의학석사 학위논문

Non-invasive prenatal testing by using next-generation sequencing of cell-free DNA from maternal plasma in multifetal pregnancies

다 태아 임신에서 모체혈장 내 유리 DNA의 차세대 염기배열 분석방법을 이용한 비 침습적 산전검사

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이 논문을 의학석사 학위논문으로 제출함

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Non-invasive prenatal testing by using next-generation sequencing of cell-free DNA from maternal plasma in multifetal pregnancies

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A thesis submitted to the Department of medicine in partial fulfillment of the requirements for the Degree of Master of Science in medicine (Obstetrics and Gynecology) at Seoul National University College of Medicine

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Abstract

Non-invasive prenatal testing by using next-generation sequencing of cell-free DNA from maternal plasma in multifetal pregnancies

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Objective

The objective of this study is to evaluate the performance of non-invasive prenatal testing (NIPT) for trisomies 21, 18 and 13 by using next-generation sequencing of cell-free DNA from maternal plasma in multifetal pregnancies.

Method

A double-blind prospective study was performed from 2014 November to 2015 June. Among a cohort of 211 high-risk pregnancies, 23 multifetal

pregnant women who were expected to undergo invasive procedures for fetal karyotype diagnosis, were recruited. At same time, invasive procedures and maternal plasma DNA sequencing were performed to detect trisomies 21 ,18 and 13. For each target chromosome 21, 18, 13 z-scores of 3 or higher were classified with trisomy high risk and z-scores of 2 over less than 3 were classified with trisomy potential high risk. The fetal karyotype of invasive test was used as gold standard to confirm the detection rate, false negative and false positive of sequencing-based non-invasive prenatal test.

Results

There were 3 monochorionic diamniotic (MCDA) twins, 19 dichorionic diamniotic (DCDA) twins and 1 trichorionic triamniotic (TCTA) triplets. There were two discordant twins of trisomy 21 confirmed by karyotyping. Plasma DNA sequencing correctly identified two cases of trisomy 21. The maternal plasma DNA sequencing for fetal trisomy 21 showed 100% accurate detection respectively.

Conclusion

Although study size is limited, this study showed that NIPT could be applicable to multifetal pregnancies with accurate detection and further supported that sequencing-based NIPT of trisomy 21 in multifetal pregnancies could be achieved with a high accurate efficiency, which could effectively avoid invasive prenatal diagnosis procedures.

Key word: Non-invasive prenatal testing, Cell-free DNA,
Multifetal pregnancies

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Introduction

In 1997, Lo et al.[1] first reported that plasma from pregnant women contained fetal cell-free DNA (cfDNA), although this is thought to be placental in origin, resulting from apoptotic trophoblasts. Fetal cfDNA can be detected as early as 4 weeks' gestation[2] and exceeds 4% of all the cfDNA in nearly all women from 10 weeks onward. The typical size of the cfDNA fragments is approximately 150 basepairs[3,4] and, importantly, the entire fetal genome is represented. It has been shown that the half-life of fetal cfDNA is very short[5]; fetal fragments are no longer detectable very soon after birth[6,7]. There is therefore no serious concern that a prenatal cfDNA test could be confounded by a prior pregnancy in multigravid women.

Non-invasive prenatal testing (NIPT) using fetal cfDNA with these characteristics has become more and more developed. Currently, the use of NIPT for detecting fetal aneuploidy has been achieved by using next-generation sequencing of cfDNA in the maternal circulation. Analysis of cfDNA in maternal blood for NIPT has been shown to be highly accurate in the detection of common fetal trisomies. About 98 to 99 percent of Down syndrome pregnancies are identified prenatally with a false positive rate as low as 0.1%[8]. Recently, NIPT by using next-generation sequencing of cfDNA in the maternal circulation has been used clinically as a screening test for fetal

aneuploidy in singleton pregnancy.[9]

However, the performance of NIPT in detecting fetal trisomy including Down syndrome in multifetal pregnancies is still unclear because of the lack of clinical validation data. Due to an increase in the use of assisted reproductive technologies (ART) over the last several decades, multiple pregnancies have become more commom. Conventional screening methods for fetal Down syndrome such a second trimester biochemistry or first trimester combined ultrasound and biochemistry in multifetal pregnancies have a false-positive rate (FPR) that is two to three times higher than that in single pregnancies[10]. Also, multifetal pregnancies are prone to complications and bear an elevated risk for procedure-related losses after amniocentesis and, chorionic villus sampling (CVS)[11]. Therefore, NIPT can offer a significant improvement for women with multifetal pregnancy.

