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# Analysis of the cell-free amniotic fluid transcriptome expressed during the euploid mid-trimester of pregnancy

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ABSTRACT

*Objective:* The amniotic fluid (AF) contains cell-free RNAs (cfRNAs), which are considered to reflect the fetal status *in utero*. However, there are limited numbers of data to examine the AF cell-free transcriptome because amniocentesis is an invasive procedure. In this study, the AF transcriptome expressed during the euploid mid-trimester of pregnancy was characterized.
*Study design:* Fourteen AF samples were collected. RNA was extracted from AF supernatant, hybridized to Affymetrix GeneChip Human arrays, and the transcriptome was analyzed by using the DAVID toolkit.
*Result:* We detected 1069 genes in the 14 AF samples. The GNF atlas mapping showed that genes present in the AF were annotated with endocrine organs and blood components, including the pancreas, adrenal gland, thyroid, ovary and monocytes. The proteins encoded by the transcriptome were localized to several organs, which are directly in contact with the AF, including the placenta, lung, skin, epithelium, and kidney. During the early fetal period, there is a bi-directional diffusion between the fetus and AF. Therefore, the AF composition is similar to that of the fetal plasma. In addition, fetal urine, swallowing, pulmonary secretion, and diffusion across the placenta contribute to produce amniotic fluid by directly excreting fluid. The KEGG pathway analysis with placenta specific genes revealed that focal adhesion and extracellular matrix receptor interaction pathways were enriched. These pathways are important for the placental development.
*Conclusion:* cfRNA in the amniotic fluid originates from placenta and fetal organs directly contacting the amniotic fluid as well as from diffusion of the fetal plasma across the placenta. AF transcriptome may reflect not only fetal development, but also placental development.


## Introduction

In 1948, Mandel and Metais reported the presence of cell-free nucleic acids (cfNAs) in human blood. The release of nucleic acids from cells into the blood is thought to be related to apoptosis and necrosis. Since their discovery, cfNAs have been studied as disease-related biomarkers in cancer patients [1]. The potential implications of cfNAs in prenatal medicine have been demonstrated by detecting fetal DNA in maternal plasma to determine fetal rhesus D genotyping and fetal gender [2–4]. In addition, karyotyping of cfNAs from the maternal blood was successfully performed and adopted for non-invasive prenatal genetic diagnosis [5].

The amniotic fluid was once thought to contain only water and electrolytes or perhaps desquamated fetal cells and various secretions. Additionally, it was reported to perform multiple functions to support fetal development and can be analyzed to determine the health status of the fetus.

Larrabee et al. demonstrated that the amniotic fluid contains cell-free RNAs (cfRNAs), which are considered to reflect the fetal

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status *in utero* [6]. However, amniocentesis is invasive. Procedure-related complications, including preterm labor, chorioamnionitis, and premature rupture of membranes, prevent the acquisition of adequate amounts of material routinely. Therefore, there are limited numbers of reports analyzing the amniotic fluid cell-free transcriptome during the mid-trimester [7–10]. These studies have been conducted by the same research group and focused on how the amniotic fluid transcriptome represents the fetal development or fetal diseases. However, genetic differences in human according to geography and ethnicity have been reported. In addition, the placenta plays an important role in producing the amniotic fluid. The origin of cfRNAs in the amniotic fluid remains unclear.

The purpose of the study was to examine the amniotic fluid transcriptome expressed during the euploid mid-trimester of pregnancy subjects. The analysis of the cell-free amniotic fluid will provide crucial information for the potential application of amniotic fluid cfRNAs and insights into early fetal development.

## Materials and methods

### *Subjects*

This study was designed as a prospective study. The participants were recruited from the Department of Obstetrics and Gynecology of CHA Gangnam Medical Center, CHA University (Seoul, Korea) between March 2014 and February 2015. The study was reviewed and approved by the institutional review board (GCI-14-11) of CHA Gangnam Medical Center in 2014, and written informed consent was obtained from all participants.

Fourteen samples were collected from 14 subjects who were in their early second trimester of pregnancy (Table 1). The median age and the median gestational age of the participants were 35 years old and 17 + 3 weeks, respectively. All participants underwent amniocentesis to exclude fetal aneuploid. Five fetuses were 46, XY and the others were 46, XX.

