Journal of Assisted Reproduction and Genetics (2018) 35:637–641
https://doi.org/10.1007/s10815-018-1114-8

TECHNOLOGICAL INNOVATIONS

Evaluation of extraction methods for methylated cell-free fetal DNA from maternal plasma

Ji Hyae Lim[1] · Bom Yi Lee[1] · Jin Woo Kim[1] · You Jung Han[2] · Jin Hoon Chung[2] · Min Hyoung Kim[2] · Dong Wook Kwak[2] · So Yeon Park[1] · Hee Back Choi[3] · Hyun Mee Ryu[1,2]

Received: 25 September 2017 / Accepted: 3 January 2018 / Published online: 8 February 2018

Abstract

Purpose Recently, fetal placenta-specific epigenetic regions (FSERs) have been identified for quantification of cell-free fetal DNA (cff-DNA) for non-invasive prenatal testing (NIPT). The aim of this study was to evaluate the efficiencies of a column-based kit and magnetic bead-based kit for quantification of methylated FSERs from maternal plasma.

Methods Maternal plasma was extracted from normal pregnant women within the gestational age of 10~13 weeks ($n = 24$). Total cell-free DNA (cf-DNA) was extracted using a column-based kit and magnetic bead-based kit from the plasma of the same pregnant woman, respectively. Methylated FSERs were enriched from the extracted total cf-DNA using a methyl-CpG-binding domain-based protein method. The four FSERs were simultaneously quantified by multiplex real-time polymerase chain reaction.

Results Methylated FSERs were detected in all samples extracted from both kits. However, the amplification of FSERs showed significant differences in the extraction efficiency of methylated FSERs between the two extraction methods. The Ct values of methylated FSERs extracted using the column-based kit were significantly lower than those obtained using the magnetic bead-based kit ($P < 0.001$ for all FSERs). The quantity of methylated FSERs was significantly higher for extracted DNA using the column-based kit than that extracted using the magnetic bead-based kit ($P < 0.001$ for all FSERs). Time and cost for the process of extraction were similar for the column kit and magnetic bead-based kit.

Conclusions Our findings demonstrate that the column-based kit was more effective than the magnetic bead-based kit for isolation of methylated FSERs from maternal plasma as assessed by FSER detection.

Keywords Cell-free fetal DNA · DNA extraction · Epigenetic markers · Non-invasive prenatal testing

Introduction

Since the discovery that cell-free fetal DNA (cff-DNA) is present in maternal plasma, it has been considered a fetal genetic source for the development of reliable non-invasive prenatal test (NIPT). All fetal genome exists in maternal blood in the form of cff-DNA [1]. It comprises short DNA fragments in the maternal circulation that originate from apoptotic placenta cells (trophoblasts) derived from the embryo [2, 3]. Moreover, cff-DNA can be detected from the 4 weeks of gestation and is cleared from the maternal circulation within 2 h after delivery [4, 5]. Therefore, cff-DNA in maternal plasma has been used for NIPT of fetal aneuploidy, paternally inherited genetic diseases, fetal RHD genotyping, and fetal sexing for X-linked disorders. However, the use of cff-DNA is still limited because cff-DNA comprises a minor proportion (approximately 10%) of total cell-free DNA (cf-DNA) in the plasma of pregnant women [6]. Therefore, the choice of method for cff-DNA extraction plays a key role in reliable downstream analyses for NIPT.

DNA methylation differences between fetal placenta and maternal blood have been exploited to develop new biomarkers to quantify cff-DNA [7–10]. The placenta is a major

✉ Hyun Mee Ryu
hmryu@yahoo.com

[1] Laboratory of Medical Genetics, Medical Research Institute, Cheil General Hospital and Women's Healthcare Center, Seoul, South Korea

[2] Department of Obstetrics and Gynecology, Cheil General Hospital and Women's Healthcare Center, Dankook University College of Medicine, 1-19, Mookjung-dong, Chung-gu Seoul 100-380, South Korea

[3] Genes Laboratory, Molecular Diagnostic Institute, Gyeonggi-do, South Korea

source of cff-DNA in maternal plasma, whereas maternal blood cells are the main contributor to background maternal cf-DNA [1, 11, 12]. Fetal placenta-specific DNA sequences, which are differentially methylated between fetal placenta and maternal blood cells, have been identified in recent years and investigated as fetal placenta-specific epigenetic markers (FSERs) for NIPT [13–15]. Quantification of FSERs has also been used in NIPT for fetal genetic disease, and excellent clinical performance has been reported [10, 14, 15]. In a previous study, we identified novel FSERs that were hypermethylated in fetal placenta samples compared to maternal blood samples using high-resolution microarray of human chromosome 21. The novel FSERs showed high diagnostic accuracies for use in NIPT of fetal trisomy 21 [10]. However, the diagnostic accuracy of this method can be improved by application of a more suitable extraction for isolating FSERs and the feasibility of its clinical application still needs to be explored.

