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Original Research

Cell-free fetal DNA in amniotic fluid supernatant for prenatal diagnosis

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Abstract: In widespread conviction, amniotic fluid is utilized for prenatal diagnosis. Amniotic fluid supernatant is usually discarded, notwithstanding being a good source of fetal DNA. The aim of the present study was to assess cell-free fetal DNA extracted from amniotic fluid supernatant for application in prenatal diagnosis such as gender determination and early diagnosis of β-thalassemia. Samples of amniotic fluid of 70 pregnant women were collected and went through routine tests along with tests for cell-free fetal DNA from amniotic fluid supernatant. The DNA in the amniotic fluid supernatant was extracted and analyzed for gender determination by PCR and Real-time PCR. ARMS-PCR was applied to test early diagnosis of IVS II-I mutation (common β-thalassemia mutation) and E7V mutation for sickle cell anemia using DNA extracted from the amniotic fluid supernatant. Using the cell-free fetal DNA extracted from the amniotic fluid supernatant, the sensitivity of PCR and Real-time PCR for gender detection was compared with the routine cytogenetic method. The fetus tested for sickle cell anemia and β-thalassemia was observed to be healthy but heterozygous for IVS II-I mutation. The findings indicated that cell-free fetal DNA from amniotic fluid supernatant can be a good source of fetal DNA and be used in early prenatal diagnosis since because of its fast and accurate application. Therefore, it would be suggested that the amniotic fluid supernatant's disposal is prevented because if the tests needs to be repeated, cell-free fetal DNA extracted from the amniotic fluid supernatant can be used as an alternative source for prenatal diagnosis.

Key words: Amniotic fluid, prenatal diagnosis, fetal DNA, thalassemia diagnosis.

Introduction

Prenatal diagnosis tests are classified into invasive and non-invasive subtypes based on risk of abortion (1). Invasive procedures such as amniocentesis and Chorionic Villus Sampling (CVS) are commonly used for prenatal genetic diagnosis, but such methods expose fetus or the mother to risk (2).

Amniocentesis with chromosomal analysis is a routine method for prenatal diagnosis of diseases associated with aneuploidy. Since long laboratory turnaround time (14-20 days) and high cost required for amniocentesis this process causes increased prenatal anxiety (3). Although this method requires a large volume and high quality of sample, there is still a possibility of failure to obtain results from chromosomal harvest of fetal cells in the amniotic fluid (4).

Plenty of free fetal DNA fragments exist in the amniotic fluid supernatant and are 100 to 200 times more concentrated than those in maternal serum (5). Free fetal DNA in the amniotic fluid supernatant shows a relatively pure embryonic cells DNAs mainly originated from fetal nucleic acids and uncontaminated by nucleic acid derived from trophoblasts or maternal nucleic acid (6, 7, 8). It was shown that cell-free fetal DNA in the amniotic fluid supernatant can be used to detect aneuploidy (9, 10). Amniotic fluid supernatant is usually discarded in the process of performing amniocentesis (11), but existence of free fetal DNA in this fluid introduces it as a potential source of genetic material for laboratory prenatal diagnosis.

Amniotic fluid is used to determine fetus's gender in the first place. This in early pregnancy can do a world of good as it can be used to consider the risk of some sex-linked disorders such as hemophilia and Duchene Muscular Dystrophy. However, when genital ambiguity exists, free fetal DNA can be utilized to determine gender (12). Another routine example of prenatal diagnosis can be made in β-thalassemia which was firstly introduced in 1975 (13). β-thalassemia is the most common autosomal recessive single-gene disorder caused by mutations in the beta globin gene (14)

In the present study, our aim was to assess whether the amniotic fluid supernatant can be a good source for DNA extraction and test whether it can be used for accurate prenatal gender determination. In addition, we also assessed the amniotic fluid supernatant's free DNA as a source for early diagnosis of β-thalassemia and sickle cell anemia in a fetus.

