligonucleotide microarrays to study large-scale gene expression in living human fetuses, with analysis of sex, gestational age, and fetal pathology as variables.

Design, Setting, and Patients Four samples of cell-free amniotic fluid were analyzed from pregnant women between 20 and 32 weeks' gestation and undergoing amnioreduction for polyhydramnios associated with twin-twin transfusion syndrome or hydrops fetalis (cases). The control consisted of 6 pooled amniotic fluid samples from women at 17 weeks' gestation and undergoing genetic amniocentesis. After extraction from the normally discarded fraction of amniotic fluid, RNA was amplified twice, labeled, and analyzed using gene expression microarrays.

Main Outcome Measure Relative mRNA expression in cell-free samples of amniotic fluid from fetuses with polyhydramnios at different gestational ages vs cell-free amniotic fluid from a pooled control.

Results Thirty-six percent of 22 283 probe sets represented on the arrays were present in the cell-free amniotic fluid, and a median of 20% of all probe sets differed between cases and the pooled control. Only male samples expressed 1 Y chromosome transcript. The expression of some developmental transcripts, such as surfactant proteins, mucins, and keratins, changed with gestational age by up to 64-fold. A water transporter gene transcript was increased up to 18-fold in both twin-twin transfusion samples. Placental gene transcripts were not present in any samples.

Conclusions This pilot study demonstrates that cell-free fetal mRNA can be extracted from amniotic fluid and successfully hybridized to gene expression microarrays. Preliminary analysis suggests that gene expression changes can be detected in fetuses of different sexes, gestational age, and disease status. Cell-free mRNA in amniotic fluid appears to originate from the fetus and not the placenta.

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Because of the inherent difficulties with research on human fetuses, the analysis of fetal gene expression has in large part been limited to the examination of tissue from human abortuses and the assessment of animal models for genes and developmental pathways that are conserved across species. Fetal monitoring in vivo is limited to noninvasive methods such as the measurement of uterine size or anatomic evaluation by fetal sonography. In addition, genetic analysis can be performed on amniotic fluid components, including amniocytes, which typically require time-consuming expansion in vitro before use, and cell-free proteins in the amniotic fluid, such as α-fetoprotein, which can serve as biomarkers for genetic anomalies. The cell-free component of the amniotic fluid is discarded after these analyses and is therefore available for research and future clinical applications.

Cell-free fetal DNA in the serum and plasma of pregnant women was first described by Lo et al[1] in 1997 after others demonstrated the presence of circulating tumor-specific DNA sequences in cancer patients.[2-4] After it was shown that cell-free fetal DNA is also present in the urine of pregnant women,[5] we hypothesized that amniotic fluid, as a reservoir of fetal urine, would contain fetal DNA. In a preliminary study, we demonstrated that much larger quantities of fetal DNA are present in amniotic fluid than in maternal serum (100- to 200-fold difference).[6]

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Cell-free fetal messenger RNA (mRNA) in maternal plasma was first demonstrated by Poon et al[7] in 2000. In 2002, Tsui et al[8] subsequently showed that fetal mRNA in peripheral blood is unexpectedly and remarkably stable. We then hypothesized that if large amounts of DNA were present in amniotic fluid, RNA would likely be present and stable enough to allow for the study of fetal gene expression. Furthermore, recent technologic advances and information from the Human Genome Project have made possible the development of gene expression microarrays, which can be analyzed for the presence and quantity of tens of thousands of gene transcripts simultaneously.[9,10]

There is no comprehensive way to examine global fetal gene expression in vivo. The purpose of this study was to determine whether it was possible to extract fetal RNA from cell-free amniotic fluid supernatant, successfully hybridize it to oligonucleotide microarrays, and analyze the arrays for the presence and quantity of thousands of different gene transcripts. Gene expression profiling of amniotic fluid by using microarrays could provide important information about the well-being, development, and potential disease status in the living fetus. This report presents preliminary results of gene expression analyses in the living human fetus by using a fraction of amniotic fluid that is normally discarded: the cell-free supernatant.

METHODS

Amniotic Fluid Collection

Approval was obtained from Tufts–New England Medical Center and Women and Infants Hospital institutional review boards to obtain amniotic fluid supernatant samples for this study. Informed consent was deemed unnecessary because discarded samples were used anonymously.

