Noninvasive Prenatal Diagnosis using Cell-Free Fetal DNA in Maternal Plasma: Clinical Applications

Young-Ho Yang, Sung-Hee Han, Kyoung-Ryul Lee

Division of Prenatal Molecular Genetics, Department of Laboratory Medicine, Seoul Medical Science Institute, Seoul Clinical Laboratories, Seoul, Korea

Owing to the risk of fetal loss associated with prenatal diagnostic procedures (amniocentesis, chorionic villus sampling), noninvasive prenatal diagnosis (NIPD) is ultimate goal of prenatal diagnosis. The discovery of circulating cell-free fetal DNA (cffDNA) in maternal plasma in 1997 has opened up new probabilities for NIPD by Dr. Lo et al. The last decade has seen great development in NIPD. Fetal sex and fetal RhD status determination by cffDNA analysis is already in clinical use in certain countries. For routine use, this test is limited by the amount of cell-free maternal DNA in blood sample, the lack of universal fetal markers, and appropriate reference materials. To improve the accuracy of detection of fetal specific sequences in maternal plasma, internal positive controls to confirm to presence of fetal DNA should be analyzed. We have developed strategies for noninvasive determination of fetal gender, and fetal RhD genotyping using cffDNA in maternal plasma, using real-time quantitative polymerase chain reaction (RT-PCR) including *RASSF1A* epigenetic fetal DNA marker (gender-independent) as internal positive controls, which is to be first successful study of this kind in Korea. In our study, accurate detection of fetal gender through gestational age, and fetal RhD genotyping in RhD-negative pregnant women was achieved. In this assay, we show that the assay is sensitive, easy, fast, and reliable. These developments improve the reliability of the applications of circulating fetal DNA when used in clinical practice to manage sex-linked disorders (e.g., hemophilia, Duchenne muscular dystrophy), congenital adrenal hyperplasia (CAH), RhD incompatibility, and the other noninvasive pregnant diagnostic tests on the coming soon. The study was the first successful case in Korea using cffDNA in maternal plasma, which has created a new avenue for clinical applications of NIPD.

Key Words: Noninvasive prenatal diagnosis, Cell-free fetal DNA, Hypermethylated *RASSF1A* gene, Maternal plasma, Fetal gender, Fetal RhD status, Single gene disorders

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Corresponding author: Young-Ho Yang, M.D., Ph.D.
Division of Prenatal Molecular Genetics, Department of Laboratory Medicine, Seoul Medical Science Institute, Seoul Clinical Laboratories, 7-14 DongBingGo-Dong, YongSan-Gu, Seoul 140-809, Korea
Tel: +82-2-330-2016; Fax: +82-2-790-6509
E-mail: yhyang102@scllab.co.kr
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Prenatal diagnosis is an integral part of obstetric practice. Prenatal genetic diagnosis relies on the sampling of fetal tissues and is dependent on invasive procedures such as amniocentesis or chorionic villus sampling (CVS), both of which carry a risk of miscarriage of around 1-2% and cannot be performed until 11 weeks of gestation. Noninvasive prenatal diagnosis (NIPD) is a goal of prenatal diagnosis. A reliable and

convenient method for NIPD has long been sought to reduce this risk of miscarriage and allow earlier testing. To stratify pregnant women according to their risk of carrying a fetus affected by chromosomal aneuploidy, several screening methods have been developed, including ultrasonography and maternal serum biochemistry[1]. These methods, however, are targeted at epiphenomena associated with the chromosomal aneuploidies, and have limited sensitivities and specificities, with strictly defined gestational age windows that must be used for specific tests. To circumvent such limitations, there is a need for the development of a new generation of non-invasive tests that target the core pathology of a fetus. From the 1960s to 1990s, many researchers have investigated a variety of approaches for the isolation of fetal nucleated cells from maternal blood[2-4]. The rarity of circulating fetal nucleated cells in maternal blood has limited the practicality of these approaches and their diagnostic sensitivity, specificity and reproducibility. The large-scale NIH-funded National Institutes of Health Fetal Cell Study (NIFTY) showed that the enrichment and analysis of circulating fetal cells was not suitable for clinical application[5]. It was clear from these figures that an alternative approach for the direct detection of fetal genetic material from maternal blood, rather than through the isolation of fetal nucleated cells in maternal blood, was needed.

Between the 1970s and 1990s, a parallel group of investigators was studying the intriguing phenomenon of cell-free DNA in the plasma of human subjects. One particularly interesting line of research was the detection of tumor-derived DNA in the plasma of women suffering from a variety of cancers[6, 7]. Inspired by such work, Lo et al. hypothesized in 1997[1] that a fetus might also release its DNA in cell-free form into the plasma of its mother. This hypothesis has led to the direct experimental demonstration of cell-free male fetal DNA in the blood of women carrying male fetuses. This discovery has opened up new possibilities for NIPD. Cell-free fetal DNA (cffDNA) originates from apoptotic placenta cells (trophoblasts) derived from the embryo[8, 9] and comprises around 3-6% of the total cell-free DNA in maternal circulation from the first trimester onwards, with concentrations that increase with gestational age[10]. In contrast to fetal cells[11], cffDNA clears very rapidly from the maternal plasma within hours after delivery[12].

Development of the technology required for safe and effective NIPD required the concerted effort of many different experts with access to clinical practice. The Special Noninvasive Advances in Fetal and Neonatal Evaluation Network (SAFE) provides these facilities[13]. SAFE was fundamental in the implementation of NIPD for fetal gender determination in parts of Europe, and has facilitated implementation of fetal RhD typing, by developing the standards required when using these new technologies in clinical practice[14]. The objective of this study is to provide an introduction to new technologies involving maternal plasma cffDNA for NIPD and to set up its clinical applications in Korea.

1. Detection of cell-free fetal DNA

1) The detection of Y-related sequences in cell-free fetal DNA

Isolating cffDNA in an overwhelming background of maternal cell-free DNA is a significant technical challenge. During early and late pregnancy, fetal DNA constitutes a mean of approximately 3-6% of total cell-free DNA in maternal plasma[10], with the obvious implication that most of the DNA in the plasma is maternal in origin. There are many general problems associated with detecting cffDNA in maternal circulation: 1) the concentration of all cell-free DNA in blood is relatively low; 2) the total amount of cell-free DNA varies between individuals; 3) the fetus inherits half its genome from the mother.

A number of methods have been developed to address these problems. Initially, the cell-free DNA must be separated from the rest of the blood. Once a sample of

maternal blood has been taken, the plasma fraction is separated from cellular matter by centrifugation. Previous studies have revealed that the way plasma is derived from maternal whole blood has important implications for the analysis of maternal plasma DNA[15]. To ensure consistent and reliable analytical results, cellular elements should be eliminated from maternal plasma either by adopting a two-step centrifugation protocol or filtration of plasma[16, 17]. Contamination of plasma with residual cellular elements could result in a fluctuant background of maternal DNA, affecting the quantitative interpretation of maternal plasma total DNA concentration[16].

