Early Fetal Gender Determination Using Real-Time PCR Analysis of Cell-free Fetal DNA During 6th-10th Weeks of Gestation

Hamid Reza Khorram Khorshid[1], Maryam Zargari[2,3], Mohammad Reza Sadeghi[4], Haleh Edallatkhah[3], Mohammad Hassan Shahhosseiny[5], and Koorosh Kamali[3]

[1] *Genetic Research Centre, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran*

[2] *Biology Department, Science and Research Branch, Islamic Azad University (IAU), Tehran, Iran*

[3] *Reproductive Biotechnology Research Centre, Avicenna Research Institute (ACECR), Tehran, Iran*

[4] *Reproductive Endocrinology and Andrology Research Centre, Avicenna Research Institute (ACECR), Tehran, Iran*

[5] *Microbiology Department, Shahr-e-Qods Branch, Islamic Azad University (IAU), Tehran, Iran*

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Abstract- Nowadays, new advances in the use of cell free fetal DNA (cffDNA) in maternal plasma of pregnant women has provided the possibility of applying cffDNA in prenatal diagnosis as a non-invasive method. In contrary to the risks of invasive methods that affect both mother and fetus, applying cffDNA is proven to be highly effective with lower risk. One of the applications of prenatal diagnosis is fetal gender determination, which is important in fetuses at risk of sex-linked genetic diseases. In such cases by obtaining the basic information of the gender, necessary time management can be taken in therapeutic to significantly reduce the necessity of applying the invasive methods. Therefore in this study, the probability of detecting sequences on the human Y-chromosome in pregnant women has been evaluated to identify the gender of fetuses. Peripheral blood samples were obtained from 80 pregnant women with gestational age between 6th to 10th weeks and the fetal DNA was extracted from the plasma. Identification of *SRY, DYS14 & DAZ* sequences, which are not present in the maternal genome, was performed using Real-Time PCR. All the obtained results were compared with the actual gender of the newborns to calculate the test accuracy. Considerable 97.3% sensitivity and 97.3% specificity were obtained in fetal gender determination which is significant in the first trimester of pregnancy. Only in one case, false positive result was obtained. Using non-invasive method of cffDNAs in the shortest time possible, as well as avoiding invasive tests for early determination of fetal gender, provides the opportunity of deciding and employing early treatment for fetuses at risk of genetic diseases.

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Introduction

Prenatal diagnostic are classified into invasive and non-procedures. Currently the invasive procedures such as amniocentesis and chorionic villous sampling (CVS) are used routinely, but each of the methods causes a small but finite risk for both fetus and the mother (1). On the other hand, in addition to the low sensitivity, in the non-invasive procedures such as ultrasonography, performed in the 2nd trimester of pregnancy, the time of diagnosis is relatively late for women especially in cases of high-risk genetic diseases (2).

In 1997 with the discovery of cell free fetal DNA (cffDNA) fragments in plasma and serum of pregnant women carrying a male fetus, reliable and accurate diagnosis using the non-invasive procedure came into reality (3). Further studies with the aid of quantitative Real-Time PCR method revealed that the concentration of cffDNA in maternal plasma which was originated from apoptotic trophoblasts of the placenta (4), reached a mean of 25.4 GEq/ml in early stage of pregnancy and 292.2 GEq/ml in late stage of pregnancy. In fact,

Corresponding Author: Koorosh Kamali
Reproductive Biotechnology Research Centre, Avicenna Research Institute (ACECR), P.O box: 19615-1177. Postal code: 1936773493, Tehran, Iran
Tel: +98 21 22432020, E-mail: k.kamali@avicenna.ac.ir

cffDNA concentrations increased with gestational age from 3.4% to 6.2% of total DNA, circulating in plasma of pregnant women (5). Also, detection of cffDNA in maternal plasma was not possible with the lapse of 4 to 30 minutes after delivery, because of its extremely rapid clearance from the maternal circulation with a mean half-life of 16.3 *min*; making it a potentially ideal source for prenatal investigation without any false effect on the results (6,7).