Each fetus in multifetal pregnancies behaves similar to that in a singleton pregnancy as far as cell-free DNA in maternal plasma is concerned, and therefore prenatal diagnosis by using next-generation sequencing of cfDNA in the maternal circulation is very likely to be applicable to twin pregnancies[12].

In this study, among a cohort of 211 high-risk pregnancies, 23 women with multifetal pregnancy who were expected to undergo invasive procedures for fetal karyotype diagnosis, were recruited. This study evaluated the performance of NIPT for detecting trisomies 21, 18 and 13 by using

next-generation sequencing of cell-free DNA from maternal plasma in multifetal pregnancies.

Methods

Study Subjects

A double-blind prospective study was performed from November 2014 to June 2015. During this period, 211 pregnant women, who were expected to undergo invasive procedures (amniocentesis, CVS or cordocentesis) for fetal karyotype diagnosis, were collected at the Miraewaheemang OB&GYN clinic. Among a cohort of 211 high-risk pregnancies, 23 womne with multifetal pregnancy were recruited and evaluated.

All women underwent conventional prenatal aneuploidy screening at accredited clinical laboratories. First-trimester screening included the measurement of serum pregnancy-associated plasma protein A (PAPP-A), total or free beta subunit of human chorionic gonadotropin (hCG), and nuchal translucency. Second-trimester screening comprised measurement maternal serum alpha-fetoprotein (AFP), hCG, unconjugated estriol and inhibin A.

The indications for invasive tests included abnormal maternal serum screening result, abnormal sonographic signs, or maternal anxiety.

Pregnant women planning to undergo invasive diagnostic testing for fetal karyotyping were asked to provide blood samples for NIPT before undergoing

the invasive test. All women who agreed to provide written informed consent and who participated in the study had multifetal pregnancies with a gestational age of at least 11 weeks and more. The institutional review board at the Miraewaheemang OB&GYN clinic approved this study.

Cell-free DNA (cfDNA) Preparation and Sequencing

About 10 mL of peripheral blood was collected from each participant in a BCT™ tube (Streck, Omaha, NE, USA). The blood sample was centrifuged at 1,200 × g for 15 min at 4°C. The plasma portion of blood was transferred to microcentrifuge tubes and centrifuged again at 16,000 × g for 10 min at 4°C. Then cfDNA was extracted from 1 mL of plasma using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Netherlands). The end-repair of the plasma cfDNA was carried out using T4 DNA polymerase, Klenow DNA polymerase, and T4 polymerase kinase. DNA libraries for the Ion Proton sequencing systems were constructed according to the protocol provided by the manufacturer (Life Technologies, SD, USA). Proton PI Chip Kit version 2.0 was used to yield an average 0.3× sequencing coverage depth per nucleotide

Data Analysis

Raw reads with different lengths derived from the Ion Torrent Suite software were trimmed from the 3' end by the sequencing quality value of >15 and filtered by read length (<50bp) and GC contents (35%-45%). Duplicate reads were identified using Picard (http://picard.sourceforge.net/) with the default parameters and an additional removal was performed with the in-house Python program. The sequence fragments from each sample were mapped to the unmasked human reference genome sequence (hg19). We evaluated several mapping software programs and acquired the results by using the Burrows-Wheeler Aligner (BWA)[13] mapping analysis algorithm. Each chromosome was divided into segments with a bin size of 300kb to calculate the z-score for detecting fetal trisomy 21. For all samples, we calculated the z-score for each chromosome to analyze fetal aneuploidy with mapped reads, as well as average mapped reads and standard deviation (SD). For example, the z-score of case 1 for chr21 was calculated as follows: $z\text{-score}_{chr21\_case1}$ = (mapped reads of $chr21_{case1}$ − mean mapped reads of $chr21_{euploid\ group}$) / (SD for mean mapped reads of $chr21_{euploid\ group}$). Sequence data has been deposited in the NIH short read archive (SRA) with the following BioProject accession number: SRP044689.

Karyotyping analysis

Invasive sampling for each case was performed after the peripheral blood was drawn. The results of karyotyping were used as the gold standard to evaluate sequencing-based NIPT.