Fetal DNA was detectable in maternal plasma and serum[1, 10, 18]. For most laboratories, plasma has become the material of choice for NIPD because it contains less maternal DNA, which is presumably released from maternal white blood cells during the clotting process. The concentration of maternal DNA derived from plasma remains stable for 6 hours[19]. These data further support the advantage of maternal plasma over serum for fetal DNA analysis and suggest that maternal blood samples collected into EDTA tubes should be processed within 6 hours.

Once the cell-free DNA fragments have been purified, small differences between the fetal and maternal DNA sequences are exploited to make a specific fetal diagnosis. To date, most studies have focused on the detection of paternally inherited sequences that are entirely absent from the maternal genotype, such as those on the Y chromosome of male fetuses. This target is particularly attractive as it comprises a large portion of DNA that is not otherwise present in women. The majority of the reports on fetal gender have used detection of the sex-determining region Y-chromosome gene (*SRY*) or DYS14 marker sequence of the testis specific protein gene (*TSPY*). The targeting of the multi-copy DYS14 sequence of *TSPY* appears to be more sensitive for quantitative assessment of the cffDNA in maternal plasma[20], but the effect of copy-number polymorphisms on quantitative analysis between different individuals has not been explored. *SRY* as a single copy gene represents the best choice for confirmatory analysis of the fetal DNA in maternal plasma samples[21].

The most common technique currently used for detection and identification of specific cffDNA sequences is PCR. A number of different types of PCR have been explored, of which the most popular is real-time quantitative PCR (RT-PCR)[22], as it combines high sensitivity with a closed detection system, thereby minimizing the risk of contamination. A reagent is used in conventional PCR, as are probes with fluorescently labeled reporter and quencher dyes. When the target gene (e.g. *SRY*) is present, exponential DNA amplification causes a proportional increase in the reporter dye fluorescence during each PCR cycle, shown after

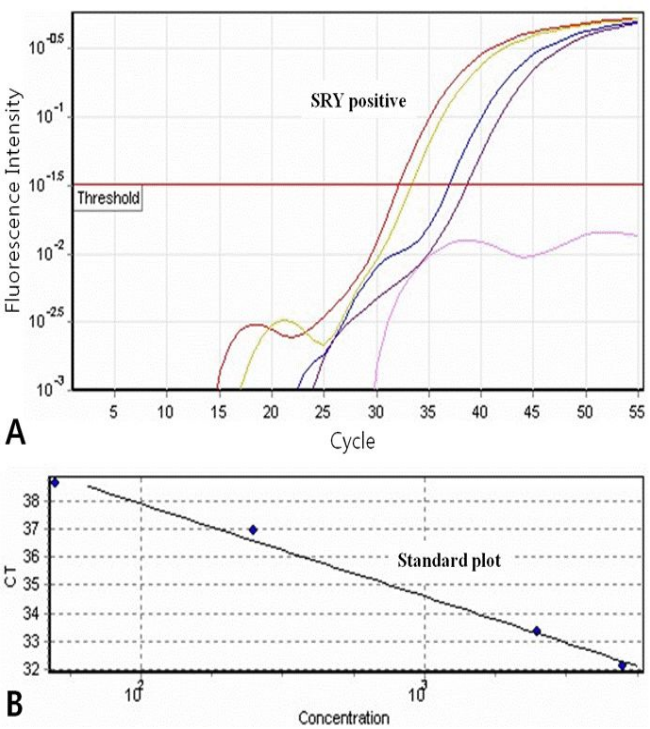

Fig. 1. Real-time quantitative PCR. (A) Amplification plots obtained using real-time quantitative PCR for the *SRY* gene. X-axis denotes the cycle number of a quantitative PCR reaction. Y-axis denotes the fluorescence intensity over the background (B) plot of the threshold cycle (Ct) against the input target quantity (common log scale). The correlation coefficient is 0.99.

Table 1. Studies Reporting the use of cffDNA in Maternal Circulation for Determination of Genetic Gender in Pregnancy[23]

Author	N	Gestation (weeks)	PCR technique	Gene	Accuracy, Males (%)	Specificity, Males (%)
Lo et al.	43	12-40	Conventional		80	100
Lo et al.	50	11-17; 37-43	Real-time	*SRY*	100	100
Zhong et al.	9	16	Nested		100	100
Honda et al.	61	10-17	Conventional	DYS14, DYZ23	87	100
Al-Yatama et al.	80	7-40	Nested	?	96	88
Costa et al.	121	8-14	Real-time	*SRY*	100	100
Sekizawa et al.	302	7-16	Real-time	DYS14	97	100
Honda et al.	81	5-10	Real-time	DYS13	100	100
Mazza et al.	18	12	Nested	*AMEL*	50	
Rijnders et al.	13	5-10	Real-time	*SRY*	100	100
Guiberts et al.	22	4-9	Real-time	*SRY*	100	100
Hromadanikova et al.	44	10-18	Real-time	*SRY*	100	100
Rijnders et al.	72	11-19	Real-time	*SRY*	97.2	100
Hyett et al.	35	7-14	Real-time	*SRY*	100	100
Boon et al.	58	11±3.3	Real-time	*SRY*	100	97.7

Abbriviations: *SRY*, sex-determining region Y gene; *AMEL*, amelogenin gene

analysis of the reaction as an amplification plot (Fig 1A). The cycle threshold (Ct) is the cycle of PCR at which the reporter dye reaches a specific level of fluorescence. During the exponential phase of the PCR, the Ct is directly proportional to the amount of target DNA present in the sample, which means that RT-PCR can also be used to quantify the amount of fetal or total (maternal and fetal) DNA present in plasma (Fig. 1B). A further advantage of RT-PCR is its sensitivity, enabling detection of very low copy numbers of DNA. This can be extremely useful in early stages of gestation. RT-PCR of the *SRY* gene was reported by numerous authors to be an accurate, fast, and reliable technique for fetal gender determination from maternal plasma samples obtained in early pregnancy (Table 1)[23]. Many groups perform replicate or triplicate analyses, and it was reported that analytical results can be interpreted with greater confidence when higher number of replicates are performed[24]. A thoroughly optimized protocol for the extraction of DNA from maternal plasma and further standardization of the RT-PCR protocol, defining the optimal number of replicates per sample, are still needed.

2) Polymorphic marker analysis in both maternal and cell-free fetal DNA

(1) Single nucleotide polymorphisms (SNPs), or point mutations

Single nucleotide polymorphisms (SNPs) or point mutationsdiffer between the maternal and paternal genomes, but may not be directly linked to a specific disease. Although there are many studies confirming that this method of detection is possible[25-27], it relies on selective enrichment of the cffDNA followed by analysis by a highly sensitive technique such as mass spectrometry, as the maternal and fetal genotypes in question only differ by a single base-pair making them challenging to distinguish. It also relies on finding SNPs that differ between the maternal and paternal genomes.