One of the applications of prenatal diagnosis is fetal gender determination; of which managing the X-linked genetic abnormality is the main reason for performing these types of tests. Determination of Fetal gender using cffDNA in maternal plasma are mainly limited to those sequences which are inherited from the father and are absent from the maternal genome; such as the *SRY*, *DYS14* and *DAZ* sequences that are located on the Y chromosome. Therefore, the only way to identify these sequences is through male-bearing pregnancies (8).

In this study, early determination of fetal gender using cffDNA can be considered as a non-invasive pre-test to achieve a reliable method with a lower risk than other methods. This method can be particularly beneficial in providing special treatment on the fetus in cases of genital ambiguity or metabolic conditions such as congenital adrenal hyperplasia. To achieve this goal, in the following study, detection of cffDNA in maternal plasma was performed on pregnant women during their 6^{th} -10^{th} weeks of pregnancy to obtain the required sensitivity, specificity and accuracy for early fetal gender determination. This method, if applied in the shortest time possible, can be the most reliable non-invasive method of fetus gender identification, in which reduce the number of invasive diagnosis by half with sparing most female fetuses.

Materials and Methods

Sample collection

Peripheral blood samples were obtained from 80 pregnant women with gestational age between 6^{th} to 10^{th} weeks, who were referred to Avicenna Infertility Clinic in Tehran, Iran during 2009-2010. Five non-pregnant women and five men were also considered as negative and positive controls consecutively in this study. Signed inform consent forms were obtained from all participants before blood sampling; the protocol of the study was approved by the Ethics Committee of Avicenna Research Institute. Five ml whole peripheral blood was collected for each case in a tube containing 200 µl of 0.5 M EDTA and immediately stored at 4˚C for further processing. Blood samples were centrifuged at 3000 g for 10 min and the upper plasma layer was carefully removed without disturbing the buffy coat, transferred into a new 1.5 ml Eppendorf tube for storage at -20˚C until further processing.

DNA extraction

Genomic DNA was extracted from 200 µl of the plasma samples using the QIAamp DNA Blood Mini kit (Qiagen, USA) as recommended by the manufacturer according to the manufacturer's "Blood and Body Fluid" protocol. The DNA was eluted in 50 µL of elution buffer.

Primers

Multicopy *DAZ* and *DYS14* genes were used for detection of sequences on the human Y-chromosome in male-bearing pregnancies. Sequence of a single copy *SRY* gene was also used as an internal control of gender determination. Analysis of the *ACTB* sequences was performed to assess the presence of sufficient cell-free DNA in an extraction. All the pairs of primers were designed using Primer3 and Gene Runner softwares (Table 1).

Quantitative real-time PCR

PCR was carried out on an ABI 7500 real-time PCR (Applied Biosystems, USA) using SYBR Green I chemistry. If the target sequence was present, SYBR green I bind to double stranded DNA which was

Table 1. Primer sequences used in Real-Time PCR.

Primer Name	Sequence (5' → 3')
SRY	Forward: AGTATCGACCTCGTCGGAAG
	Reverse: TCTTGAGTGTGTGGCTTTCG
DYS14	Forward: AGCCCTGATCACTGACGAAG
	Reverse: TGCAGAGATGAACAGGATGC
DAZ	Forward: TACCTCCAAAGCACCAGAGC
	Reverse: AATCTACCCATTCCCGAACC
ACTB	Forward: GATGGTGGGCATGGGTCAGAAGGA
	Reverse: CATTGTAGAAGGTGTGGTGCCAGAT

synthesized in the PCR amplification and as a result, fluorescence was emitted. The melting temperature of amplified DNA is available by measuring the fluorescence intensity. The PCR was carried out in 40 cycles, and amplification reactions were set in a total volume of 10 µl containing 2 µl of plasma DNA (10 ng/µl), 0.2 µl of each primer (10 mM), 5 µl of SYBR® Premix Ex Taq™ (Takara, Japan), 0.2 µl Rox reference Dye II and 2.4 µl of deionised water. Also, a non template control, containing DNA free water was added in each reaction to recognize the contamination.