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Materials and Methods

Subjects

Amniotic fluid was obtained from 70 women in 14-15 weeks of pregnancy. All of the women have given an informed consent. Based on standard protocols of cytogenetic laboratory of Tabriz Genetic Analysis Center, the samples were centrifuged and went through chromosomal harvest. 10 ml of the amniotic fluid supernatant was used for DNA extraction and molecular tests.

DNA extraction of amniotic fluid supernatant and quantity and quality control

Amniotic fluid supernatant samples were vortexed for 15 second. In order to remove any possible remaining cell, 500 µl of the amniotic fluid supernatant was centrifuged in 13500g and 200 µl of it was used for DNA extraction. The chelex100 (15) and QIAamp DNA mini kit protocol (16) were used for DNA extraction. DNA quantitation was carried out using spectrophotometry.

For the quality assessment of the extracted DNA, however, GAPDH gene (a housekeeping gene) was analyzed by PCR. After ensuring of the DNA presence in the samples, PCR and Real-time PCR were carried out.

Sex-determination of extracted DNA samples by PCR

Male-specific SRY marker which is located in the sex-determining region of Y chromosome was amplified by using designed primers. (Table 1) PCR reaction was performed in a final volume of 20 µl including 10 µl of PCR master mix (containing DNA polymerase, salts, magnesium, dNTPs and optimized reaction buffer), 2 µl of each primers, 4 µl of sample DNA and 2 µl of distilled water. The conditions of the reaction are shown in table 1.

Sex determination of extracted DNA samples by Real time PCR

Real-time PCR reaction was performed in the Real-time smart cycler and in a final volume of 25 µl including 12.5 µl of Tag DNA master mix, 0.15 µl of probe, 0.6 µl of each primers, 5 µl of extracted DNA and 6.15 µl of distilled water. The conditions of reaction are illustrated in Table 2.

Tests for early diagnosis of thalassemia and sickle cell anemia in fetus using DNA sample extracted from supernatant

In the target family mother was carrying IVS II-I heterozygous mutation and father was carrying E7V heterozygous mutation. IVS II-I mutation and E7V mutation in beta hemoglobin chain were analyzed in the fetus by ARMS-PCR method using the DNA sample extracted from amniotic fluid supernatant. Compared repetitive sequences used in the PCR included AvaII, Hinf, 3`and XMNI used primers and conditions reaction is given in Table 3.

Results

The results showed that DNA extracted by chelex100 failed to have the desired quality and efficiency and GAPDH gene was identified only in samples extracted by Qiagen kit. However, high quality DNA samples were extracted using a small amount of amniotic fluid supernatant. Sex determination was conducted by conventional PCR, the results of which were quite identical to those of cytogenetic indicating a 100% sensitivity of the PCR reaction. Fetus gender was also tested by Real-time PCR and the results were similar to conventional showing that a 100% sensitivity of Real-time PCR (figure 1).

Table 1. Primers and PCR reactions that were used for this project.

Target Gene	Primer(5`->3`)	PCR condition	Product size
GAPDH	Forward CATGGCCTCCAAGGAGTAAG Reverse GCTTGAGCACAGGGTACTTTA	94 ºC, 2 min; 35 cycles of 95 ºC, 30 s; 60 ºC, 30 s; 72 ºC, 30 s	219 bp
SRY	Forward GTAACAAAGAATCTGGTAGA Reverse TTTCAGTGCAAAGGAAGGAA	95 ºC, 15 min; 35 cycles of 94 ºC, 30 s; 57 ºC, 30 s; 72 ºC, 30 s	212 bp

Table 2. Taqman probe and primers and Real time-PCR reactions.

Target Gene	Primer(5`->3`)	Probe	PCR condition
SRY	Forward TGGCGATTAAGTCAAATTCGC Reverse CCCCCTAGTACCCTGACAATGTATT	AGCAGTAGAGCAGTCAGGGAGGCAGA	212 bp95 ºC, 10 min; 45 cycles of 95 ºC, 15 s; 60 ºC, 60 s

Table 3. Used primers and PCR reactions for ARMS PCR.