To date, various DNA extraction methods have been used to isolate cff-DNA from maternal plasma. However, effective extraction methods to obtain methylated cff-DNA for appropriate detection of FSERs have not yet been identified. Therefore, in this study, we compared the ability of commonly used isolation kits (column-based kit and magnetic bead-based kit) to effectively isolate methylated cff-DNA from maternal plasma as assessed by the amplification efficiency of FSERs.

Materials and methods

Ethics approval

Appropriate ethical approval for this study was obtained from the ethics committee of the institutional review board at Cheil General Hospital, Korea (CGH-IRB-2016-5). All participants have agreed the written informed consents before collection of blood samples.

Study participants and samples

Blood samples were collected from 24 pregnant women during the first trimester. All pregnancies were singletons at or before 13 weeks of gestation. The singleton pregnancy and gestational age were confirmed by ultrasonography. The women delivered normal babies at term without maternal or fetal complications. Cheil General Hospital's electronic medical record system collected maternal, fetal, and infant clinical records. Participants did not have preexisting histories of disorders such as chronic kidney disease, liver disease, hypertension, or diabetes mellitus.

Isolation of plasma and cf-DNA

Immediately after maternal peripheral blood (10 mL) was collected from each participant, the blood samples were centrifuged at 1600 g for 10 min at 4 °C. The supernatant plasma portion was re-centrifuged at 16,000 g for 10 min and then stored at − 80 °C until examined. Cf-DNA was extracted from 2 mL maternal plasma using a column-based kit (QIAamp DSP Virus Kit, Qiagen, Hilden, Germany) and magnetic bead-based kit (NextPrep-Mag™ cfDNA Isolation Kit, Bioo Scientific, Austin, USA) according to the manufacturer's recommendations. Each cf-DNA sample was eluted in 80 μL sterile DNase-free water.

Enrichment of methylated cf-DNA

Methylated cf-DNA was isolated from cf-DNA extracted from maternal plasma using the MethylMiner™ methylated DNA enrichment kit (Invitrogen, Carlsbad, USA). The methyl-CpG-binding domain (MBD) protein of 3.5 μg was treated to 10 μL Dynabeads M-280 Streptavidin. The capture reaction of methylated cf-DNA was performed by MBD magnetic bead conjugates and 80 μL extracted cf-DNA. The methylated cf-DNA bound in MBD magnetic bead conjugates was eluted in a step-wise elution series using increasing NaCl concentrations in the elution buffer (450, 600, 1000, and 2000 mM NaCl). Finally, eluted methylated cf-DNA was concentrated in a final volume of 30 μL using a DNA concentrator (Zymo Research Corp., Irvine, USA). MBD capture reaction was validated using methylated DNA and unmethylated DNA included in the kit.

Multiplex qPCR

The amount and Ct values of methylated cff-DNA were quantified by multiplex qPCR using four FSERs. Multiplex qPCR was performed using the QuantStudio 7 Flex Real-Time PCR System (Life Technologies, Carlsbad, USA). The sequences of PCR primers, hydrolysis probes, and amplicons of the selected regions are shown in Table 1. The multiplex qPCR was performed in a volume of 20 μL containing 2X Real-Time PCR Master Mix (Genes Laboratories, Gyeonggi-do, Republic of Korea), 400 nmol/L primers, 100 nmol/L hydrolysis probe, and 10 μL of methylated cf-DNA. The amplification program consisted of an initial denaturation step of 37 °C for 10 min and then 95 °C for 5 min, followed by 50 cycles of 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 1 min. Sample reactions were performed in triplicate.

Statistical analysis

The difference in efficiency of methylated cff-DNA isolation of the column kit versus the bead kit was determined by comparison of the levels (copies/mL) and Ct values of four FSERs.