Results

Among a cohort of 211 high-risk pregnancies, 23 women with multifetal pregnancy were assessed in this study. Table I shows the clinical characteristics of these 23 women with multifetal pregnancy. The maternal age ranged from 29 to 42 years with a median of 34 years, and 52% (12/23) of women were aged 35 years or older. The median gestational age at the time of blood sampling for NIPT was 13.5 weeks, and 74% (17/23) of women were in the 1st trimester period.

There were 3 monochorionic diamniotic (MCDA) twins (13%), 19 dichorionic diamniotic (DCDA) twins (83%), and one trichorionic triamniotic (TCTA) triplets (4%). There was only one case with natural pregnancy, and 22 cases with use of Assisted assisted reproductive technologies (ART). Twenty-two cases underwent amniocentesis for diagnostic karyotype test and only one case underwent CVS.

Figure 1 shows the chromosome 21, 18, and 13 results of z-scores for these 23 multifetal pregnancies. Table II shows all clinical data as well as the NIPT

z-score for these 23 cases. The z-scores of chromosome 21 were elevated in two twin pregnancies affected by Down syndrome (Table II). Z-score of case 1 affected by Down syndrome was more than over 3, however, Z-score of case 2 affected by Down syndrome was more than 2 but less than 3. The result of NIPT in both cases was high risk for Down syndrome.

All NIPT reports were available within 14 days after sampling. The result was negative for Cases 3-23, and it was positive for Case 1 and case 2 (Table II), which were confirmed to be cases of discordant fetal trisomy 21 by karyotyping.

High risk cases of trisomy 21 high risk on the cases of conventional prenatal aneuploidy screening test included 11 multifetal pregnancies. Only case 2 was confirmed as having trisomy 21. Another case of trisomy 21, Case 1, had low risk of trisomy 21 on the conventional prenatal aneuploidy screening test. In this study, sensitivity and specificity of the conventional prenatal screening test for trisomy 21 were 50% and 47%, respectively; however, the sample size was limited (Table III). As shown above, NIPT had higher sensitivity, specificity (p-value=0.004), positive predictive value (PPV), and negative predictive value (NPV) than the conventional prenatal aneuploidy screening test (Table III).

In this study, the detection rate of trisomy 21 was 100% and the false positive rate was 0%. The z-scores of chromosomes 18 and 13 were less than 3 in all enrolled women with multifetal pregnancies including twins pregnancies

affected by Down syndrome.

Discussion

Prenatal screening for aneuploidy has changed significantly over the last 30 years, from being age-based to maternal serum and ultrasound based techniques. Even now, prenatal screening of fetal aneuploidy is still changing and it needs to be developed further. Conventional fetal aneuploidy screening tests in multifetal pregnancies especially those based on maternal serum markers, perform worse than in singleton pregnancies with a lower detection rate and a higher false positive rate[12,14].

Invasive test in multifetal pregnancies is also more difficult than in singleton pregnancies because of the higher incidence of procedure-related loss, sampling errors, and more complicated techniques[12].

Therefore, we need a better method for detecting fetal aneuploidy, especially in multifetal pregnancies.

NIPT for detecting fetal aneuploidy by using next-generation sequencing of cfDNA in the maternal circulation has been proven to be highly accurate and useful in singleton pregnancy. Although validation data is lacking, NIPT application in twin pregnancies could be implemented theoretically. It has been shown that the sensitivity of NIPT by using next-generation sequencing of cfDNA in detecting fetal aneuploidy from maternal plasma is limited only

by counting statistics which in turn is determined by the sequencing depth and fetal DNA concentration[15].

In monochorionic (MC) twin pregnancies, almost always the two fetuses are concordant in karyotyping and therefore the MC twin could be considered as one singleton pregnancy. Since MC twin pregnancies are associated with higher cell free fetal DNA concentration in maternal plasma[16,17], the use of NIPT in detecting fetal Down syndrome in MC twins are most likely to be as useful as in singleton pregnancies.

In dichorionic (DC) twin pregnancies affected by Down syndrome, usually only one of the fetuses, that is discordant fetal aneuploidy, is affected. In our study, two case of discordant fetal trisomy 21 were detected by NIPT and were confirmed by the invasive test.