(2) Variable regions of repeated DNA (short tandem repeats or STRs)

Variable regions of repeated DNA (short tandem repeats or STRs) can be used to identify paternally inherited sequences by comparing the number of re-

peats present with the paternal and maternal sequences. Amplification of these STR sequences will therefore result in two majorproducts corresponding to the maternal alleles and one minor product corresponding to the paternally inherited fetal allele. Paternal alleles on the autosomal chromosomes can also be detected, if they are known to be absent in the maternal genome, but this requires detailed sequence knowledge of the paternal genotype of interest, as well as detection methods that can distinguish DNA sequences that might only differ by a single nucleotide. Further, as informative fetal markers will only be present at 1.5% of maternal marker, the assay sensitivity needs to be high. According to Gail et al., these markers have proved informative in around 40% of cases[28].

3) Universal fetal DNA markers (fetal epigenetic markers)

The limitations of the two approaches above have led to the search for a specific fetal marker that could be used universally in NIPD. Such candidates include loci that are subjected to differential epigenetic modification in the mother and fetus. Recent work has revealed that the promoter region of two tumor suppressor genes—*maspin*[29] and *RASSF1A*[30] are differentially methylated in the placenta relative to maternal cells, providing the first truly universal markers for fetal DNA. *Maspin* is preferentially methylated in maternal DNA and hypomethylated in fetal (placental) DNA[29]. However, the preferential detection of unmethylated fetal sequence is more tedious and less sensitive than the detection of methylated material[29]. Fortunately, in the *RASFF1A* promoter, it is the fetal DNA that is preferentially hypermethylated and maternal DNA unmethylated. Chan et al.(2006) recently reported a unique approach to this issue[30]. The methylation pattern of the *RASSF1A* promoter in the placenta and maternal blood cells allows the use of methylation-sensitive restriction enzyme digestion for specifically cutting the maternally derived background *RASFF1A* sequences while leaving the fetal derived *RASSF1A* sequence intact. Because BstU I enzyme is a methylation-sensitive restriction enzyme, the hypomethylated DNA sequence, such as the *RASSF1A* molecules derived from maternal blood cells, is expected to be digestible and not detectable after enzyme digestion. Without enzyme digestion, *RASSF1A* sequences were detectable in both the placental tissues and maternal blood cells. After enzyme digestion, only *RASSF1A* molecules from the placenta were detected. An internal control system was devised for the detection of incomplete enzyme digestion, which could potentially lead to false-positive results[30]. This internal control system consisted of a RT-PCR assay targeting the beta-actin gene promoter. Because the digestion efficiencies of unmethylated beta-actin and *RASSF1A* sequences are similar, undetectable beta-actin signal should reflect the completeness of the enzyme digestion of unmethylated *RASSF1A* sequences.

RASSF1A is a tumor suppressor gene and its inactivation could be connected with development of cancer. Inactivation is most often caused by transcriptional silencing of the gene by inappropriate promoter methylation. Hypermethylation of the *RASSF1A* promoter plays an important role in pathogenesis of hepatocellular carcinoma and the detection of hypermethylated *RASSF1A* in the serum one year after tumor resection was associated with disease recurrence[30]. The use of hypermethylated *RASSF1A* sequences as the universal fetal marker is limited only for women with no prior malignancies in medical history as the hypermethylated *RASSF1A* sequence can originate from residual cancer cells rather than from fetal part of placenta and can be fetal non-specific.

2. Clinical applications of noninvasive prenatal diagnosis

There are many discrete clinical applications of cffDNA analysis for NIPD. These applications fall into three categories of clinical antenatal care:

I. High genetic risk families where there is a family history of heritable genetic disorders

– Fetal gender determination: X-linked disorders, such as hemophilia A, Duchenne muscular dystrophy, certain endocrine disorders, such as congenital adrenal hyperplasia (CAH)

– Certain autosomal single gene disorders: paternal disease causing the allele to differ from the maternal one, e.g., achondroplasia, Huntington's disease

II. Routine antenatal screening for all pregnancies at population level: Down's syndrome and other aneuploidies (coming soon)

III. Management of pregnancies at risk for specific complications: Fetal RhD genotyping in RhD-negative women

1) Fetal gender determination

Clinical indications for fetal gender determination include 1) risk of X-linked genetic disorders, such as hemophilia A or Duchenne muscular dystrophy, 2) a family history of conditions associated with ambiguous development of the external genitalia, 3) some fetal ultrasound findings and, occasionally, discrepancy between genetic sex and the appearances of the external genitalia on fetal ultrasound. The most studies using cffDNA for gender determination use RT-PCR to detect genes on the Y chromosome of male fetuses: most detect the *SRY*.

Early identification of fetal gender is important for couples at risk for fetal X-linked diseases as identification of a male fetus indicates hemizygosity for the X chromosome and potential disease if the mother is a carrier. Male offspring of women carriers of an X-linked disorder have a 50% chance of inheriting the condition, whereas female offspring will, in most conditions, not manifest the disorder. Carrier mothers of serious X-linked conditions, such as Duchenne muscular

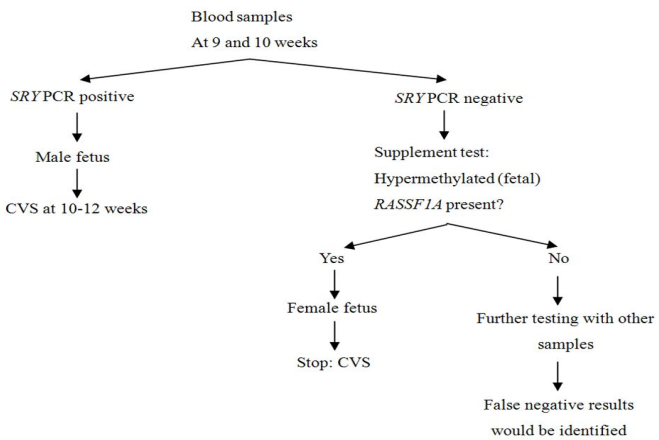

Fig. 2. New strategy protocol for carriers of X-linked recessive disease[49].

