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Detection of short tandem repeats at 5 loci and amelogenin with cell-free fetal DNA as a specimen in the development of prenatal paternity diagnostic tests

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Abstract

Introduction: A prenatal paternity test is one widely-used method of determining the paternity of an unborn child. Such tests using chorionic villus or amniocentesis may increase the risk of harm to both mother and foetus. In the present day, a prenatal paternity test using circulating cell-free fetal Deoxyribonucleic Acid (DNA) is one alternative method due to it being non-invasive and safe for both mother and foetus.

Aim: The aim of this study is to detect Short Tandem Repeat (STR) at 5 Loci (vWA, TH01, D13S317, D18S51, and D21S11) and amelogenin genes in circulating cell-free fetal DNA in paternity tests.

Materials and methods: Forty-one samples of maternal blood were obtained from pregnant woman. Circulating free fetal DNA was subsequently extracted. A paternity test was conducted using an STR test at loci vWA, TH01, D13S317, D18S51, D21S11 in circulating free fetal DNA. An analysis of the paternity test between loci > 200 bp and < 200 bp was also conducted to establish the sensitivity of the test.

Results: There was a significant difference between maternal blood DNA and circulating cell-free fetal DNA ($p = 0.000$ D13S317; $p = 0.000$ D21S11; $p = 0.000$ D18S51; $p = 0.000$ vWA; $p = 0.000$ TH01; and $p = 0.000$ amelogenin genes). The locus < 200 bp also had a higher sensitivity than locus > 200 bp.

Conclusion: Circulating free fetal DNA can be used as an alternative sample for prenatal paternity tests because of its similarity with maternal DNA.

Keywords: Early detection, Circulating free fetal DNA, Non-invasive technique, Prenatal paternity test

Introduction

A prenatal paternity test is one popular method of determining the paternity of an unborn child which requires direct chorionic villus or amniotic fluid sampling via amniocentesis (Guo et al. 2012; Ryan et al. 2013). Amniocentesis carries a 1 in 300 to 1 in 500 risk of miscarriage and very limited risk of other complications (Seeds 2004; Elchalal et al. 2004). Chorionic villus sampling carries a similar risk of miscarriage and a 1 in 3000 risk of fetal limb reduction defects, especially when performed within the first 10 weeks of gestation

(Brambati et al. 1992). Due to this risk, a non-invasive method of carrying out prenatal paternity tests has been developed.

Circulating cell-free fetal DNA diagnostic technique is a non-invasive technique which uses circulating cell-free fetal DNA contained in maternal blood at the 8-week stage of pregnancy or later (Guo et al. 2012). This technique was intended to meet the “challenge” of developing diagnostic methods in the field of gynecology obstetrics for prenatal cases mainly related to genetic disorders which have been of concern in the foeto-maternal field. Although this technique was first reported more than decade ago, the development of and reporting on this study remains limited (Ryan et al. 2013).

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The use of fetal cell-free DNA in obstetrics gynecology and the urgent need for samples based on circulating cell-free fetal DNA in forensic cases in the future is inevitable. Prenatal paternity tests may successfully address the complexity of unborn child identification in abortus provocatus or rape cases. The presence of residual cell-free fetal matter derived from previous pregnancies and the technical challenges associated with the amount of fetal DNA in early pregnancy which is usually less than 20% of the total DNA circulating freely are investigated here (Barrett et al. 2011; Lo et al. 1998). The aim of this study is to detect paternity tests using Short Tandem Repeat (STR) at 5 Loci (vWA, TH01, D13S317, D18S51, and D21S11) and amelogenin genes gender determination in cell-free fetal DNA.

Materials and methods

Peripheral blood was collected from 41 women during the first, second and third trimesters of their pregnancy. This research has been approved with ethical clearance from the Committee of Ethical Clearance of Health Research, Faculty of Dental Medicine, Universitas Airlangga (No. 014/ HRECC.FODM/II/2017). Participating volunteers signed a consent form and their right to privacy was consistently observed.

Plasma was obtained by centrifugation of maternal blood samples contained in 2 ml Eppendorf tubes at 3000 rpm for 10 min. Plasma was obtained by means of careful separation with a buffycoat before being re-centrifuged at 6000 rpm for 10 min. DNA extraction was performed by means of an organic method using DNAzol (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). DNA concentration and purity were measured in a sample that had been previously extracted with a UV-spectrophotometer (Shimadzu UV-16, Kyoto, Japan). DNA amplification was performed with PCR-STR (Polymerase Chain Reactions-Short Tandem Repeat) at five loci of CODIS (Combined DNA Index System) to the FBI standard (Federal Bureau of Investigation) D13S317, vWA, TH01, D18S51, D21S11 and gene amelogenin for gender determination (cat. K562, GoTaq® Green Master Mix, Promega Corp, Madison, USA). The results were obtained through PCR-STR and subsequently analyzed to determine the pattern of “exclusion-inclusion” between the sample and comparison groups. Data analyses were visualized using Gel Polyacrylamide 6% gel (cat. K562, GoTaq®, Green Master Mix, Promega Corp, Madison, USA) and statistical methods relating to independent samples on SPSS 17.0 for Windows with $p < 0.01$.

Results

DNA was extracted from the peripheral blood of 41 pregnant women. Circulating cell-free fetal DNA and maternal DNA were separated and their concentration and purity measured. The comparison of DNA concentration from

circulating cell-free fetal DNA and maternal DNA can be seen in Fig. 1.

The mean percentage (%) of fetal DNA contained in maternal DNA was then calculated at $16.24 \pm 1.67\%$. The mean score was subsequently subjected to statistical analysis and the results compared with the successful analysis of loci D13S317, D18S51, D21S11, vWA, TH01 and amelogenin. The results are shown in Table 1.