For the study, we adopted a set of exclusion criteria, including abnormal karyotype, suspected congenital anomaly by ultrasonography, multifetal pregnancy, and pre-existing maternal medical condition.

### *Cell-free RNA extraction*

The amniotic fluid from the second-trimester was centrifuged at 350 × *g* for 10 min at room temperature to remove amniocytes. The cfRNA was extracted from 5 to 10 mL of amniotic fluid supernatant. To extract cfRNAs, the QIAamp® Circulating Nucleic Acid (Qiagen, Hilden, Germany) kit was used according to the manufacturer's instructions. The RNA was purified and concentrated with the RNeasy®MinElute® Cleanup kit (Qiagen) and eluted with RNase-free water.

### *Microarray*

Gene expression was analyzed with the GeneChip®PrimeView™ array (Affymetrix, Santa Clara, CA, USA). Biotinylated complementary RNA was prepared according to the standard Affymetrix protocol from 100 ng total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). Following fragmentation, 12 μg of amplified RNA were hybridized for 16 h at 45 °C on a GeneChip® Human array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GeneChip® Scanner 3000 7G. Data were analyzed with Robust Multi-array Analysis (RMA) using the Affymetrix default analysis settings and global scaling as a normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalized, and log transformed intensity values were then

**Table 1**
Subject demographics.

| No. | Sample | Age (years) | G. age at amniocentesis | Parity | Indication of amniocentesis | Others | Fetal sex | Mode of delivery | Indication for C/S | G. age at delivery | Birth weight | Apgar score | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | 1 min | 5 min |
| 1 | CHA1 | 38 | 17 + 3 | 3 | AMA | | 46, XY | VD | | 39 + 4 | 3700 | 8 | 9 |
| 2 | CHA3 | 27 | 17 + 0 | 1 | Positive screening test for Down syndrome | | 46, XX | C/S | Breech | 39 + 0 | 2930 | 8 | 9 |
| 3 | CHA4 | 31 | 17 + 2 | 1 | Positive screening test for Down syndrome | | 46, XY | VD | | 38 + 1 | 3110 | 7 | 8 |
| 4 | CHA44 | 36 | 17 + 3 | 2 | Single umbilical artery, bad obstetric history (IUFD) | Placenta previa | 46, XX | C/S | Placenta previa | 37 + 6 | 3290 | 8 | 9 |
| 5 | CHA52 | 40 | 18 + 6 | 2 | AMA, bad obstetric history (IUFD) | | 46, XX | | | | | | |
| 6 | CHA80 | 31 | 17 + 6 | 1 | Positive screening test for Down syndrome | | 46, XX | | | | | | |
| 7 | CHA85 | 34 | 17 + 2 | 2 | Bad obstetric history (Turner syndrome) | | 46, XX | | | | | | |
| 8 | CF497 | 39 | 17 + 2 | 1 | AMA | | 46, XX | C/S | Breech | 39 + 4 | 3600 | 8 | 9 |
| 9 | CF514 | 43 | 17 + 1 | 1 | Positive screening test for Down syndrome | IVF, GDM | 46, XX | C/S | Elderly primigravida | 38 + 5 | 3580 | 8 | 9 |
| 10 | CF521 | 31 | 17 + 5 | 1 | Positive screening test for Down syndrome | | 46, XX | VD | | 40 + 4 | 3490 | 8 | 9 |
| 11 | CF611 | 36 | 16 + 1 | 1 | AMA | | 46, XX | VD | | 41 + 0 | 3710 | 8 | 9 |
| 12 | CF418 | 35 | 17 + 5 | 2 | Positive screening test for Down syndrome | Preterm delivery | 46, XY | VD | | 36 + 1 | 3010 | 6 | 7 |
| 13 | CF462 | 35 | 16 + 5 | 2 | Positive screening test for Down syndrome | | 46, XY | C/S | Previous C/S | 39 + 0 | 3270 | 8 | 9 |
| 14 | CF492 | 31 | 16 + 5 | 1 | Positive screening test for Down syndrome | | 46, XY | VD | | 40 + 5 | 3380 | 8 | 9 |

G. age, gestational age; AMA, advanced maternal age; VD, vaginal delivery; C/S, cesarean section; IVF, *in vitro* fertilization; GDM, gestational diabetes mellitus; IUFD, intrauterine fetal death.