Table 2. Prenatal Diagnosis on Pregnancies at Risk of Hemophilia. Gestations Stated are at which Both Invasive Testing Gave Results[33]

Case	Gestation	US Scan	cffDNA	CVS	Outcome
1	11^{+5}	Male	Male	Declined, (?mutation)	TOP
2	12^{+0}	Male	Male	46XY, affected	TOP
3	12^{+2}	Male	Male	Declined	LB, affected male
4	12^{+3}	Male	Male	Declined	LB, affected male
5	13^{+0}	Male	Male	46XY, unaffected	LB, unaffected male
6	14^{+1}	Male	Male	Declined	LB, unaffected male
7	11^{+2}	Female	Female	Declined	LB, female
8	12^{+0}	Female*	Female	Declined	20-week scan, female
9	12^{+4}	Female	Female	Declined	20-week scan, female
10	12^{+6}	Female	Female	Declined	LB, female

Abbreviations: CVS, Chorionic villus sampling; TOP, termination of pregnancy; LB, live birth
*Represents where repeat testing was required

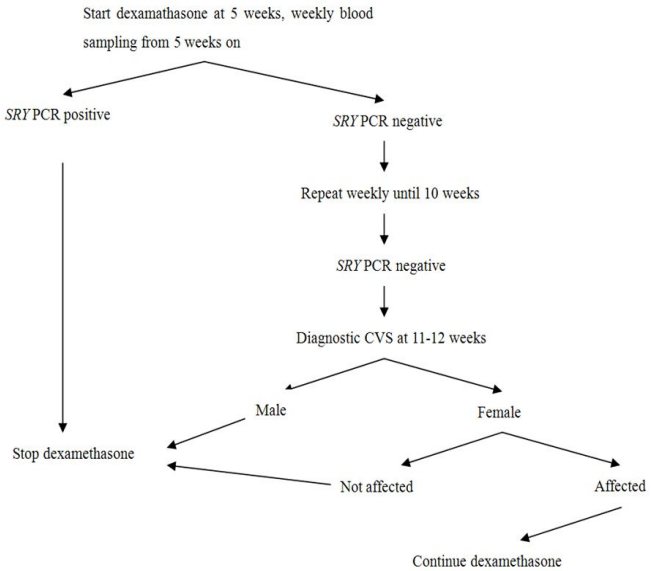

Fig. 3. Protocol for pregnancies at risk for congenital adrenal hyperplasia in the fetus[36].

dystrophy, Hunter's disease, and adrenoleukodystrophy, which confer significant morbidity or mortality, require genetic prenatal diagnosis only when the fetus is male. Diagnostic testing for many of these conditions is available by molecular testing of chorionic villi obtained following CVS, which can be performed from around 11 weeks gestation.

Early fetal gender determination using cffDNA could reduce the number of invasive diagnoses required for each specific disease by half, with the particular advantage of sparing most female fetuses from unnecessary invasive diagnostic testing[31] (Fig. 2). In a recent British national audit of fetal gender determination performed using cffDNA in women requesting prenatal genetic testing for X-linked disorders, there was a 45% reduction in invasive testing, with the most women carrying female fetuses opting not to undergo CVS[1, 32]. Noninvasive fetal gender determination by ultrasound and analysis of cffDNA in maternal plasma also provide a reliable way of avoiding invasive testing for carriers of hemophilia[33] (Table 2).

The other major use of NIPD for fetal sex determination is in the management of CAH. CAH is a group of genetic disorders resulting from abnormalities of adrenal steroid production. Most are due to defects in the *CYP21A2* gene, which encodes 21-hydroxylase, and result in overproduction of adrenal androgens. The development of male external genitalia is unaffected by the excess androgens, but varying degrees of virilization occur in affected female fetuses after exposure to high levels of androgens[34]. Virilization begins from early as 8 weeks gestation, but maternal administration of dexamethasone, which can cross the placental barrier, suppresses the hypothalamic-pituitary-adrenal (HPA) axis and can reduce or prevent virilization[35]. Despite being advised that starting dexamethasone by 6 weeks' gestation prevents virilization, many women are unwilling to take steroids, both because of the theoretical risk of side effects and because seven out of eight pregnancies that are treated will be unaffected. Women at risk of CAH requesting gender determination before

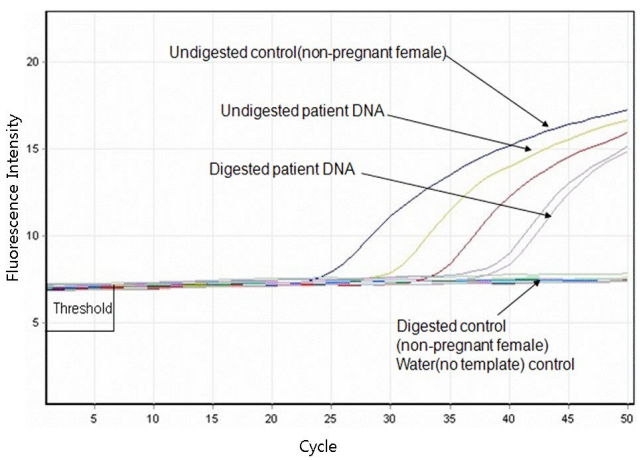

Fig. 4. Hypermethylated *RASSF1A* test system to confirm the presence of cffDNA in *SRY*-negative samples testing; multiplex RT-PCR for the *RASSF1A* promoter gene using FAM-labeled probes and using DNA both undigested and digested with BstU1[49]. The beta- actin gene was used as an internal control to show that enzyme digestion was complete. The analysis data show the signals crossing the threshold for these samples: undigested control DNA from a non-pregnant female. Note that after digestion, the signal does not cross the threshold. Undigested patient DNA - this signal represents amplification of *RASSF1A* from plasma-derived cffDNA and maternal DNA. Digested patient DNA - this signal above threshold remaining after BstU1 digestion shows the cffDNA is present, as the fetal gene is hypermethylated and enzyme-resistant; samples typing *SRY*-negative are predictive of a female fetus.

Table 3. Strategy for Noninvasive First Trimester Fetal Gender Determination using cffDNA from Maternal Plasma in Korea[49)]

Case	Gestation (wks)	*β-actin*	*RASSF1A*	*SRY* RQ Result	*SRY* CN	Fetal sex
1	5	Undetected	Detected	(−)		F
2	5	Undetected	Detected	(+)	2.44	M
3	5	Undetected	Detected	(−)		F
4	5	Undetected	Detected	(−)		M
5	6	Undetected	Detected	(+)	44.88	M
6	6	Undetected	Detected	(−)		F
7	6	Undetected	Detected	(+)	9.54	M
8	6	Undetected	Detected	(−)		F
9	7	Undetected	Detected	(+)	9.13	M
10	7	Undetected	Detected	(−)		F
11	7	Undetected	Detected	(+)	6.61	M
12	7	Undetected	Detected	(−)		F
13	7	Undetected	Detected	(+)	3.74	M
14	7	Undetected	Detected	(−)		F
15	7	Undetected	Detected	(+)	18.88	M
16	7	Undetected	Detected	(+)	0.1	M
17	8	Undetected	Detected	(+)	10.39	M
18	8	Undetected	Detected	(+)	34.54	M
19	8	Undetected	Detected	(+)	35.69	M
20	8	Undetected	Detected	(+)	25.39	M
21	8	Undetected	Detected	(−)		F
22	8	Undetected	Detected	(−)		F
23	8	Undetected	Detected	(+)	16.73	M
24	8	Undetected	Detected	(−)		F
25	8	Undetected	Detected	(+)	23.33	M
26	9	Undetected	Detected	(+)	15.87	M
27	9	Undetected	Detected	(−)		F
28	9	Undetected	Detected	(+)	25.54	M
29	9	Undetected	Detected	(+)	7.49	M
30	10	Undetected	Detected	(−)		F
31	10	Undetected	Detected	(+)	10.96	M
32	11	Undetected	Detected	(−)		F
33	11	Undetected	Detected	(−)		F
34	12	Undetected	Detected	(+)	33.22	M
35	12	Undetected	Detected	(−)		F