At the end amplification plots with melting curve data were collected and analysed using the ABI 7500 system software. To record the results, observing the amplification of each *SRY*, *DYS14* and *DAZ* sequence in fetal DNA was considered as a positive reference, so that in cases where more than two amplification were observed for the mentioned sequences in fetal DNA, similar to positive control DNA sample, as a result would considered the male gender. On the other hand, in the absence of Y chromosome sequences, if there was no amplification in fetal DNA similar to negative control DNA sample, it would considered as female gender.

Anti-contamination measures

An aerosol-resistant pipette tips were used for all liquids, and separate areas were considered for all steps of the analysis as an anti-contamination measure; only female operators were selected in all procedures using a laminar flow hood to eliminate contact with exogenous male DNA**.**

Statistical analysis

Each pregnancy was considered as one study subject; in case of multiple gestations only the numbers of pregnancies were considered regardless of the number of the fetuses, in which the identification of the Y chromosome sequences in male-bearing pregnancy was based as a positive result. Finally, all the results were analyzed and compared with the actual gender of the newborns to calculate the sensitivity, specificity and accuracy of the applied method. Kappa coefficient of agreement was also calculated to evaluate the precision of the method used, parameters such as Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were also analysed. Statistical analysis was performed using the SPSS 11.5.

Results

From the total of 80 samples with an age range of 18 to 46 years (30.4±5.8) and pregnancy week range from 6 to 10 (7.7±1.1), abortion was observed in 5 cases; so their data were excluded from statistical analysis due to lack of knowledge for the actual fetal gender. Among the 80 samples, 16 cases with multiple gestations (9 identical twins, 6 non-identical twins and 1 triplet) were observed and without considering the numbers of the fetuses, only the numbers of these pregnancies were considered in statistical analysis.

Real-time PCR results

In all the obtained samples, *ACTB* were amplified indicating the presence of sufficient DNA in the extracted samples. Comparison of the obtained results with the fetal gender at birth indicates that from 38 baby girl born, 37 fetal genders were correctly diagnosed and only in one case, positive result was obtained. Also from 37 baby boy born, by using the *SRY* and *DAZ* sequences respectively 36 and 35 genders were correctly diagnosed. While using the *DYS14* sequence, gender of all newborn boys were identified correctly (Figure 1).

Sensitivity, Specificity and kappa coefficient of agreement in gender determination test is given in table 2 (with 95% confidence intervals) using *SRY*, *DYS14* and *DAZ* sequences. Also, *p* values = 0.001 were statistically significant in each analysis using *SRY*, *DYS14* and *DAZ* sequences. The PPV and NPV of the method used respectively were 97.3% (95% CI= 0.862-0.995) and 97.4% (95% CI=0.865-0.995).

Table 2. Quantitative parameters of gender determination using sequences of *SRY*, *DYS14* and *DAZ*.

Sequences	Result by Real-Time PCR				*P* Value	Kappa Value	Sensitivity	Sensitivity with 95% Confidence Intervals	Specificity	Specificity with 95% Confidence Intervals
	Female		Male							
	Correct	Incorrect	Correct	Incorrect						
SRY	37	1	36	1	0.001	0.947	0.973	0.862 to 0.995	0.973	0.865 to 0.995
DYS14	37	1	37	0	0.001	0.973	1.000	0.905 to 1.000	0.973	0.865 to 0.995
DAZ	37	1	35	2	0.001	0.920	0.945	0.823 to 0.985	0.973	0.865 to 0.995
Total Gender at Birth	38		37							

