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# An Enrichment Method of Cell-free Fetal DNA from Mothers in the 11th Week of Pregnancy; On The Way of Non-invasive Prenatal Diagnosis of Beta-thalassemia as a Single Gene Disorder

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## Abstract

The aim of this study was to examine the feasibility of using an economic and practical method in order to perform non-invasive prenatal testing of thalassemia as a sing gene disorder.Sixteen **(**16) pregnant mothers in the 11$^{th}$ week of pregnancy who were referred for prenatal diagnosis of thalassemia were selected. The parents had one of IVSII-1, IVSI-5 or FR codon 8/9 mutations. Enrichment of cffDNA was performed by a modified whole genome amplification. Based on the relative mutation dosage assay, wild and mutant alleles were compared by allele specific and Taqman allele specific real time PCR. The results obtained were compared with the results of invasive CVS. When both paternal and maternal mutations were identical IVSII-1 or FR codon 8/9, all three major thalassemic fetuses were detected by significant minus ΔCts (Ct M-CtW) but no different ΔCts was observed in seven cases in which fetuses were normal or carrier. In two cases with identical IVSI-5 parental mutations, the two major thalassemic fetuses could not be detected. In four cases with different paternal and maternal mutations, all three carrier fetuses were detected and in one major fetus, only paternal mutation was detected.This innovative method showed the detection of three of the five major thalassemic fetuses when the parental mutations were identical. Furthermore, paternal mutation inheritance could be determined in carrier or major thalassemic fetuses when the parental mutations were different. Further studies on fetuses in late gestational age may have more successful results.

**Keywords**: Non-invasive prenatal testing; Beta-thalassemia; Enrichment; Modified whole genome amplification; Relative mutation dosage.

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## Introduction

Thus far, the presence of cell free fetal DNA in maternal plasma has shown many applications. However non-invasive prenatal testing (NIPT) for single gene disorders is straggling [1] Thalassemia has been considered as disease model to progress strategies for NIPT of monogenic traits [2]. One approach is detection or exclusion of paternally inherited mutation in fetus that are absent in mothers' genome [3]. Detection of maternally inherited mutations in maternal plasma requires to access to highly sensitive DNA quantification methods. Digital PCR, next generation sequencing and relative haplotype dosage (RHDO) have been used most often in molecular techniques [1]. Digital PCR with relative mutation dosage (RMD) among these methods showed the ability to detect fetal genotyping directly even where parental mutation are identical [4]. The cost and complex data analysis of NGS and RHDO or digital PCR and RMD could be reduced by enrichment methods [5]. Furthermore, enrichment methods of cffDNA based on whole genome amplification with or without size based selections, have been reported in some other studies [6-8]. In the present study, we aimed to combine both RMD and enrichment approaches to determine an economic simple and available method for NIPT of the first three common mutations of thalassemia in Iran.

## Materials and Methods

Blood samples were collected from 16 mothers in the 11$^{th}$ week of pregnancy, who were referred for prenatal diagnosis of thalassemia to one of our partner labs. Sampling was done before CVS performance. Parents were selected to have one of IVSII-1, IVSI-5 or FR codon 8/9 mutations. Blood samples were used for plasma isolation.

### *Plasma isolation*

About 12ml of blood samples were collected in 15ml falcon tubes. First step centrifugation was performed at 800g for 10 min to separate plasma from the cellular fraction. The plasma fraction was removed and centrifuged again at 16,000g for 10 min to further remove any contaminating cellular particles. This fraction was then frozen in 2ml cryotubes at -080°C.

### *DNA extraction from plasma*

Samples were subjected to DNA extraction using the Qiagen QiAamp Blood Maxi Kit (Cat # 51194) and eluted in a final volume of 500µL.

### *Whole genome amplification*

We modified the protocol for the GenomePlex® Complete Whole Genome Amplification (WGA) Kit (Sigma, Cat#WGA2-50rxn) as follows: Because of fragmented nature of cffDNA in maternal plasma and to prevent larger maternal sequences from fragmenting for subsequent amplification, we eliminated the fragmentation step. Second modification performed to increase amplification of short fragments which include more fetal DNA ratio versus long ones. This step done by dividing the amplification step into two parts after the denaturation step at 95°C for 3min: the first 12cycles : 95°C for 15 sec and 65°C for 5min and then 10 cycles : 88 °C for 15 sec and 65°C for 5min.

### *Taqman real time PCR*

Sex determination of eight enriched plasma samples performed by Taqman realtime PCR. Primers and probes for SRY (Gene ID: 6736) and HBB (Gene ID: 3043) genes were designed by the Beacon designer software trial version (Premier Biosoft Co) which was ordered from Metabion company. The detection of SRY and HBB in the enriched DNA was performed in a 10µl doublex reaction which contained 0.5 µM of primers and 0.2 µM of probes, 5µl of 2x Rotor-Gene Multiplex PCR Master Mix (Qiagene) and 2 µl of enriched DNA. The reaction was conducted in a thermo cycler Realtime PCR Rotorgen 6000 as follows; denaturation step in 95°C for 5min and 45 cycles of 95°C for 15 sec and 60°C for 15 sec.