Abbreviations: RQ, real-time quantitative; CN, copy number

7 weeks because they wish to avoid dexamethasone treatment can be offered testing with cffDNA, but must be advised of the limitations of accuracy at early gestations, and should be offered retesting after 7 weeks[31)]. Report by Rijnders et al., proposed starting treatment and testing at a gestational age of 5 weeks and performing serial testing up to 11 weeks or until male DNA is detected[36)]. In male fetuses, dexamethasone treatment can be stopped, and invasive diagnostic tests are unnecessary. Once the diagnosis of CAH can be made, unaffected female fetuses could also be withdrawn from therapy without CVS (Fig. 3). Most patients suffering from CAH are compound heterozygotes, having a different mutation on each copy of chromosome 6[37)]. In the future, PCR analysis of fetal DNA could be used to establish the molecular diagnosis of CAH, by searching for paternal inherited mutations or deletions in the CYP locus of chromosome 6. Other indications for fetal gender determination using cffDNA include the identification of abnormal genitalia in fetal ultrasound, apparent

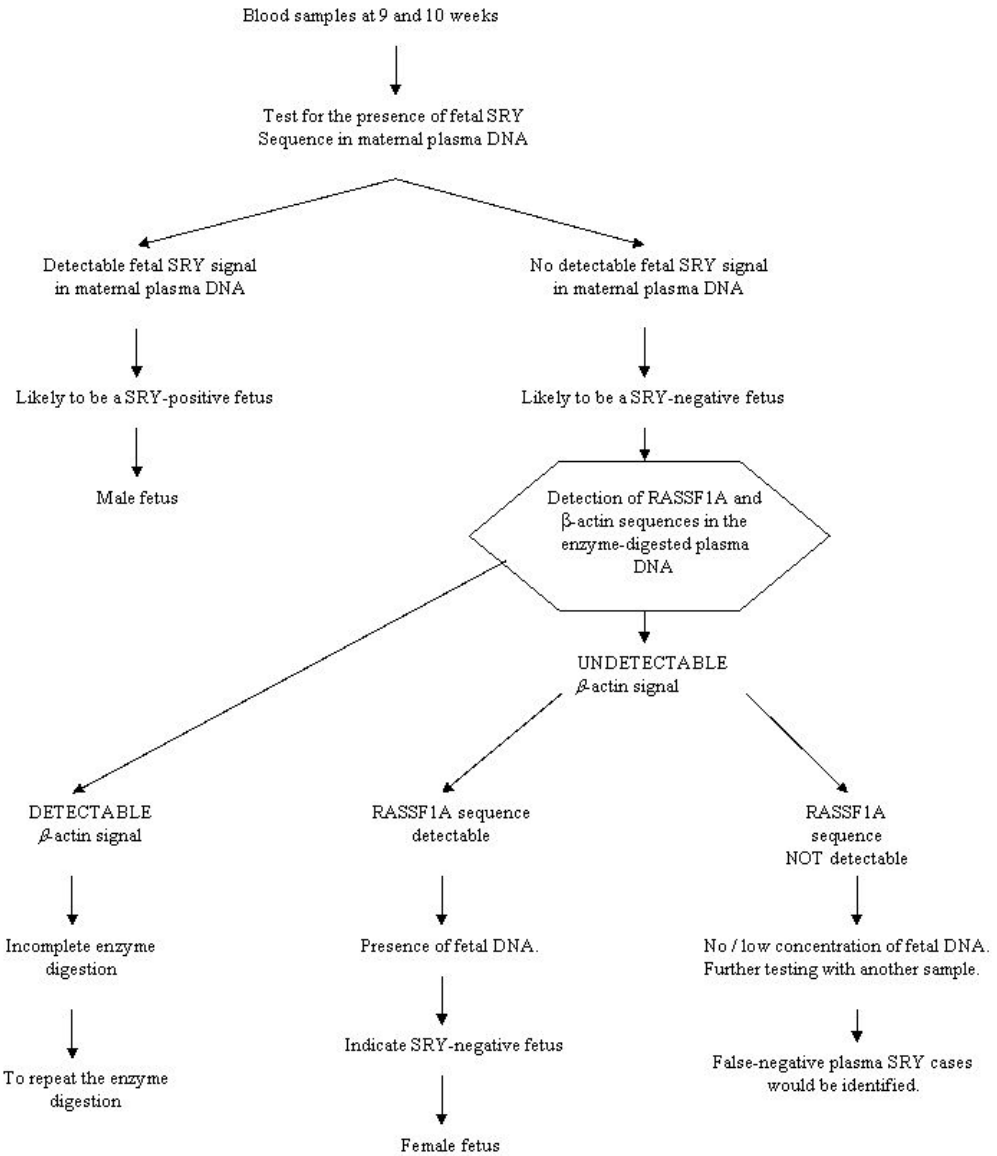

Fig. 5. Strategy for accurate noninvasive first trimester fetal gender determination using cffDNA from maternal plasma: Modified from Chan et al. 2006[49].

Table 4. Statistical Parameters at Different Gestational Ages[49]

Statistical parameters (%)	First trimester (35) M/F=20/15	Second trimester (22) M/F=9/13	Third trimester (19) M/F=10/9	Whole pregnancy (76) M/F=39/37
Sensitivity	100	100	100	100
Specificity	100	100	100	100
Positive predictive value	100	100	100	100
Negative predictive value	100	100	100	100
Accuracy	100	100	100	100

Abbreviation: M/F, number of male/female-bearing pregnancies analyzed
Sensitivity: number of male-bearing pregnancies correctly identified/total of male-bearing pregnancies tested. Specificity: number of female-bearing pregnancies correctly identified/total of female-bearing pregnancies tested. Positive predictive value: number of male-bearing pregnancies correctly identified/total of positive results at RT-PCR. Negative predictive value: number of female-bearing pregnancies correctly identified/total of negative results at RT-PCR. Accuracy: total of male-and female-bearing pregnancies correctly identified/total of pregnancies tested

discrepancies between genetic gender as determined following CVS or amniocentesis, and occasionally when ultrasound findings suggest the possibility of a genetic syndrome in which genital ambiguity or sex reversal can be a diagnostic feature.