Cases

In healthy pregnancies, between 10 and 30 mL of amniotic fluid can safely be removed from the fetal sac, but only about 8 to 15 mL of supernatant remains after clinical testing, including karyotype analysis and α-fetoprotein measurement. Preliminary experiments showed that this remaining volume of amniotic fluid from a normal singleton fetus might not contain a sufficient quantity of mRNA for microarray analysis. Therefore, 5 large-volume amniotic fluid samples were obtained from 4 pregnant women undergoing therapeutic amnioreduction for polyhydramnios. Two of these women had a fetus with hydrops (gestational ages of 29⁴⁄₇ weeks and 32 weeks), 1 had a fetus with twin-twin transfusion (TTT) syndrome (gestational age of 20 weeks), and another woman with fetal TTT underwent amnioreduction at 2 gestational ages (21⁶⁄₇ weeks and 24³⁄₇ weeks) and thus provided 2 samples. Cell-free supernatant was obtained by centrifugation of at least 350*g* for 10 minutes.

Controls

To obtain sufficient RNA from healthy fetuses for comparison, multiple 10-mL samples of frozen, archived amniotic fluid supernatant were combined to form larger pools. These samples were obtained from pregnant women who were between 17 and 18 weeks' gestational age and underwent routine genetic amniocentesis for advanced maternal age. Six samples from male fetuses and 6 from female fetuses were selected according to known normal karyotypes and similar gestational age. These control samples were combined by sex, with each 60-mL pool representing amniotic fluid supernatant of an average 17-week fetus. Cell-free supernatant was obtained by centrifugation of 350*g* for 10 minutes.

Oligonucleotide Hybridization

After centrifugation, total RNA was extracted from all samples using the QIAamp Viral RNA Vacuum Protocol for Large Sample Volumes (Qiagen Inc, Valencia, Calif), with modification. Volumes of viral lysis buffer (AVL) and ethanol were increased to 20 mL for each 5 mL of amniotic fluid per column, and 60-mL syringes were attached to the columns to accommodate the large volume samples. The mRNA was amplified twice and converted to complementary RNA (cRNA) by in vitro transcription in the presence of biotinylated nucleoside triphosphates following the GeneChip Eukaryotic Small Sample Target Labeling Technical Note (Affymetrix Inc, Santa Clara, Calif). Samples were further purified by phenol-chloroform extraction by using Phase Lock Gels (Eppendorf AG, Hamburg, Germany). To verify the quantity and quality of biotinylated cRNA, samples were analyzed using gel electrophoresis and fragmented before hybridization to Affymetrix Test3 oligonucleotide arrays according to the manufacturer's documentation. Subsequently, 15 to 75 µg of biotinylated cRNA was hybridized to Affymetrix U133A arrays, which are composed of 22 283 probe sets and more than 500 000 distinct oligonucleotide features, representing 14 239 of the best-characterized human genes. Accession numbers herein are from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Microarray Data Analysis and Statistical Analysis

Each array was scanned at 570 nm by a confocal scanner (Agilent, Palo Alto, Calif) with a resolution of 3 µm per pixel. Pixel intensities were measured, and expression signals were extracted and analyzed by using Microarray Suite 5.0 (Affymetrix). All microarrays were scaled to the same target signal of 50 by using the "all probe sets" scaling option so that the expression signals from all experiments could be directly compared.

Comparison analyses were performed with the Wilcoxon signed rank test via the Microarray Suite 5.0 software between each of the TTT or hydrops cases and the pooled male control. Data from the TTT3 sample at 21⁶⁄₇ weeks and the pooled female sample were not used because of noisy data (see "Results"). Data were copied into Excel files (Microsoft, Redmond, Wash) and sorted for probe sets called "present" in either the case or control. Data sets for each case were then narrowed to transcripts that were increased or decreased relative to the pooled male con-

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Table 1. Quantities of RNA Extracted and Amplified From Amniotic Fluid

	Pooled Male Control	Pooled Female Control	TTT2	TTT3	TTT1*	Hydrops 1	Hydrops 2
Gestational age, wk	17	17	20	21 6/7	24 3/7	29 4/7	32
Amniotic fluid volume, mL	57	62	120	120	90	90	180
Total RNA eluted, ng	46	77	105	22	17	36	20
Biotinylated cRNA after amplification, µg	59	36	84	54	61	25	65

Abbreviations: cRNA, complementary RNA; TTT, twin-twin transfusion.
*TTT3 and TTT1 represent 2 samples from the same woman.