Using Y-chromosomal associated gene as a marker for fetal DNA, it has been shown that the cell-free fetal DNA concentration in maternal plasma is the same between singleton pregnancies and twin pregnancies with mixed-fetal sex[16,18], suggesting that each of the fetuses in a twin pregnancy is producing a similar amount of cell-free DNA as in singleton pregnancies[12]. Therefore, if one of the fetuses is affected, the situation actually is similar to an affected singleton pregnancy, and therefore the efficacy of next-generation sequencing of cfDNA in the maternal circulation for Down screening in DC twin is likely to be similar to that in singleton pregnancies[12]. However, direct evidence is limited.

The strengths of this study were as follows: double-blind prospective study and blood sampling time for NIPT. For 74 percent of women in the groups, the gestational ages at blood sampling for NIPT was from the first trimester (11−14 completed weeks). However, there was a limitation in sample size.

We correctly classified the 21 normal pregnancies and the two cases with discordant fetal Down syndrome. Although the sample size was small, the results suggested that prenatal screening for fetal Down syndrome by using next-generation sequencing in cfDNA of maternal plasma is possible. However, the NIPT approach will only enable the detection of "an affected" fetus in a twin pregnancy, but cannot identify exactly which of the two fetuses is the affected one.

Nonetheless, the high specificity enables the correct detection of the far majority of pregnancies with normal fetuses, and thus avoiding the need for invasive tests. Due to limited number of studies, it is still unclear whether NIPT by using maternal plasma DNA can be offered as a routine clinical service to women with twin pregnancies. However, all available evidence suggested that such an approach is likely to be as effective as in singleton pregnancies. Therefore, this test may be offered to selected women with twin pregnancies, especially to those who have been screened as high risk by the conventional test[19], after extensive counseling by experts who have significant experience in this specialized area.

Conclusion

Although the sample size was limited, this study further confirmed that sequencing-based NIPT for detecting trisomy 21 in multifetal pregnancies could achieve a high accuracy, which could effectively avoid invasive prenatal diagnostic procedures

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Table I Characteristics of the 23participants

Maternal age	
Median (year)	34
Range (year)	29-42
Advanced maternal age (≥35year) (n, %)	12(52%)
25–29year (n, %)	1(4%)
30–34year (n, %)	10(44%)
35–39year (n, %)	10(44%)
40-44year (n, %)	2(8%)
Gestational age	
Median (week)	13.5
Range (week)	11-21
1st Trimester (n, %)	17(74%)
2nd trimester (n, %)	6(26%)
Chorionicity (n, %)	
MCDA	3(13%)
DCDA	19((83%)
TCTA	1(4%)
Type of pregnancy (n, %)	
Assisted pregnancy	22 (96%)
Natural pregnancy	1(4%)

MCDA Monochorionic diamniotic twins,

DCDA Dichorionic diamniotic twins

TCTA Trichorionic triamniotic triplets

Table II Clinical details of 23 multifetal pregnancies

Cases	Age	GA (wks)	Chorionicity	Type of pregnancy	Karyotypes Fetus 1 Fetus2 Fetus3	Invasive test	NIPT z-score			Conventional Test	
							Trisomy 21	Trisomy 18	Trisomy 13	Seq #1	Seq #2
Case 1	31	17+2	DCDA	IVF-ET	47,XY,+21 46,XY	Amnio	4.38	0.16	-0.54	Low risk	not done
Case 2	35	21+0	DCDA	IUI	47,XX,+21 46,XY	Amnio	2.27	-0.4	-1.31	not done	Down High risk
Case 3	33	12+0	DCDA	IUI	46,XX 46,XY	Amnio	-0.99	-0.92	-0.99	Low risk	Down High risk
Case 4	36	16+5	DCDA	IUI	46,XX 46,XY	Amnio	-0.349	-1.278	-1.789	not done	not done
Case 5	35	12+3	DCDA	IVF-ET	46,XX 46,XX	Amnio	1.17	0.02	0.4	Low risk	not done
Case 6	35	12+2	DCDA	IVF-ET	46,XY 46,XX	Amnio	1.25	-0.55	-0.28	Low risk	not done
Case 7	31	11+3	DCDA	IVF-ET	46,XX 46,XX	Amnio	-0.01	-0.56	-0.78	Down High risk	not done
Case 8	36	17+5	DCDA	IVF-ET	46,XY 46,XY	Amnio	-0.27	1.45	1.62	not done	not done