In our study, for fetal gender determination, 76 pregnant women participated. Maternal blood samples from gestations ranging from 5 to 38 weeks were collected to determine the most appropriate week to perform the test. The medical ethical committee of the hospital approved the study, and all patients provided informed consent. The confirmation methods used were conventional prenatal genetic testing, ultrasound scan or clinical diagnosis at birth.

We have incorporated the use of a *RASSF1A* system and the existing *SRY* system as internal positive controls for fetal gender determination, which is the first successful study of this kind in Korea (Table 3, Fig. 4). Fig. 4 shows a hypermethylated *RASSF1A* test system used to confirm presence of cffDNA in *SRY*-negative sample testing.

The flow chart of the procedures and results is presented in Fig. 5. As Fig. 5 shows, the *SRY*-negative results with detectable *RASSF1A* sequence indicate a female fetus. Any *SRY*-negative results with negative detection of enzyme-digestion-resistant *RASSF1A* should be regarded as inconclusive and further testing with another plasma sample would be indicated. Our findings show that the hypermethylated *RASSF1A* sequences were detectable in all 76 plasma samples derived from 37 pregnant women carrying females. We analyzed a total 152 maternal plasma samples from 76 pregnant women at different gestational stages in a blind manner; 39 of those were carrying male fetuses, and 37 were carrying females. The results of 35 maternal plasma samples from pregnant women in the first trimester are shown in Table 3. Among them, 20 were *SRY*-positive and 15 were *SRY*-negative. The hypermethylated *RASSF1A* sequences were detectable in all 15 pregnant women with *SRY*-negative, and they confirmed the fetal gender as female.

Table 5. Quantitation of Fetal DNA in Maternal Plasma Relationship with Gestational Age[49]

	SRY concentration (copies/mL)			
	First trimester (35) M/F=20/15	Second trimester (22) M/F=9/13	Third trimester (19) M/F=10/9	Whole pregnancy (76) M/F=39/37
Range	0.1-44.9	1.1-96.1	1.4-117.0	0.1-117.0
Mean	17	28.4	40	28.5
Median	13.4	6.4	16.9	13.4

Abbreviation: M/F, number of male/female-bearing pregnancies analyzed
First trimester, 7-12 wks; Second trimester, 13-24 wks; Third trimester, 25 wks-

Table 6. Representative Selection of Recent Studies using Real-time PCR for Non-invasive Prenatal Diagnosis of Fetal RhD Genotyping[45]

Author (Date)	Country	N	Gestation (weeks)	Sensitivity (%)	Specificity (%)
Rijinders (2004)	Holland	72	11-19	100	96.6
Brojer (2005)	Poland	230	5-39	100	98.6
Gautier (2005)	France	283	8-35	100	100
Rouillac-Le Sciellour (2007)	France	300	10-34	100	97.5
Minon (2008)	Belgium	563	10-38	100	99.5
Finning (2008)	UK	1997	8-38	99.8	99.2

The *SRY* sequence was detected by RT-PCR in all pregnant women bearing male fetuses. The sensitivity of the determination of male gender from maternal plasma was 100%, the specificity was 100%, and the accuracy was 100% (Table 4).

Quantitative fetal DNA data from the maternal plasma relationship with gestational age is summarized in Table 5. These data show that the concentrations of fetal DNA in plasma are higher in late gestation than in early gestation. The mean concentration of fetal DNA in maternal plasma is 11.5 times higher in the third trimester than in the first trimester. The absolute concentrations of fetal DNA in maternal plasma were similar in individual cases. We showed that fetal DNA concentration increased as pregnancy progressed. The success of this technology by *SRY* marker with *RASSF1A* using RT-PCR has resulted in the implementation of noninvasive fetal gender determination for pregnant women at risk of X-linked disorders and CAH into routine clinical practice.

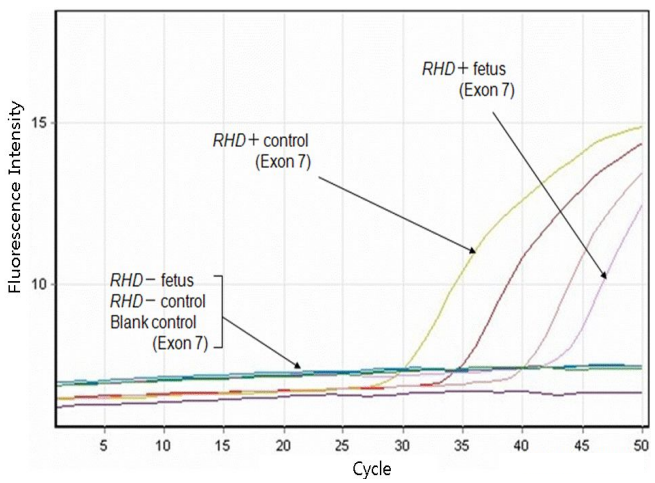

Fig. 6. Detection of fetal-derived RhD exon 7 sequences in an RhD-negative pregnant woman, by fluorescence versus cycle number and showing assay controls[49]. Representative amplification curves showing real-time monitoring of fluorescence intensity for PCR RhD exon 7 as a function of the number of cycles. RhD+ control: plasma sample from an RhD-positive woman; RhD-positive fetus: plasma sample from pregnant woman carrying an RhD-positive fetus; RhD- control: plasma sample from an RhD-negative man; RhD-negative fetus: plasma sample from a pregnant woman carrying an RhD-negative fetus.

In clinical audits in Europe, fetal gender determination using cffDNA can be performed efficiently in samples taken after 7 weeks of gestation using stringent reporting criteria. Parents should be advised of the small risk of discordant results and of the possibility that repeat testing may be required to resolve inconclusive results. Ultrasound should be used to confirm the gestation and check for twins before taking maternal blood and should also be offered to confirm the fetal gender reported using cffDNA[38].

2) Fetal RhD determination (Fetal RhD genotyping)

Hemolytic diseases of fetuses and newborns (HDFN) still contribute to perinatal morbidity and mortality, in spite of widespread immnoprophylaxis[39, 40]. Currently, the main problem in the management of RhD-negative women is how to avoid invasive investigations. Rh antigens are the main cause of HDN. Because of its strong immunogenicity, RhD is the most important antigen of the polymorphic Rh system.