Table 2. Selection of Genes in TTT Samples With the Most Significantly Different (>4-Fold Difference) Expression Levels in Amniotic Fluid*

Category and Accession No.	Gene Description	Fold Difference TTT2 (20 wk)	Fold Difference TTT1 (24 3/7 wk)
Cell division			
AA807529	MCM5 minichromosome maintenance deficient	–5	–8
Embryogenesis/organogenesis			
NM_004306	Annexin A13	–9	–16
BC003169	Calpain 3	–6	–11
NM_002166	Inhibitor of DNA binding	–6	–4
Ion/molecule transport			
NM_000040	Apolipoprotein C-III	–13	–16
AL518391	Aquaporin I	20	18
NM_000111	Solute carrier family 26	–11	–128
AF162690	Transthyretin (prealbumin)	–6	–32
Metabolism			
BF195998	Aldolase B, fructose-bisphosphate	–5	–16
NM_024308	Hypothetical protein MGC4172	–11	–16
NM_005327	L-3-Hydroxyacyl-coenzyme A dehydrogenase	–6	–5
NM_003704	Phosphatidic acid phosphatase	–15	–4
AF119873	Serine protease inhibitor (α_1-antitrypsin)	–49	–32
U09024	Sulfotransferase family, cytosolic, 2A	–9	–16
Signaling/communication			
211889_x_at	Carcinoembryonic antigen-related cell adhesion molecule	18	13
NM_001443	Fatty acid binding protein 1, liver	–7	–32
206896_s_at	Guanine nucleotide binding protein (G protein)	18	13
NM_004616	Transmembrane 4 superfamily	–52	–16
203868_s_at	Vascular cell adhesion molecule	9	5
Unclassified			
217622_at	Expressed tag sequences	6	7
NM_022097	Hepatocellular carcinoma antigen gene	–4	–32
220595_at	Hypothetical protein DKFZp434B0417	34	13
NM_014129	PRO0478 protein	181	49
NM_000295	Serine (or cysteine) proteinase inhibitor	–10	–26

*Fetuses with twin-twin transfusion (TTT) compared with the pooled control.

trol by a 2-fold or greater difference. The 2 remaining TTT data sets were then compared with each other, as were the 2 hydrops data sets, to detect genes consistently increased or decreased in both cases, with the same disease compared with the pooled control. Finally, expression levels of selected genes of interest, such as Y chromosome genes, surfactant, mucin, keratin, aquaporin, and placental genes, were reviewed in all cases relative to the pooled male control.

RESULTS

RNA Extraction and Hybridization to Microarrays

TABLE 1 shows the volumes of amniotic fluid used for extraction, amount of RNA eluted, and quantities of biotinylated cRNA available for the microarrays after amplification. Five of the 7 samples hybridized well to the arrays, as measured by scale factors within 3-fold of one another, as recommended by the manufacturer. Therefore, data are presented only for these 5 samples (TTT1, TTT2, hydrops 1, hydrops 2, and the pooled male control). The other 2 samples (TTT3 and the pooled female control) had low signals, as shown by higher scale factors, and were therefore not included in this analysis. The average background level of the images (median, 55.37 units; range, 49.64-61.50) was highly similar across all the arrays (typical values range from 20-100). Noise, a measurement that reflects sample quality and electrical noise of the scanner, was also comparable across the arrays (median, 2.21 units; range, 2.06-2.37).

For the 5 analyzed samples, a median of 36% (range, 11%-44%) of the probe sets represented on the microarrays was detected as "present," 62% (range, 54%-88%) was not detectable (ie, "absent"), and 2% (range, 1%-2%) was "marginal." There was evidence of low-level false or cross-hybridization according to the presence of randomly distributed probe sets; these results were not statistically significant and were therefore not included for analysis. Within individual samples, there was some variation in the 3′/5′ ratios of the internal control genes (glyceraldehyde-3-phosphate dehydrogenase and actin) that are used to assess RNA sample and assay quality. When these control genes were compared across all samples, certain control genes consistently had a normal (ie, less than 3) 3′/5′ ratio in every sample, whereas other control genes always had a high 3′/5′ ratio (10 to 100).