Cases	Age	GA (wks)	Chorionicity	Type of pregnancy	Kayrotypes Fetus 1 Fetus2 Fetus3)	Invasive test	NIPT z-score			Conventional Test	
							Trisomy 21	Trisomy 18	Trisomy 13	Seq #1	Seq #2
Case10	30	11+5	DCDA	IUI	46,XY,t(1;10) 46,XY,inv(9)	Amnio	-0.92	-0.28	-0.87	Down High risk	not done
Case11	32	12+1	DCDA	IVF-ET	46,XX 46,XX	Amnio	-1.75	-0.12	-1.07	not done	Down High risk
Case12	32	12+3	DCDA	IVF-ET	46,XY 46,XY	Amnio	-0.47	0.29	-0.33	Low risk	Down High risk
Case13	31	12+2	DCDA	IVF-ET	46,XY 46,XY	Amnio	-1.91	-0.61	-1.15	Down High risk	not done
Case14	42	11+5	DCDA	natural	46,XX 46,XX	Amnio	0.28	-1.81	-1.49	Down High risk	not done
Case15	31	11+2	DCDA	IVF-ET	46,XX 46,XY	Amnio	-1.39	-1.83	-1.72	Low risk	not done
Case16	39	17+1	DCDA	IVF-ET	46,XY,inv(9)(p12q13) 46,XX	Amnio	-0.5	-3.482	-3.554	not done	not done
Case17	33	11+6	TCTA	IVF-ET	46,XX 46,XX 46,XY	Amnio	-0.73	-0.49	-0.82	not done	not done

Cases	Age	GA (wks)	Chorionicity	Type of pregnancy	Karyotypes Fetus 1 Fetus 2 Fetus 3	Invasive test	NIPT z-score			Conventional Test	
							Trisomy 21	Trisomy 18	Trisomy 13	Seq #1	Seq #2
Case19	37	11+3	DCDA	IVF-ET	46,XX 46,XY	Amnio	-0.2	-0.37	-0.91	Low risk	not done
Case20	36	17+0	DCDA	IVF-ET	46,XX 46,XY	Amnio	-0.73	-1.24	-1.52	Low risk	not done
Case21	38	13+1	MCDA	IVF-ET	46,XY	CVS	-1.36	-0.24	-1.21	Down High risk	not done
Case22	38	11+0	MCDA	IVF-ET	46,XX	Amnio	-0.15	-1.63	-1.45	Low risk	low risk
Case23	29	12+4	MCDA	IVF-ET	46,XX	Amnio	-1.64	-0.9	-1.66	Low risk	Down High risk

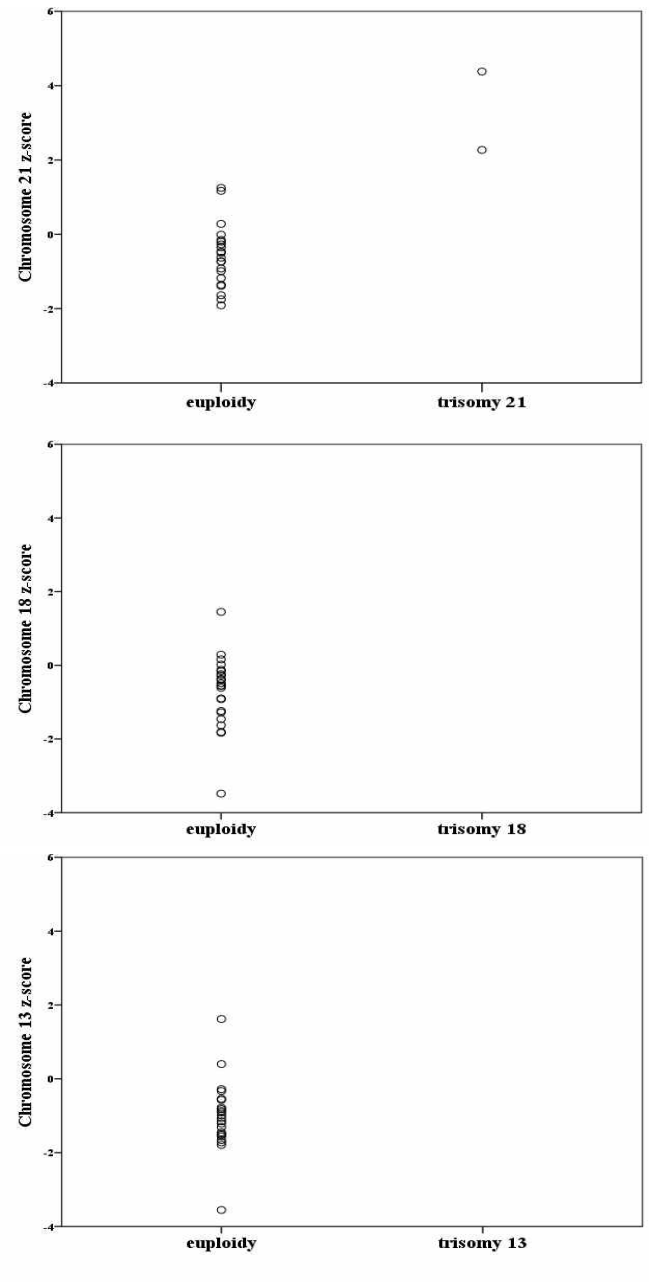
GA ;Gestational age, wks; weeks, seq#1; sequential #1, seq#2; sequential #2, DCDA; dichorionic diamniotic twins, TCTA; trichorionic triamnionic triplets, MCDA; monochorionic diamnionic twins, IVF-ET; in vitro fertilization- embryo transfer, IUI; intra uterine insemination, amnio; amniocentesis, CVS; chorionic villus sampling