The advent of NIPD using cffDNA to determine fetal D status offers significant potential for a change in the way RhD-negative women are managed in pregnancy. Since the RhD gene is usually completely absent from the genome of RhD-negative mothers, the detection of the fetal RhD gene in maternal blood implies that the fetus must be RhD-positive. When the father is heterozygous at the RhD locus, the fetus has a 50% chance of being RhD-negative and for this reason it is unaffected. In an immunized mother, when the fetus is predicted to be RhD-negative, the need for further invasive techniques is reduced. Transplacental hemorrhage with deterioration of clinical features and fetal losses related to procedures are avoided. Additional investigations and unnecessary immune globulin anti-D administration are avoided in non-immunized pregnant women. Noninvasive prenatal genetic determination of fetal RhD status in RhD-negative mothers could provide the solution to this problem and was first shown to be

feasible in 1998 by Lo et al.,[41] when it was extensively developed and widely applied. The majority of NIPD studies of Rhesus factor using cffDNA was a RT-PCR[42] to identify the presence of the RhD gene in RhD-negative mothers. Many laboratories prefer to include an amplification of exon 7, which appears to provide a higher affinity reaction than exon 10[43, 44]. In the UK, determination of fetal RhD status is now accepted for management of high-risk, sensitized pregnancies, and fetal gender determination is also becoming increasingly commonplace in specialist centers for pregnancies at risk of specific inherited disease.

There are multiple reports of high degrees of accuracy for the NIPD of the fetal D status in RhD-negative mothers (Table 6)[45], and this was introduced as a routine service by several European centers[25, 46, 47]. The benefits of testing antenatally for fetal D status by analysis of cffDNA in maternal plasma of RhD-negative mothers could be significant. It is likely to prove cost-effective, as the cost of the test is substantially less than the cost of anti-immunoglobulin. There would be no requirement to give women with RhD-negative fetuses anti-D immunoglobulinprenatally after potential sensitizing events, such as amniocentesis or other trauma. If the test proved accurate enough, there would be no need to test cord red cells serologically for RhD. Most importantly, it eliminates unnecessary treatment of pregnant women with blood products and the associated inconvenience, discomfort, and perceived risks of infection from pooled donor blood products that such injections entail[48].

In our study, we analyzed a total of 44 maternal plasma samples from 22 pregnant women with RhD-negative in serologic testing. Analysis of DNA extracted from buffy-coat samples from 22 women revealed no RhD DNA, a finding in agreement with the serologic results. The RhD RT-PCR assay can be achieved by including an amplification of exon 7. Representative RT-PCR

Table 7. Noninvasive Fetal Gender and RhD Genotyping using Plasma from RhD-negative Pregnant Women in Korea[49]

Case	*β-actin*	*RASSF1A*	*SRY* RQ Result	*SRY* CN	RhD result	RhD CN
1	Undetected	Detected	(-)		Positive	159.38
2	Undetected	Detected	(-)		Positive	2106.3
3	Undetected	Detected	(+)	0.06	Positive	2637.0
4	Undetected	Detected	(-)		Positive	1.35
5	Undetected	Detected	(+)	0.06	Negative	0
6	Undetected	Detected	(-)		Positive	511.42
7	Undetected	Detected	(-)		Positive	8.05
8	Undetected	Detected	(-)		Positive	15.52
9	Undetected	Detected	(-)		Negative	0
10	Undetected	Detected	(+)	0.82	Positive	16387.7
11	Undetected	Detected	(-)		Positive	3194.13
12	Undetected	Detected	(-)		Positive	119.7
13	Undetected	Detected	(-)	0.08	Positive	472.95
14	Undetected	Detected	(+)	0.07	Positive	136.55
15	Undetected	Detected	(-)		Positive	602.17
16	Undetected	Detected	(+)	0.23	Negative	0
17	Undetected	Detected	(-)		Negative	0
18	Undetected	Detected	(-)		Positive	245.16
19	Undetected	Detected	(-)		Positive	2.84
20	Undetected	Detected	(-)		Positive	6154.68
21	Undetected	Detected	(+)	36.9	Positive	20962.9
22	Undetected	Detected	(+)	47.34	Positive	21017.3

Abbreviations: RQ, real-time quantitative; CN, copy number

amplification plots of exon 7 are shown in Fig. 6. For each group of samples, Ct values for exon 7 assay were determined. Plasma samples from pregnant women carrying an RhD-positive fetus gave Ct values in the range of 35-40 cycles, according to the gestational age of pregnancy, whereas no Ct values were observed when the fetus was RhD-negative. The fetal RhD genotype was invalidated when Ct values were in the range of 26-30 cycles.

We have concurrently incorporated the use of the *RASSF1A* system and the existing *SRY* system as internal positive controls for fetal RhD genotyping, making this the first study of this kind in Korea (Fig. 7, 8). The fetal RhD-negative genotype is interpreted as RhD negative when a maternal plasma sample negative for RhD is shown to be positive for hypermethylated *RASSF1A*. The study showed that false-negative diagnosis was avoided in samples that were negative for both RhD and *RASFF1A* sequences. The failure to detect hypermethylated *RASSF1A* sequences signified the lack of fetal DNA in the maternal plasma sample. The fetal RhD genotyping result could not be interpreted (Fig. 7).

Fig. 8 shows the hypermethylated *RASSF1A* test system to confirm presence of cffDNA in samples testing as RhD-negative and *SRY*-negative. The detection of hypermethylated *RASSF1A* sequences shows that samples typing RhD-negative and *SRY*-negative are predictive of a female RhD-negative fetus.

Among the 22 fetuses, 18 were RhD-positive and 4 were RhD-negative on serologic analysis of cord blood or PCR testing of amniotic fluid. The results of the fetal RhD genotyping by RT-PCR assay from 22 RhD-negative pregnant women are shown in Table 7. There was complete concordance between results of the fetal RhD genotyping with the use of the RhD RT-PCR assay of maternal plasma samples and the results obtained from genotyping of amniotic fluid or serologic testing of cord blood. Among the 4 RhD-negative results, case

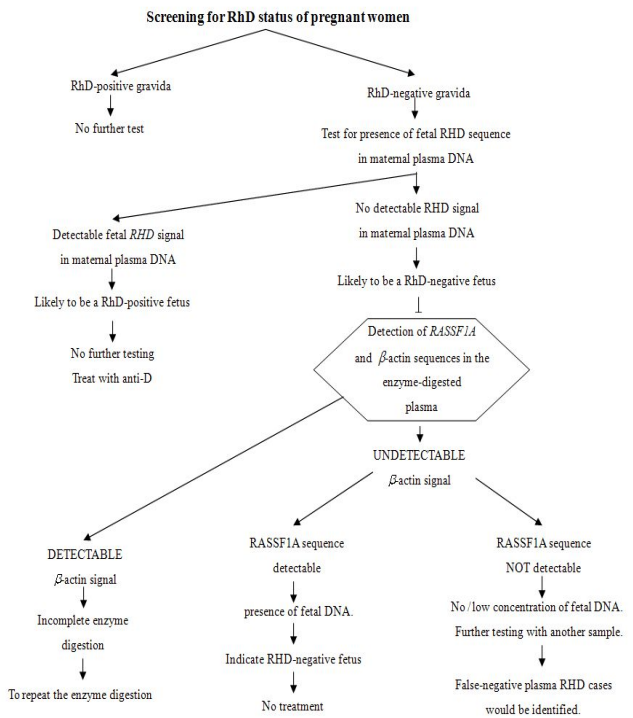

Fig. 7. The strategy for screening for fetal RhD status of RhD-negative pregnant women using cffDNA from maternal plasma: Modified from Chan et al., 2006[49].