Table 1 Sequences of primers, probes, and amplicons used for multiplex qPCR

Target	Sequence information	
FSER-E	GenBank accession no. NC_000021.9	
	Forward primer	5′-ACA GCT GAA GCT GGG CCG-3′
	Reverse primer	5′-ATG GCA GAT GCC ATC AGA CG-3′
	Probe	5′-(FAM) TGT TGA GAC GAG TGA GTG CAG AGC CT (BHQ1)-3′
FSER-U1	GenBank accession no. AP001745.1	
	Forward primer	5′-ACC CCA CGT GCA CTG AGC G-3′
	Reverse primer	5′-CAC TTC TGC CCT CTG CCC G-3′
	Probe	5′-(Cy3) ACT CTG CCG GAA CTG GGG CGG GAC T (BHQ2)-3′
FSER-U2	GenBank accession no. AP001745.1	
	Forward primer	5′-GGT GCA CGC AAG GAG CTA TCG-3′
	Reverse primer	5′-TGG TGC ACA CGG CTG CTT CCG-3′
	Probe	5′-(Cy5) TGC TCC CAA CTG GCC GCT TGC TTG T (BHQ1)-3′
FSER-R	GenBank accession no. NM_007182.4	
	Forward primer	5′-GAG CCT GAG CTC ATT GAG CTG-3′
	Reverse primer	5′-ACC AGC TGC CGT GTG G-3′
	Probe	5′-(HEX) ACC CGG CTG GAG CGT GCC AAC GC (BHQ1)-3′

Accession numbers are for GenBank (http://www.ncbi.nlm.nih.gov/genbank)

FSER-E, fetal placenta-specific epigenetic DNA region located in intragenic region of the *ERG* gene; *FSMR-U1*, one of two fetal placenta-specific epigenetic DNA regions located in the promoter region of the *UMODL1* gene; *FSMR-U2*, the second of two fetal placenta-specific epigenetic DNA regions located in the promoter region of the *UMODL1* gene; *FSER-R*, fetal placenta-specific epigenetic DNA region located in the intragenic region of the *RASSF1A* gene; *qPCR*, real-time quantitative PCR

Time and cost for the process of extraction of the two kits were also compared. All data are presented as medians (interquartile ranges). The Mann-Whitney *U*-test was performed to estimate the significance of the difference in methylated cf-DNA extraction using SPSS version 12.0 (SPSS, Chicago, USA). In all tests, P value < 0.05 was considered to indicate statistical significance.

Results

The population included in the study was singleton pregnancy and ranged in maternal age from 35 to 40 years. At maternal blood sampling, gestational age was from 10 to 13 weeks. Among the 24 plasma samples collected, 10 samples corresponded to male fetuses and 14 samples corresponded to female fetuses.

For comparison purposes, cf-DNA was extracted from 24 plasma samples by both column and bead kits. No failure of MBD capture was observed. In methylated cf-DNA isolated by MBD capture, methylated cff-DNA was detected using four FSERs. Levels of the four FSERs were simultaneously measured in all samples using multiplex qPCR; amplification reactions for FSERs were also confirmed in all samples.

The isolation efficiencies of the kits for FSERs were compared based on the levels and Ct values. The levels and Ct values of FSERs are provided in Table 2. The Ct values of FSERs isolated using the column kit showed lower values than those of the bead kit ($P < 0.001$ for all FSERs). Levels of FSERs were higher when methylated cff-DNA was

Table 2 Efficiencies of kits based on FSERs detection

FSER	Magnetic bead-based kit	Column-based kit	*P* value
Ct			
FSER-E	35.5 (34.2–36.9)	33.3 (32.5–34.3)	< 0.001
FSER-U1	32.5 (32.2–35.1)	30.8 (30.4–31.6)	< 0.001
FSER-U2	34.4 (33.8–36.4)	32.1 (31.8–32.9)	< 0.001
FSER-R	35.4 (34.7–36.3)	35.4 (34.7–36.3)	< 0.001
Average	34.6 (33.7–36.0)	32.6 (32.1–33.0)	< 0.001
Quantity (copies/mL)			
FSER-E	13.3 (4.8–25.6)	58.4 (39.0–99.0)	< 0.001
FSER-U1	14.4 (5.5–17.7)	48.4 (35.5–65.9)	< 0.001
FSER-U2	8.6 (3.2–13.3)	44.3 (34.7–36.3)	< 0.001
FSER-R	9.7 (4.1–15.1)	35.1 (29.5–51.2)	< 0.001
Average	10.8 (5.4–17.9)	45.7 (35.0–63.1)	< 0.001

Data are expressed as medians (interquartile ranges). The Ct and levels of the FSMRs were compared using the Mann-Whitney *U*-test

FSER-E, fetal placenta-specific epigenetic DNA region located in intragenic region of the *ERG* gene; *FSMR-U1*, one of two fetal placenta-specific epigenetic DNA regions located in the promoter region of the *UMODL1* gene; *FSMR-U2*, the second of two fetal placenta-specific epigenetic DNA regions located in the promoter region of the *UMODL1* gene; *FSER-R*, fetal placenta-specific epigenetic DNA region located in intragenic region of the *RASSF1A* gene

isolated using the column kit than the bead kit ($P < 0.001$ for all FSERs).