Table III Comparison Conventional test between NIPT

		Conventional test For trisomy 21	NIPT for trisomy 21
Sensitivity	case/n	1/2	2/2
	95% CI[1]	50 (0.09,0.91)	100 (0.34,1)
	p-value	-	-
Specificity	case/n	8/17	21/21
	95% CI[1]	47.06 (0.26,0.69)	100 (0.85,1)
	p-value[2]	-	0.004
PPV	case/n	1/10	2/2
	95% CI[1]	10 (0.02,0.4)	100 (0.34,1)
	p-value	-	-
NPV	case/n	8/9	21/21
	95% CI[1]	88.89 (0.57,0.98)	100 (0.85,1)
	p-value[3]	-	-

[1]95% C.I. with Wilson score method
[2]Exact McNemar test

Figure I Chromosome 21, 18, 13 z-score of the 23participants



국문초록

목적: 다 태아 임신에서 모체 혈장 내 DNA의 차세대 염기서열 분석법을 이용하여 다운 증후군, 에드워드 증후군, 파타우 증후군의 비침습적 산전 검사의 유용성을 평가하고자 하였다.

방법: 이중 맹검 전향적 연구로 2014년 11월부터 2015년 6월까지 시행되었다. 211명의 고위험산모들 중 염색체 확진 침습적 검사를 계획하고 있는 23명의 다태 임신 산모를 모집되었다. 염색체 확진 침습적 검사 시행 동시에 비 침습적 검사도 시행되었다. 염색체 21번 18번 13번의 염기서열분석법을 통해 z-score를 산출하여 3보다 클 때 위험군 2에서 3미만인 경우 잠재적 위험군으로 분류하여 보고 되었다. 태아 염색체 염기서열 분석방법을 이용한 비침습적 산전 검사의 염색체 이상 발견률 위양성율, 위음성율의 확인하는데 태아 염색체 확진검사가 이용되었다.

결과: 23명의 다태아 임산모 중 3명의 단일융모막 쌍태아, 19명의 두융모막성 쌍태아, 1명의 삼융모막성 삼태아가 포함되었다. 염색체 확진검사에서 2명의 다운증후군 태아가 발견되었다. 쌍태아 중 한명만 다운증후군이고 다른 한명은 정상인 쌍태아를 임신한 두명의 산모의 비 침습적 산전검사 상 염색체 21번의 z-core가 높게 나와 고위험군으로 보고 되었고, 정확하게 다운증후군을 100%예측하였다.

결론: 연구의 모집된 다태임신 산모의 수의 한계점이 있지만, 다태임신에서도 단태임신에서 와 같이 차세대 염기서열 분석법을 이용한 비침습적 산전 검사의 적용가능성 및 유용성을 확인하였다. 이는 향후 다태임신에서의 불필요한 침습적 검사를 피할 수 있는 높은 정확도 유용성을 가진 비침습적 산전 검사의 대규모 연구에 뒷받침 될 수 있을 것이다.

주요어: 비 침습적 산전검사, 다태임신, 세포 유리 DNA,
염기서열 분석 방법

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