### *Allele specific real time PCR for detection of three mutations of thalassemia*

Allele specific primers were designed for mutant and wild alleles of IVSII-1(rs33945777) and the FR codon 8/9(rs35699606). Allele specific primers and one probe were designed for IVSI-5 (rs33915217).

Realtime PCR for IVSII-1 and FR codon 8/9 were performed as a 12 µl reaction which contained 6µl of RealQ Plus 2x Master Mix Green (Ampliqon Co), 0.5 µM of primers, 2µl of enriched DNA in a thermo cycler Realtime PCR Rotorgen 6000 after 15min denaturation at 95°C and 40 cycles of 91°C for 10 sec and 61°C for 20 sec.

Taqman Realtime PCR for IVSI-5 was performed as a 12 µl reaction which contained 6µl of 2x Rotor-Gene MultiplexPCR Master Mix, 0.5 µM of primers and 0.2 µM of probes and 2µl of enriched DNA in a thermo cycler Realtime PCR Rotorgen 6000 after 5min denaturation at 95°C and 45 cycles of 95°C for 15 sec and 60°C for 15 sec.

## Results

According to the request of some parents to check for aneuploidy, we had eight parents with known fetal sex determined by quantitative florescent PCR on CVS samples. The sex determination of eight extracted DNA from maternal plasma samples was performed by Taqman realtime PCR using the primers and probe set shown in Table 1 and all cases were confirmed as four male and four female fetuses.

Based on the different genotypes of paternal and maternal alleles, we had samples as follows (Table 2).

In nine (9) cases with identical parental mutations of IVSII-1 which have been detected to have 2 major, 5 carriers, and 2 normal fetuses by CVS, two major fetuses were detected by ΔCts (Ct of mutant allele –Ct of wild allele)-7.0 and -5.5. The carrier and normal fetuses did not show any significant difference in ΔCts.

In Two cases with identical parental mutations of IVSI-5 which have been detected to have two major fetuses by CVS, cffDNA assay of maternal plasma did not show any significant difference in ΔCts.

In One case with identical parental mutations of Fr codon 8/9 which have been detected to have a major fetus by CVS the major fetus was detected by ΔCts -3.0.

In four cases with different parental mutations which have been detected to have one major and three (3) carrier fetuses by CVS ;

One carrier fetus with paternal IVSII-1 mutation were detected. One carrier fetus had maternal IVSI-5 mutation in which paternal allele was detected as normal. One major fetus could not be detected as major but the paternal IVSII-1 mutation was detected. One carrier fetus had maternal IVSII-1mutation in which the paternal allele detected as normal.

From a total of 6 major fetuses, 3 of them with identical parental mutations were successfully detected and from other three major cases, the paternal mutation could be detected in one case. From a total of 8 carrier cases, in 4 cases with different parental mutations, the status of the fetus could be detected.

## Discussion

NIPT for beta-thalassemia and other single gene disorders has most often been based on the detection of paternally inherited alleles [9-11]. Recently, some studies showed the successful genotyping of fetus when parental mutations were identical. They developed methods using expensive digital PCR, Next generation equipment's complex bioinformatics and relative haplotype dosage analysis [4, 12-14].

We aimed to develop a way of determining fetal status using cffDNA independent of parental inheritance by a simple and practical method which can be used as routine in the clinic. So to start, we considered β-thalassemia as one of the most common single gene disorders and the first three common mutations in Iran.

The success of digital PCR approach to genotyping of fetus seems related to the RMD assay to some extent. In theory, if we can reach a desirable concentration of cffDNA by enrichment methods, the RMD assay would be useful by a simple method such as Real time PCR. It is possible to use co-amplification at a lower denaturation temperature-PCR (COLD-PCR)cffDNA at a temperature at which maternal DNA would remain double-stranded(12). Therefore, considering COLD PCR and whole genome amplification techniques, we could find a new approach for enrichment [6, 16, 17]. Recently in 2017, a new study was performed like our