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Differences in Gene Expression Between Samples

Of the 22 283 probe sets present on the microarray, a median of 20% (range, 15%-29%) had significant differences in their levels of expression between the cases and the pooled male control. TABLE 2 and TABLE 3 show a selection of genes with the most statistically significant different levels of expression (larger than 4-fold) in both TTT fetuses and both hydrops fetuses, respectively, compared with the pooled control. One Y chromosome probe set (accession NM_001008) was present in all 4 samples from male fetuses (TTT2, hydrops 1, hydrops 2, and the pooled male control) but not in the sample from the female fetus (TTT1).

To determine whether genes involved in fetal development showed differential expression with increasing gestational age of the fetal samples, specific developmental gene families were investigated and compared with the pooled male control. Statherin (accession NM_003154), a gene involved in saliva secretion and ossification, was up to 28 times more concentrated in the older fetuses compared with the 17-week pooled control. Surfactant genes (TABLE 4) increased from only 3 transcripts present in the pooled control to all 9 present in hydrops 1 (29 4/7 weeks' gestational age). Only a few transcripts in the mucin gene family (TABLE 5) were present in any sample, but certain transcripts such as tracheobronchial/gastric mucin and salivary mucin were increased up to 56-fold in the older fetuses compared with the 17-week pooled control. Most keratin genes decreased with increasing gestational age (TABLE 6); several transcripts had up to a 4-fold decrease compared with the control.

Other genes were reviewed in the context of fetal pathology or maternal-fetal trafficking of cell-free nucleic acids. One transcript for aquaporin 1, a water transporter, was elevated up to 18-fold (TABLE 7) in both TTT fetuses but not the hydropic fetuses compared with the pooled control. Aquaporin 3 expression varied minimally in the cases compared with the pooled control, with 2 of

Table 3. Selection of Genes in Hydrops Samples With the Most Significantly Different (>4-Fold Difference) Expression Levels in Amniotic Fluid*

Category and Accession No.	Gene Description	Fold Difference: Hydrops 1 (29 4/7 wk)	Fold Difference: Hydrops 2 (32 wk)
Cell division			
NM_004454	ets Variant gene 5	5	6
AF247704	NK3 transcription factor related	5	20
Embryogenesis/organogenesis			
BC003169	Calpain 3	–13	–11
AI694562	Collagen, type IV	11	4
AU160004	IGF-II mRNA-binding protein	–6	–5
NM_006121	Keratin 1	–16	–5
AI521646	Mucin 5, tracheobronchial	5	5
BC001060	Paired box gene 8	6	5
NM_003154	Statherin	9	28
Ion/molecule transport			
X02162	Apolipoprotein A-I	–39	–5
NM_000483	Apolipoprotein C-II	–7	–16
NM_000040	Apolipoprotein C-III	–111	–169
NM_016321	Rhesus blood group, C glycoprotein	7	34
NM_001038	Sodium channel, nonvoltage-gated 1α	4	5
NM_003982	Solute carrier family 7	–23	–11
AI627943	Solute carrier family 12	4	9
NM_000111	Solute carrier family 26	–26	–11
AI300520	Stanniocalcin I	5	6
Metabolism			
BC005314	Aldolase B, fructose-bisphosphate	–111	–52
NM_000050	Argininosuccinate synthetase	–7	–4
NM_000790	Dopa decarboxylase	–5	–20
AF087942	Glycogenin	–13	–11
AV711904	Lysozyme	17	12
NM_000295	Serine proteinase inhibitor (α_1-antitrypsin)	–30	–52
L25275	Sulfotransferase family, cytosolic, 1A	–7	–4
U08024	Sulfotransferase family, cytosolic, 2A	–30	–10
Signaling/communication			
NM_001443	Fatty acid binding protein 1, liver	–34	–21
AI653981	L1 cell adhesion molecule	21	30
NM_005739	RAS guanyl releasing protein 1	12	17
NM_016321	Rhesus blood group, C glycoprotein	7	34
AA502643	Tyrosine 3- and tryptophan 5-monooxygenase activation protein	–5	–5
Structure/motility			
NM_004306	Annexin A13	–42	–28
AW026491	Cyclin D2	–7	–28
NM_006498	Lectin, galactoside-binding	–42	–23
Unclassified			
NM_022097	Hepatocellular carcinoma antigen gene	–42	–16
BE328312	Hypothetical protein MGC12103	–5	–7
NM_014951	KIAA0844 protein	4	5
NM_022129	MAWD binding protein	–11	–8
NM_002443	Microseminoprotein, β	5	9
U22178	Microseminoprotein, β	13	34
AA886335	Serologically defined breast cancer antigen	–42	–23

*Fetuses with hydrops compared with the pooled control.