Target Gene	Primer(5`->3`)	Product size	PCR condition
AvaII	Mutant GAACTTCAGGGTGAGTCTATGGGACC Normal GAACTTCAGGGTGAGTCTATGGGACG Common GATCCTGAGACTTCCACACTGATGC	152 bp	95 ºC, 2 min; 29 cycles of 95 ºC, 30 s; 64.9 ºC, 30 s; 72 ºC, 20 s
IVS II-I	Mutant AAGAAAACATCAAGGGTCCCATAGACTGAC Normal AAGAAAACATCAAGGGTCCCATAGACTGAT Common ACCTCACCCTGTGGAGCCAC	634 bp	95 ºC, 2 min; 35 cycles of 95 ºC, 30 s; 64 ºC, 30 s; 72 ºC, 70 s; 72 ºC, 5min
E7V	Mutant CCCACAGGGCAGTAACGGGACACTTCTGCA Normal CCCACAGGGCAGTAACGGCAGACTTCTGCT Common ACCTCACCTGTGGAGCCAC	207 bp	95 ºC, 2 min; 14 cycles of 95 ºC, 30 s; 69.4 ºC, 30 s; 72 ºC, 110 s, and 19 cycles of 95 ºC, 30 s; 62.4 ºC, 30 s; 72 ºC, 110 s

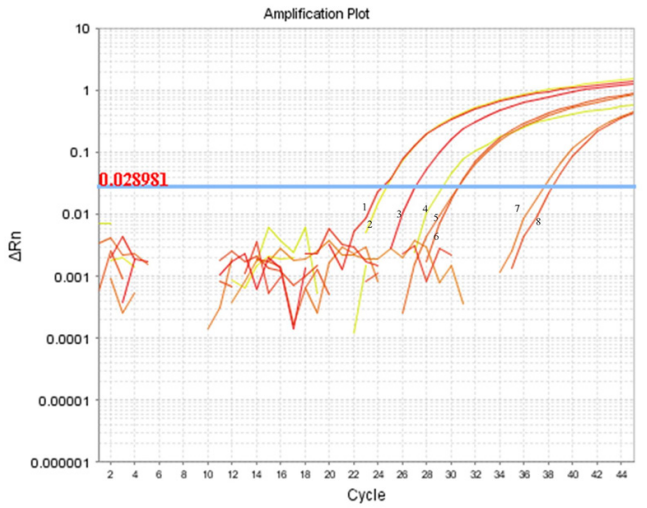

Figure 1. Real time-PCR analysis by the SRY gene to determine fetal gender using of DNA extracted from Blood and amniotic fluid supernatant. Curves 1: male DNA extracted from Blood (positive control), 2: diluted human genome (1/100), 3: male DNA extracted from Blood (positive control), 4: diluted human genome (1/1000), 5 and 6: male DNA extracted from amniotic fluid supernatant, 7 and 8 : female DNA extracted from Blood (negative controls). Female DNAs extracted from amniotic fluid supernatant were also not detected.

Using fetal DNA extracted from amniotic fluid supernatant, tests for prenatal diagnosis of thalassemia and sickle cell anemia were carried out. Similar to the mother, the embryo was heterozygous for IVS II-I mutation, but was normal for E7V mutation (Figure 2, Figure 3). DNA sample was confirmed to be fetal by using Ava II site on the β-globin gene (Figure 4).

Discussion

Cell-free fetal DNA is present in the amniotic fluid at high levels (11). Zhong et al. (17) examined the relationship between the amount of free fetal DNA in maternal plasma and Amniotic fluid in 12 samples. The authors demonstrated that the amount of DNA in maternal plasma is lower than that of the amniotic fluid. Makrydimas and et al.as in 2008 also confirmed this results by comparing cell-free fetal DNA concentration in maternal serum, amniotic fluid and celomic fluid (18).In addition, it was shown that free fetal DNA in amniotic fluid can be readily used for molecular karyotype production (4, 19).Fetal mRNA extracted from amniotic fluid has also been used for gene expression (20, 21).Amniotic fluid supernatant was usually ignored before 1997 with unawareness that it could be used for prenatal diagnosis (22, 23, 24, 25, 26).