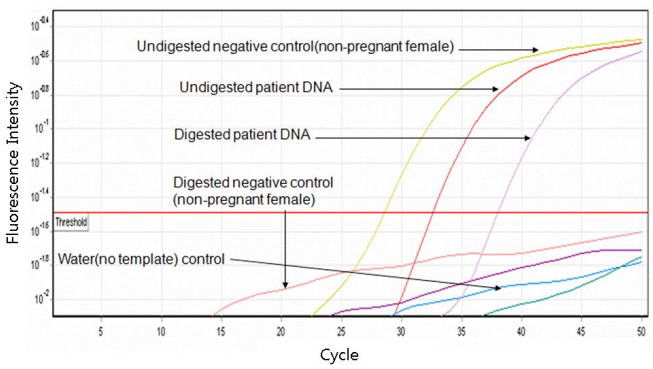

Fig. 8. Hypermethylated *RASSF1A* test system to confirm presence of cell cffDNA in samples testing as RhD- and *SRY*-negative; multiplex RT-PCR for the *RASSF1A* promoter gene using FAM-and VIC-labeled probes and using DNA both undigested and digested with BstU1[49]. The beta- actin gene was used as an internal control to show that enzyme digestion was complete. The analysis data show the signals crossing the threshold for undigested control DNA from a non-pregnant female. Note that after digestion, the signal does not cross the threshold. Undigested patient DNA - this signal represents amplification of *RASSF1A* from plasma-derived cffDNA and maternal DNA. Digested patient DNA - this signal above threshold remaining after BstU1 digestion shows the cffDNA is present, as the fetal gene is hypermethylated and enzyme-resistant; samples typing RhD- and *SRY*-negative are predictive of a female RhD-negative fetus.

9 and 17 were *SRY*-negative and *RASSF1A*-detected. The positive detection of the *RASSF1A* sequences in these cases confirmed the fetal DNA, and the RhD-negative and *SRY*-negative results could be confidently interpreted. Incorporation of fetal markers such as *RASSF1A* gene to verify the fetal DNA in RhD-negative fetuses can reduce the possibility of a false-negative result.

We confirmed the fetal DNA in all samples from pregnant women including RhD-negative female fetuses. In this study, we show that the assay is sensitive, easy, and reliable. It is the best method for assessing RhD fetal status in RhD-negative mothers and is the first step in identifying fetuses at risk for HDFN at the time of the first affected pregnancy or any subsequent ones. Noninvasive testing of fetal RhD genotyping provides a robust method of assessing of fetal RhD status and is an alternative to amniocentesis for the management of RhD-negative women and it alleviates the need for prophylactic Rhogam administration in this population of pregnant mothers.

In our study, accurate detection of fetal gender through pregnant gestational age, and fetal RhD genotyping in RhD-negative pregnant women was achieved. In this assay, we show that the assay is sensitive, easy, and reliable. These developments improve the reliability of the applications of circulating fetal DNA when used in clinical practice to manage sex-linked disorders, CAH, RhD incompatibility, and the other noninvasive pregnant diagnostic tests on the coming soon. The study was the first successful case in Korea using cffDNA in maternal plasma, which has created a new avenue for clinical applications of NIPD.

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국문초록

현재 사용되고 있는 침습적 산전진단법(양수천자, 융모막샘플링)은 1-2%의 태아 손실이 초래되어, 비침습적 산전진단법이 산전진단의 궁극적인 목표로 대두되어 왔다. 1997년 Dr. Lo에 의해서 임신부 혈장 내에 세포 유리 태아 DNA (cffDNA)의 존재가 발견된 후 비침습적 산전진단의 새로운 가능성이 열렸으며, 과거 10년간 이에 대한 연구의 많은 진전을 보여주고 있다. 최근에 cffDNA를 이용한 Hemophilia A와 듀센형 근이영양증 등 반성 유전병(X-linked disorders) 진단에 필수적인 산전 태아의 성 판정과 RhD-음성 임신부에서 태아의 RhD유전자 핵형 분석 등이 이미 외국에서 임상적으로 적용되고 있으나, 한국에서는 아직 실용화되지 않고 있다.

CffDNA의 임상 사용에는 여전히 많은 제약점이 있으며, 이는 임신부 혈장 내 cffDNA 양에 비해 많은 양의 모태 DNA가 존재하고, 종래에 사용되었던 특이적인 Y염색체 유전자(Y-specific gene)는 남아 태아 임신 시에만 적용된다는 것에 기인한다. 따라서 모든 태아에 적용할 수 있는 태아 성과 무관한 마커(sex-independent universal fetal marker as internal positive controls)가 요구되며, 이를 이용하여 정확한 태아 DNA를 검출할 수 있다.

본 연구진은 국내 처음으로 임신부 혈장 내에 cffDNA를 이용하여 *SRY* 유전자, RhD-exon 7, 태아 성과 무관한 DNA마커(universal fetal DNA marker)로써 *RASSF1A* 유전자를 실시간 중합효소연쇄반응(RT- PCR)을 사용하여 뛰어난 결과를 얻었다. 이는 한국에서 처음으로 성공적으로 시도된 것이다. 연구결과에서 산전 태아 성 판별과 산후 태아의 성이 100% 일치하였으며, 임신 주기별 *SRY* 수치는 임신이 진행할수록 증가함을 확인할 수 있었다. 따라서 이러한 방법은 혈우병 A, 듀센형 근이영양증, 선천성 부신증식증과 연골 무형성증의 진단과 치료 상담에 이용할 수 있으며 50%에서 침습적인 방법을 줄일 수가 있다. 또한, RhD-음성 임신부 대상으로 태아의 성 판정과 RhD 태아 유전자형을 분석한 결과 RhD-음성 태아를 정확히 검출함으로써 앞으로 기존 양수천자 등 침습적 검사를 대체할 수 있을 것이다. 특히 이는 치료가 필요 없는 RhD-음성 태아에서 RhD-면역글로불린의 예방적 치료를 사전에 막을 수 있어, 임신부 건강을 보호하고 의료 비용을 줄일 수 있는 큰 장점을 가진다.

한국에서 최초로 시도된 임신부 혈장 내 cffDNA를 이용한 본 연구의 성공은 비침습적 산전진단 임상 적용의 새 길을 제시하였다. 따라서 이를 각 유전질환의 산전진단에 유용하게 활용하는 것은 태아와 임신부의 건강 증진과 의료비용 절약 등 개인과 국가에 많은 기여를 할 것으로 사료된다.

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