Time and cost of cf-DNA isolation were also compared between the column and bead kits (Table 3). The total time required for cf-DNA isolation was similar between the column and bead kits (33 min for the column kit and 26 min for the bead kit). The cost per case based on 2 mL maternal plasma was higher using the column kit ($26.4) than the bead kit ($16.32).

Discussion

Since the discovery of cff-DNA in maternal plasma, many workers have debated the best way to use this new source of fetal DNA for NIPT of trisomy 21. The absolute concentration of cff-DNA in pregnant women carrying fetuses affected by aneuploidy has been reported to be elevated by a number of groups [16–18]. The recent development of FSERs for quantifying cff-DNA in maternal plasma has opened up the new possibility of NIPT. The development of FSERs could allow demonstration of the presence of amplifiable cff-DNA without contamination of maternal cf-DNA in a maternal plasma sample [19]. Moreover, FSERs are useful for detecting cff-DNA in maternal plasma, regardless of fetal gender [19]. However, the amount of cff-DNA in maternal plasma is extremely low in comparison to maternal background DNA. Moreover, cf-DNA and cff-DNA are present proportionally in maternal plasma [20]. Therefore, when a small amount of cf-DNA is extracted from maternal plasma, the FSER such as cff-DNA is also detected to be low. This can increase the frequency of no-call results or false negative and reduce the accuracy of subsequent experiments. Therefore, effective enrichment methods of cff-DNA in maternal plasma are needed to apply these approaches in the clinical setting.

We evaluated the isolation efficiencies of the column and bead kits for methylated cff-DNA as assessed by downstream amplification of FSERs. FSERs were detected in methylated cf-DNA extracted by the two kits in maternal plasma samples collected during the first trimester of pregnancy. Of these two kits, the column kit was effective at extracting methylated cff-DNA for FSER analysis. Despite the fact that carrier RNA used in the column kit can lead to high background readings during Qubit-based DNA quantitation, carrier RNA is recommend to extract low copy number DNA (< 10,000 GE/mL) according to the manufacturer's instructions. Therefore, column kits using carrier RNA are used routinely for the detection and quantification of low copies of viral nucleic acids in clinical samples and allow PCR detection at low target concentration as five copies per milliliter. Shaw et al. previously showed that DNA yield can be improved by the ideal ratio of carrier RNA to DNA [21]. Our results also showed that the column kit using carrier RNA is a more suitable extraction method for isolating a low fraction of methylated cff-DNA for the amplification of FSERs. Moreover, carrier RNA used in the column kit is not an issue in the multiplex qPCR-based quantification of FSERs. However, there is the potential risk of cross-contamination and reduction in overall costs and hands-on time is required. In comparison, a magnetic bead kit is cheaper and quicker to use than a column kit when isolating cf-DNA from a small volume of plasma. However, it is not appropriate for isolating low copies of methylated cff-DNA for the downstream detection of FSERs.

In conclusion, we evaluated the suitability of using a column kit and magnetic bead-based kit to extract methylated cff-DNA for the downstream amplification of FSERs in maternal plasma samples collected during the first trimester of pregnancy. The column kit performed better than the magnetic bead-based kit. This approach is robust enough to obtain methylated FSERs from maternal plasma samples collected during the first trimester of pregnancy. However, the procedure requires further modification to reduce costs and simplify its introduction into the daily routine of clinical diagnostic laboratories. Therefore, a larger-scale study is needed to evaluate the diagnostic accuracy of FSERs using established methods.

Table 3 Total processing times and costs of kits

	Magnetic bead-based kit	Column-based kit
Total process time: min	26	33
Cost: $/case (based on 2 mL plasma)	16.32	26.4

Acknowledgments We thank our staff who took the time and effort to participate in this study: Da Eun Lee, Ju Yeon Park, Do Jin Kim, Shin Young Kim, Hyun Jin Kim, Yeon Woo Lee, Ah Rum Oh, Shin Young Lee, So Min Seo, Hwa Jin Choi, and Kyoung Mee Han.