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3 transcripts not detected in any fetus. Placenta-specific transcripts, including corticotropin-releasing hormone, chorionic somatomammotropin hormone 1 (placental lactogen), and the β subunit of chorionic gonadotropin, were not present in any of the samples.

COMMENT

To our knowledge, this is the first in vivo study of global gene expression in the living human fetus by oligonucleotide microarray analysis of fetal mRNA isolated from cell-free amniotic fluid. Cell-free fetal RNA was successfully extracted from this typically discarded component of amniotic fluid, amplified, labeled, and hybridized to oligonucleotide microarrays. Our analyses revealed important information about the presence and level of gene expression in living human fetuses. In addition, these preliminary data appear to show that observed gene expression patterns correlated with known variables (sex, gestational age, and disease status) between the cases and control. Although validating our results with real-time quantitative reverse-transcriptase polymerase chain reaction would have been optimal, it was impossible because of limited sample template. However, the presence of 1 Y chromosome transcript in all 4 male samples but not the female sample provided physiologic validation of the data.

Next, expression differences were evaluated in gene families known to be important in fetal development to look for changes with gestational age. Significant differences were observed in several genes expressed in lung, intestine, and skin epithelial cells, which are all in contact with amniotic fluid. For example, it is well known that the type and quantity of surfactant genes expressed in human fetal lungs increase during development.[11] Messenger RNA for surfactant proteins B and C is detectable as early as 13 weeks, and by 24 weeks, the levels are 50% and 15%, respectively, of adult levels. Surfactant protein D mRNA is first detectable in the second trimester, with expression increasing throughout fetal and postnatal development. Surfactant protein A expression begins only after about 30 weeks and reaches maximum near term. The findings of the current study are consistent with the published data. All of the fetuses older than 24 weeks produced increased amounts of surfactant proteins B and C compared with the 17-week control, and surfactant proteins D and A were observed only after 29 weeks. The fact that the 20-week fetus and the 32-week fetus produced fewer surfactant transcripts than some of the more immature fetuses may be due to differing hybridization efficiencies but may also be explained by their severe disease status.

Many differences with gestational age were observed in expression of proteins produced by epithelial cells and assumed to be in contact with the amniotic fluid. Mucins are filamentous glycoproteins present at the interface of epithelia and extracellular environments in the gastrointestinal tract, lungs, or urogenital tract.[12] As fetuses mature, they produce mucin in increasing amounts to protect their epithelia in preparation for life outside the womb. This is consistent with the current study,

Table 4. Surfactant Gene Transcripts by Patient and Gestational Age*

Surfactant Protein Transcripts	Accession No.	Pooled Male Control (17 wk), Detection	TTT2 (20 wk)		TTT1 (24⅗ wk)		Hydrops 1 (29⅘ wk)		Hydrops 2 (32 wk)	
			Detection	Fold Difference	Detection	Fold Difference	Detection	Fold Difference	Detection	Fold Difference
A2	NM_006926	–	–		–		+	20	–	
B	J02761	+	–		+	3	+	4	+	
B	4901244	+	–		+	3	+	5	+	
C	NM_003018	–	–		–		+	64	–	
C	BC005913	–	–		–		+	49	–	
C	AA633841	–	–		–		+	49	–	
C	AI831055	–	–		–		+	5	–	
C	4878786_RC	+	–		+	3	+	14	+	6
D	NM_003019	–	–		–		+	3	+	7

*Fetuses with twin-twin tranfusion (TTT) or hydrops relative to the pooled male control. Where not indicated, changes in transcript detection were less than 1-fold different.

Table 5. Mucin Gene Transcripts, by Patient and Gestational Age*

Mucin Gene Transcripts	Accession No.	Pooled Male Control (17 wk), Detection	TTT2 (20 wk)		TTT1 (24⅗ wk)		Hydrops 1 (29⅘ wk)		Hydrops 2 (32 wk)	
			Detection	Fold Difference	Detection	Fold Difference	Detection	Fold Difference	Detection	Fold Difference
1, Transmembrane	NM_002456	–	+		+		+	4	+	
1, Transmembrane	AI610869	+	–		+	1	+	4	+	3
5, Tracheobronchial/ gastric	AW192795	–	–		–		+	20	–	
5, Tracheobronchial/ gastric	AI521646	–	–		–		+	5	+	5
7, Salivary	L13283	+	–		+	11	+	20	+	56

*A selection of transcripts with the most significant differences. Fetuses with twin-twin transfusion (TTT) or hydrops relative to the pooled male control. Where not indicated, changes in transcript detection were less than 1-fold different.