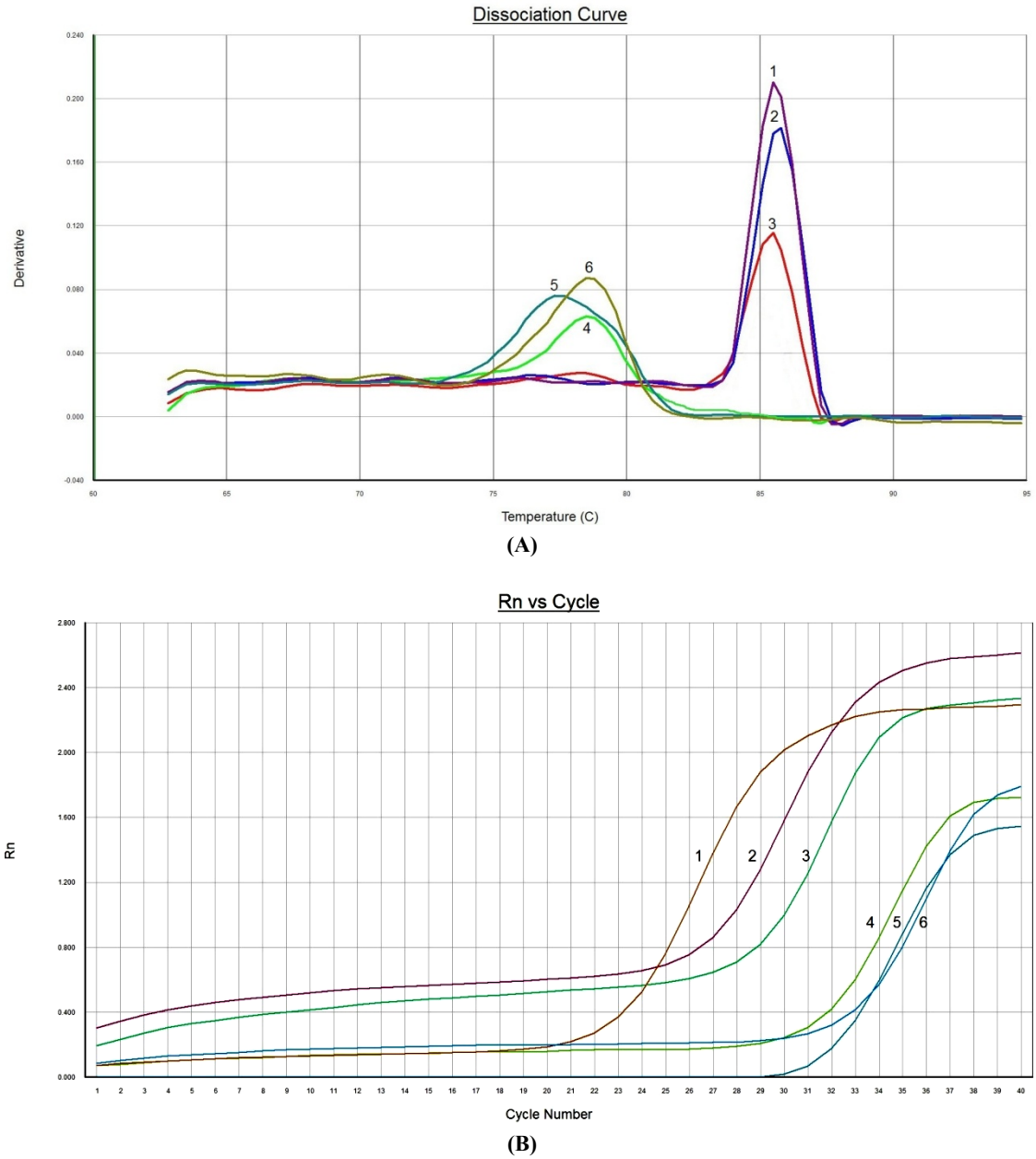

Figure 1. Real-Time PCR Analysis in some fetal DNA samples extracted from the maternal plasma (A) Dissociation curve of *SRY* (B) Amplification Plot of *DYS14*, Curve 1: Male DNA as a positive control, Curve 2,3 & 4: fetal DNA, Curve 5: non pregnant women DNA as a negative control and Curve 6: PCR reaction negative Control (Water).
Signals in sample 2 & 3 indicate the gender of the fetuses is male, and lack of amplification in the rest of the samples indicate the female gender.