Funding information This research was supported by a grant (HI16C0628) from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea.

Compliance with ethical standards Appropriate ethical approval for this study was obtained from the ethics committee of the institutional review board at Cheil General Hospital, Korea (CGH-IRB-2016-5). All participants have agreed the written informed consents before collection of blood samples.

Conflict of interest The authors declare that they have no competing interests.

References

1. Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N, et al. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. Prenat Diagn. 2007;27:415–8.

2. Lun FM, Chiu RW, Chan KC, Leung TY, Lau TK, Lo YM. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem. 2008;54:1664–72.
3. Lo YM, Chan KC, Sun H, Chen EZ, Jiang P, Lun FM, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Sci Transl Med. 2010;2:61ra91.
4. Illanes S, Denbow M, Kailasam C, Finning K, Soothill PW. Early detection of cell-free fetal DNA in maternal plasma. Early Hum Dev. 2007;83:563–6.
5. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet. 1999;64:218–24.
6. Norton ME, Brar H, Weiss J, Karimi A, Laurent LC, Caughey AB, et al. Non-Invasive Chromosomal Evaluation (NICE) Study: results of a multicenter prospective cohort study for detection of fetal trisomy 21 and trisomy 18. Am J Obstet Gynecol. 2012;207:137.e1-8.
7. Old RW, Crea F, Puszyk W, Hultén MA. Candidate epigenetic biomarkers for non-invasive prenatal diagnosis of Down syndrome. Reprod BioMed Online. 2007;15:227–35.
8. Papageorgiou EA, Fiegler H, Rakyan V, Beck S, Hulten M, Lamnissou K, et al. Sites of differential DNA methylation between placenta and peripheral blood: molecular markers for noninvasive prenatal diagnosis of aneuploidies. Am J Pathol. 2009;174:1609–18.
9. Chu T, Burke B, Bunce K, Surti U, Allen Hogge W, Peters DG. A microarray-based approach for the identification of epigenetic biomarkers for the noninvasive diagnosis of fetal disease. Prenat Diagn. 2009;29:1020–30.
10. Lee DE, Lim JH, Kim MH, Park SY, Ryu HM. Novel epigenetic markers on chromosome 21 for noninvasive prenatal testing of fetal trisomy 21. J Mol Diagn. 2016;18:378–87.
11. Lui YY, Chik KW, Chiu RW, Ho CY, Lam CW, Lo YM. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex mismatched bone marrow transplantation. Clin Chem. 2002;48:421–7.
12. Lim JH, Park SY, Ryu HM. Non-invasive prenatal diagnosis of fetal trisomy 21 using cell-free fetal DNA in maternal blood. Obstet Gynecol Sci. 2013;56:58–66.
13. Tong YK, Jin S, Chiu RW, Ding C, Chan KC, Leung TY, et al. Noninvasive prenatal detection of trisomy 21 by an epigenetic-genetic chromosome-dosage approach. Clin Chem. 2010;56:90–8.
14. Papageorgiou EA, Karagrigoriou A, Tsaliki E, Velissariou V, Carter NP, Patsalis PC. Fetal-specific DNA methylation ratio permits noninvasive prenatal diagnosis of trisomy 21. Nat Med. 2011;17:510–3.
15. Lim JH, Kim SY, Park SY, Lee SY, Kim MJ, Han YJ, et al. Non-invasive epigenetic detection of fetal trisomy 21 in first trimester maternal plasma. PLoS One. 2011;6:e27709.
16. Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM, et al. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. Clin Chem. 1999;45:1747–51.
17. Zhong XY, Bürk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. Prenat Diagn. 2000;20:795–8.
18. Farina A, LeShane ES, Lambert-Messerlian GM, Canick JA, Lee T, Neveux LM, et al. Evaluation of cell-free fetal DNA as a second-trimester maternal serum marker of Down syndrome pregnancy. Clin Chem. 2003;49:239–42.
19. Lo YM. Fetal DNA in maternal plasma: progress through epigenetics. Ann N Y Acad Sci. 2006;1075:74–80.
20. Lim JH, Park SY, Kim SY, Kim DJ, Choi JE, et al. Effective detection of fetal sex using circulating fetal DNA in first-trimester maternal plasma. FASEB J. 2012;26:250–8.
21. Shaw KJ, Thain L, Docker PT, Dyer CE, Greenman J, Greenway GM, et al. The use of carrier RNA to enhance DNA extraction from microfluidic-based silica monoliths. Anal Chim Acta. 2009;652:231–3.