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in which the level of several mucin transcripts increased with advancing gestational age. The kidney, intestine, and skin also produce keratins. In the skin, keratin proteins are first produced in the intermediate layer during the 11th week of human fetal development. During the fifth month of gestation, this layer develops into definitive layers of keratinocytes, and as cells progress from the basal layer of stem cells to the outer horny layer, they stop production of keratins, which are then bundled and cross-linked. By arrival of the top layer, metabolic activity of the cells has ceased, with the scalelike terminally differentiated keratinocytes forming the horny protective layer.[13] The reason for the observed gestational age–related decrease in keratin expression could be that as the fetal skin matures, fewer keratin-producing cells are in direct contact with the amniotic fluid and may not release their mRNA into the cell-free fraction. Rather, the layer of hard, cross-linked keratin itself may protect the buried keratin-producing cells from releasing mRNA into amniotic fluid.

Next, aquaporin genes, a family of water transporters, were reviewed because the large differences in aquaporin 1 levels between fetuses with TTT and the control were highly significant. In fetal sheep hearts, aquaporin 1 expression has been shown to be developmentally regulated with fetal age and also in response to chronic anemia, a condition associated with interstitial fluid generation and hydrops.[14] In human fetal membranes, there is evidence that aquaporin 1 is present on the apical aspect of the chorionic plate amnion but aquaporin 3 is not active.[15] It has been postulated that aquaporin 1 may play a role in water movement from the amniotic cavity across the placenta into the fetal circulation. Our findings support the presence of aquaporin 1 and the relative lack of aquaporin 3 in amniotic fluid. The significant increase of aquaporin 1 in TTT patients suggests that it might play a role in the polyhydramnios associated with TTT but not hydrops.

Several placenta-specific genes, including corticotropin-releasing hormone, chorionic somatomammotropin hormone 1, and chorionic gonadotropin, were also examined. The presence of these genes in the plasma of pregnant women has been presented as proof

Table 6. Keratin Gene Transcripts, by Patient and Gestational Age*

Keratin Gene Transcripts	Accession No.	Pooled Male Control (17 wk), Detection	TTT2 (20 wk)		TTT1 (24⅗ wk)		Hydrops 1 (29⅖ wk)		Hydrops 2 (32 wk)	
			Detection	Fold Difference	Detection	Fold Difference	Detection	Fold Difference	Detection	Fold Difference
4	X07695	+	+	–3	+	–2	+		+	
5	NM_000424	+	–		+	–1	+	2	+	
6A	AL569511	+	–		+		+	2	+	
6B	J00269	+	–		+	–2	+	1	+	
6B	AI831452	+	+	–4	+		+		+	
7	BC002700	+	–		+	–2	+		+	
8	U76549	+	+	–3	+	–3	+	–2	+	–5
10	NM_000421	+	–		+	–3	+	–2	+	–2
10	M19156	+	–		+	–2	+	–2	+	–3
10	X14487	+	+	–2	+		+		+	–3
13	NM_002274	+	+	–6	+		+		+	
14	BC002690	+	+		+		+	3	+	2
15	NM_002275	+	–		+		+	2	+	
16	AF061812	+	+	–6	+	–2	+	1	+	
17	NM_000422	+	+	–3	+	–3	+	–2	+	–5
17	Z19574	+	+		+	–3	+		+	
18	NM_000224	+	+	–3	+	–3	+	–2	+	–3
19	NM_002276	+	+		+		+		+	–2
23	NM_015515	+	–		–		+	–5	+	
24	NM_019016	+	+		+	–17	+	1	+	

*A selection of transcripts with the most significant differences. Fetuses with twin-twin transfusion (TTT) or hydrops relative to the pooled male control. Where not indicated, changes in transcript were less than 1-fold different.