**Table 1.** Primer/probe sequences

| Target | | Primer/probe sequence | Amplicon size (bp) | Annealing T (ºC) |
|---|---|---|---|---|
| HBB | F | CCTCTTATCTTCCTCCCA | 158 | 60 |
| | R | AAGCGAGCTTAGTGATAC | | |
| | Probe | 5' HEX-CATTAGCCACACCAGCCACC-3'BHQ-1 | | |
| SRY | F | GCTTCTGCTATGTTAAGC | 127 | 60 |
| | R | CACACTGATACTTAGAGTTAC | | |
| | Probe | 5' 6-FAM- CAACAGCGATGATTACAGTCCAGC-3'BHQ-1 | | |
| IVSII-1 | N | ATCAAGCGTCCCATAGACTCAC | 123 | 61 |
| | M | CATCAAGCGTCCCATAGACTCAT | | |
| | R | TTAGTGATGGCCTGGCTCAC | | |
| IVSI-5 | F | CCTCAAACAGACACCATG | 134 | 60 |
| | N | ACCTGTCTTGTAACCTTGATGC | | |
| | M | ACCTGTCTTGTAACCTTGATGG | | |
| | Probe | 5' HEX-CCTGAGGAGAAGTCTGCCGTTACTGCC-3'BHQ-1 | | |
| Fr 8-9 | N | AGGGCAGTAACGGCAGACT | 138 | 61 |
| | M | GGGCAGTAACGGCAGACC | | |
| | R | GGCTGGGCATAAAAGTCAGGG | | |

**Table 1.** Results from cell free fetal DNA analysis and invasive method (CVS)

| | # | Maternal Mutation | Paternal mutation | Fetal phenotype (by CVS) | Fetal phenotype (by cffDNA) |
|---|---|---|---|---|---|
| Parents with different mutations | *1* | *IVSI-5/WT* | *IVSII-1/WT* | Carrier *IVSII-1/WT* | Carrier *IVSII-1* |
| | *2* | *IVSI-5/WT* | *IVSII-1/WT* | Major | Carrier *IVSII-1* |
| | *3* | *IVSI-5/WT* | *IVSII-1/WT* | Carrier *IVSI-5/WT* | Normal/carrier(not paternal) |
| | *4* | *IVSII-1/WT* | *Fr8-9/WT* | Carrier *IVSII-1/WT* | Normal/carrier(not paternal) |
| Parents with identical mutations | 5 | IVSI-5/WT | IVSI-5/WT | Major | Normal/carrier |
| | 6 | IVSI-5/WT | IVSI-5/WT | Major | Normal/carrier |
| | 7 | IVSII-1/WT | IVSII-1/WT | Carrier | Normal/carrier |
| | 8 | IVSII-1/WT | IVSII-1/WT | Normal | Normal/carrier |
| | 9 | IVSII-1/WT | IVSII-1/WT | Carrier | Normal/carrier |
| | 10 | IVSII-1/WT | IVSII-1/WT | Carrier | Normal/carrier |
| | 11 | IVSII-1/WT | IVSII-1/WT | Carrier | Normal/carrier |
| | 12 | IVSII-1/WT | IVSII-1/WT | Carrier | Normal/carrier |
| | 13 | IVSII-1/WT | IVSII-1/WT | Normal | Normal/carrier |
| | *14* | *IVSII-1/WT* | *IVSII-1/WT* | Major | Major |
| | *15* | *Fr8-9/WT* | *Fr8-9/WT* | Major | Major |
| | *16* | *IVSII-1/WT* | *IVSII-1/WT* | Major | Major |

assay to enrich cffDNA based on PCR at a lower denaturation temperature. They added linker fragments and constructed libraries from whole genome. The experiment was conducted on mothers in the 18$^{th}$ week of pregnancy and the enrichment was investigated by real time PCR. The results were hopeful for use in routine clinics but it was not tested practically on patients [18]. In comparison, our enrichment method seems simpler because we only modified current protocol of WGA kit. In addition our study was performed in lower gestational age, however more investigations are required to increase detection rate.

Circulating cell-free fetal DNA originates from the apoptotic trophoblastic cells. The increasing gestational age causes the large size of placenta and the increasing number of apoptotic trophoblastic cells. Fetal DNA comprise approximately 3–13% of the total cell free DNA in most of the samples and has to be estimated with real-time quantitative PCR. Over 8% is considered satisfactory for most NIPT methods, while a 4–8% is considered marginal [19].

Since the gestational age and maternal weight significantly affected the ratio of cffDNA and our study was performed in the 11th week of pregnancy, the cffDNA concentration may not be enough in some patients to detect the fetal status [20].

NIPT of single gene diseases which could detect maternal mutations of fetus in maternal plasma require highly sensitive DNA measuring techniques like NGS or digital PCR .Our results showed feasibility of a new simple method for detection of major thalassemia fetuses by real time PCR, when parents have identical mutations. According to the high ratio of identical mutations in consanguineous marriages, the number of fetuses which could be detected by this method in populations with this feature would be considerable.

When parental mutations were different, we could detect if carrier fetuses have paternal alleles or not and the paternal allele of the major fetus could also be detected. Therefore, this new method will be useful in reducing the number of cases referred to do CVS.

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