Discussion

With the discovery of cffDNA in maternal plasma since 1997, an outstanding revolution in non-invasive prenatal diagnosis was created. Since then and during the last decade, substantial advances have been made in the enrichment and isolation of cffDNA as a reliable fetal genetic source among the alternative approaches in non-invasive prenatal diagnosis like fetal gender determination (9). In this study, the obtained sensitivity level to identify male fetuses, using *SRY, DYS14* and *DAZ* sequences (Table 2), is very close to sensitivity of the results achieved in the past and in most cases, even significantly higher sensitivity was achieved. Previous investigations have shown sensitivity of 97% (Rijinders *et al*., 2001), 86% (Hwa *et al*., 2004), 89% (Davalieve *et al.,* 2006), 80% (Pichiassi *et al*., 2008) and close to 100% (Lo *et al*., 1997) (9-13). However in these

findings, longer time range of pregnancy were considered; which led to high probability of identifying cffDNA because of the gradual increase of its concentration with increasing gestational age (14). In comparison, the results obtained in this study in the 6th to 10th weeks of pregnancy led to significantly better results. Overall, the differences observed between the results of previous studies and this study can be explained through the use of different methods of fetal DNA extraction, low concentration of cffDNA at an early gestational age, number of population, time range of the pregnancy, efficiency of the methods used and existence of potential contamination.

In this study, false positive result was observed only in one case that the gender of a female fetus, which was at the age of six weeks, was diagnosed as a male. Citing the theory of vanishing twins within the first 7 weeks of gestation in 0.3%-0.7% of pregnancies (15), it can be concluded that during the time of sampling in the 6th week of gestation, there was a male twin that was disappeared in the subsequent weeks of pregnancy and only baby girl was born. On the other hand, some differences were observed in identification results of male fetuses using the *SRY, DYS14* and *DAZ* sequences that are caused by different cases of repetitions and copies of the relevant genes. In this regard, *SRY* is a single copy gene while *DYS14* and *DAZ* genes have multiple copies, also the differences between *DYS14* and *DAZ* genes with equal 4 copies arise from their repetitions and the number of repetitions varies in different people (16). Since the target *DAZ* gene primers were designed for these repetitive regions, therefore, different results were obtained than *DYS14* assay. Also, the findings of Zimmerman in 2005 showed that the concentration levels of cffDNA could be quantified with high precision by the multicopy *DYS14* assay as opposed to the single copy *SRY* measurements, so its application in identification of male fetuses could have higher sensitivity and specificity than the single copy *SRY* gene. Hence remarkable consistency is obtained from the comparison of results in this study with the findings of Zimmerman (17).

In comparison between total numbers of fetuses that were correctly diagnosed and total numbers of the infant born, 97.3% accuracy was achieved in fetal gender determination. Also according to the Kappa coefficient of agreement, which is in "almost perfect" agreement range between 0.81 and 0.99 (18), it can be concluded that the results are remarkably consistent with the actual gender of the babies.

To check whether the method used in this study is suitable for clinical application or not, the parameters of PPV and NPV were calculated. The PPV and NPV indicate that if test results are positive and the fetus is diagnosed as a boy, there is 97.3% probability of being born a boy and 97.4% probability to be a girl if the test results are negative. Therefore, this method can be used as a clinical method in determining the fetal gender due to its high probability of correct prediction, prior to applying invasive methods. In conclusion, we assume that non-invasive early determination of fetal sex using cffDNAs in maternal plasma could be presented to women who are at a higher risk of having an infant with an X-linked genetic disorder in the shortest time possible to avoid unnecessary invasive testing and hopefully even in the near future, this method could be applied for identification of specific genetic diseases such as chromosomal aneuploidies, metabolic conditions, *etc*.

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