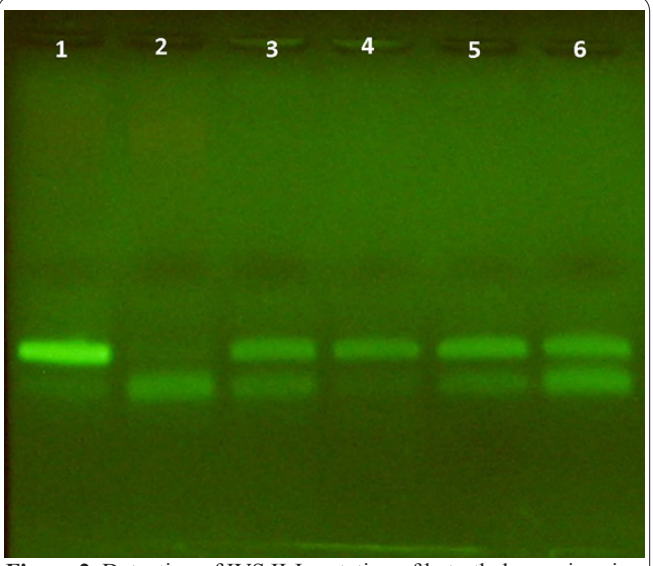

Figure 2. Detection of IVS II-I mutation of beta-thalassemia using ARMS-PCR.
Line 1-2: Normal and Mutant bands for IVS II-I mutation from blood (Father, Normal). Line 3--4: Normal and Mutant bands for IVS II-I mutation from blood (Mother, Heterozygote). line 5-6 Normal and Mutant bands for IVS II-I mutation from amniotic fluid supernatant (Fetus, Heterozygote) respectively.

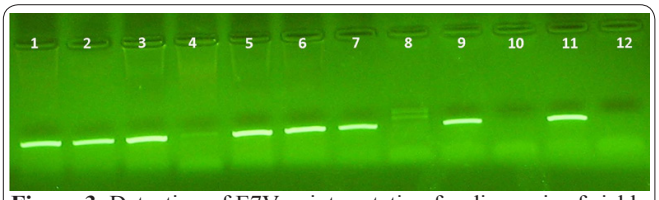

Figure 3. Detection of E7V point mutation for diagnosis of sickle cell anemia using ARMS-PCR.
Line 1-2: Normal and Mutant bands of E7V mutation from blood sample (Father, Heterozygote). Line 3-4: Normal and Mutant bands of E7V mutation from blood sample (Mother,Normal). line 5-6: Normal and Mutant bands of E7V mutation from blood sample (Affected son, Heterozygote). line 7-8: Normal and Mutant bands of E7V mutation from amniotic fluid sample (Fetus, normal), line 9-10: Normal and Mutant bands of E7V mutation from amniotic fluid supernatant sample (Fetus, normal), line 11-12 Normal and Mutant bands of E7V mutation from CVS sample (Fetus, normal). CVS: Chorionic villus sampling.

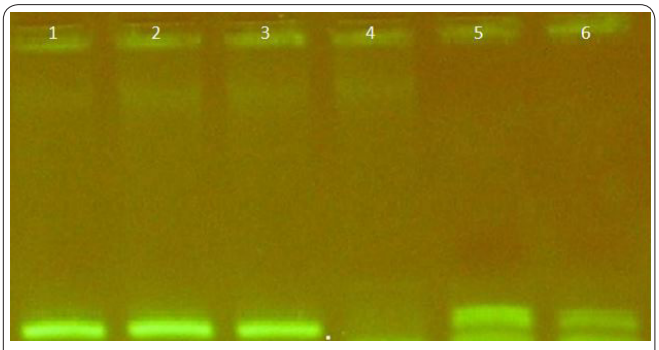

Figure 4. Gel electrophoresis of ARMS PCR for normal and mutant primers for Ava II site on the β-globin gene.
Line 1-2: PCR of normal and mutant sample of father. Line 3--4: PCR of normal and mutant sample of mother, line 5-6: PCR of normal and mutant sample of amniotic fluid supernatant.