Table 7. Aquaporin 1 and 3 Gene Transcripts, by Patient and Gestational Age*

Aquaporin Transcripts	Accession No.	Pooled Male Control (17 wk), Detection	TTT2 (20 wk)		TTT 1 (24⅗ wk)		Hydrops 1 (29⅖ wk)		Hydrops 2 (32 wk)	
			Detection	Fold Difference	Detection	Fold Difference	Detection	Fold Difference	Detection	Fold Difference
1	NM_000385	–	+		+		–		+	3
1	AL518391	–	+	20	+	18	–		–	
3	NM_004925	–	–		–		–		–	
3	4855867_RC	+	–		+	–2	+		+	
3	4855868	–	–		–		–		–	

*Fetuses with twin-twin transfusion (TTT) or hydrops relative to the pooled male control. Where not indicated, changes in transcript detection were less than 1-fold different.

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of fetal-maternal trafficking of cell-free RNA.[16,17] The absence of these placental transcripts in amniotic fluid suggests that fetal-maternal transplacental trafficking of nucleic acids is primarily unidirectional toward the mother. Previous work has shown that cell-free fetal DNA in maternal plasma is significantly more concentrated relative to cell-free maternal DNA in the fetal plasma.[18]

This study demonstrated that for the majority of samples, cell-free RNA from amniotic fluid successfully hybridized to microarrays. However, some samples hybridized less well, possibly because the RNA was degraded, which could occur from delays before sample processing or introduction of a freeze/thaw cycle. However, freezing and thawing did not appear to be detrimental to the male control, which hybridized well despite its composition of archived amniotic fluid samples that had been stored at –80°C. Additionally, it has been demonstrated that a single freeze/thaw cycle produces no significant effect on the cell-free RNA concentration in plasma or serum.[8] It is possible that cell-free RNA is inherently degraded and therefore has different properties than RNA extracted from whole cells. RNA is labile, so it is surprising that any cell-free RNA in amniotic fluid survives until extraction. There is evidence that circulating RNA in plasma is associated with stabilizing particles.[19] In this study, certain internal control genes had normal 3′/5′ ratios in every sample, whereas ratios of certain other genes were always high. This discrepancy suggests a pattern of preservation of specific RNA transcripts, which could be related to alteration and packaging of mRNA during apoptosis. Housekeeping genes vary significantly in their expression patterns between various tissues and organisms,[20] and these patterns in cell-free RNA in amniotic fluid are unknown.

The low levels of nonsignificant false positives observed in this study could be due to cross-hybridization of the short oligonucleotide probes on the arrays with different mRNAs that have short sequences in common. However, the algorithms take this into account and have largely eliminated this source of error.[21] Additionally, the cases and control had different genetic backgrounds and other variables (gestational age and disease status) because amniocentesis is not generally performed on healthy fetuses older than 19 to 20 weeks. Further, small amounts of maternal contamination could possibly confound the results. Therefore, the data on diversity of fetal gene expression must be interpreted with caution at this stage of investigation, and further study is necessary.

For this pilot study, large-volume samples were used to demonstrate feasibility. However, it appears that amniotic fluid samples from healthy fetuses contain a higher concentration of cell-free mRNA than amniotic fluid samples from fetuses with polyhydramnios (Table 1). Our technical improvements are directed toward improving extraction of mRNA from routinely collected amniotic fluid samples (<30 mL) so that individual fetuses may be studied.

In summary, this study demonstrates that cell-free fetal mRNA can be isolated from amniotic fluid and successfully detected using oligonucleotide microarrays. Preliminary gene expression analyses appear to show gene expression patterns that vary among fetuses of different sexes, gestational age, and disease state. The entire study was conducted by using a portion of amniotic fluid that is typically discarded and thus is readily available for use. The intriguing gene expression differences observed suggest that this technology could facilitate the advancement of human developmental research, as well as the cultivation of new biomarkers for assessment of the living fetus.

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Study concept and design: Lai, Bianchi.
Acquisition of data: Larrabee, Johnson, Ordovas, Cowan, Tantravahi.
Analysis and interpretation of data: Larrabee, Johnson, Lai, Ordovas, Bianchi.
Drafting of the manuscript: Larrabee, Johnson, Bianchi.
Critical revision of the manuscript for important intellectual content: Larrabee, Johnson, Lai, Ordovas, Cowan, Tantravahi, Bianchi.
Statistical analysis: Larrabee, Johnson.
Obtained funding: Bianchi.
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Study supervision: Johnson, Bianchi.
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