This study was designed to test the quality of fetal DNA extracted from the amniotic fluid supernatant. The results showed that high quality DNA can be extracted from a low volume of amniotic fluid supernatant using kits. Gender determination by the amniotic fluid supernatant and the amniotic fluid as sources were shown to be 100% similar with 100% sensitivity. The results for detection of IVS II-I and E7V mutation in the targeted family confirmed the amniotic fluid supernatant is useful in early diagnosis of this disease.

IVS II-I mutation and E7V mutation were analyzed using the samples extracted from the amniotic fluid supernatant. Healthy for sickle cell anemia was not detected in the fetus carrying the IVS II-I heterozygous mutation. These results were confirmed using the routine

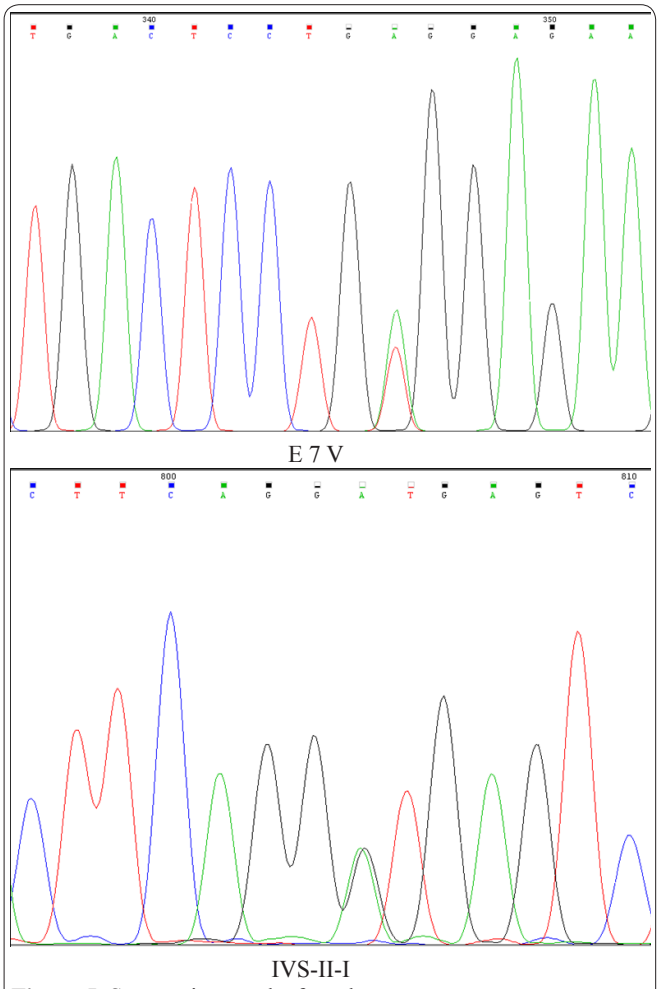

Figure 5. Sequencing results from heterozygote cases.

methods. Therefore, our results showing the fact that using cell-free fetal DNA in the supernatant of amniotic fluid is fast and accurate.While the amniotic fluid is used as the main source for prenatal diagnosis our finding suggest that by including amniotic fluid supernatant as a potential and even more efficient material for prenatal diagnostic tests. As conclusion, the findings of the present study suggest that the cell-free fetal DNA found in the amniotic fluid supernatant are applicable for prenatal diagnosis and can be used as an additional material for diagnosis of genetic disorders and gender determination.

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