Table 1 showed the PCR-STR at loci D13S317, D18S51, D21S11, vWA, TH01 and the amelogenin derived from circulating cell-free fetal DNA inherited from parental allele. This result meant that a technical analysis of the results of an STR examination on circulating cell-free fetal DNA could be conducted. Table 2 shows a comparison between the loci > 200 bp and < 200 bp in length. There was a significant difference between the loci above and below 200 bp in length. Locus < 200 bp has a higher sensitivity than locus > 200 bp.

Discussion

Extracting maternal plasma during pregnancy for the purposes of molecular analysis has become increasingly common in recent times. The identification of fetal DNA in the maternal bloodstream represents a promising new hope for the development of non-invasive, prenatal diagnostic techniques which still encounter many problems in their application (Lo et al. 1998). The use of circulating cell-free fetal DNA found in maternal plasma enables forensic examination to be performed in paternity cases (Rong et al. 2012; Sarasola et al. 2006). Detection by means of circulating cell-free fetal DNA is capable of determining the gender of the foetus after 8 weeks of pregnancy (Colmant et al. 2013).

In this study, the respective DNA concentrations of circulating cell-free fetal DNA and maternal blood were compared, with significant differences being identified. This percentage indicates that the quantity of DNA derived from circulating cell-free fetal is high. DNA derived from circulating cell-free fetal DNA can be used as a specimen for the purposes of DNA examination by means of PCR method. This is due to the requirement that the volume of DNA specimens used in forensic DNA profiling must be at least 20 ng/ml (Notosoehardjo 1999).

The average purity of both maternal blood DNA or circulating cell-free fetal DNA is 1.79, allowing DNA to be used in PCR amplification (Muladno 2002). DNA purity can be classified as high if it falls within the range of 1.8–2.0. This study shows DNA purity to fall outside that range possibly because protein contamination might have occurred during the isolation of the DNA sample. The purity level of forensic DNA samples does not pose a significant problem if it remains above 1. The purity of DNA ranging from 1 to 2 for forensic DNA examination can still be used for DNA typing using PCR (Notosoehardjo 1999; Muladno 2002).

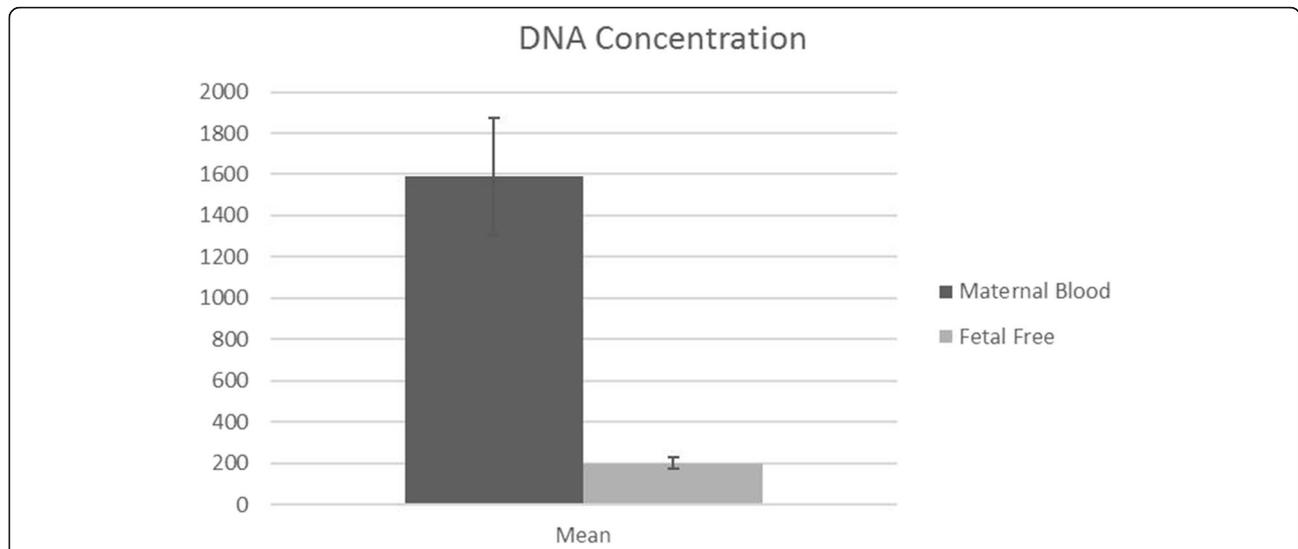


Fig. 1 Comparison of DNA concentration from circulating cell-free fetal DNA and maternal blood using T-test. There was a significant difference between circulating cell-free fetal DNA and maternal blood with $p = 0.000$ ($p < 0.01$)

PCR is a sensitive method of DNA amplification. In forensic science, particularly the field of DNA typing, this method is also applied due to its ability to amplify small quantities of DNA (Kashyap et al. 2004). However, the limited amount of target DNA in certain circumstances does not affect the success of PCR. This research used several STR loci, such as loci vWA, TH01, D132317, D18S51, and D21S11, in addition to amelogenin genes for gender examination. It can be concluded from this study that the detection of forensic DNA of a length less than 400 bp was successful. The possibility of DNA fingerprinting using cell-free fetal DNA specimens was also confirmed. Nevertheless, the cell-free fetal DNA contained in certain samples failed the DNA fingerprinting process for one sample of circulating cell-free fetal DNA during the examination of loci D18S51 and D21S11. This occurrence might have been due to the fact that circulating cell-free fetal DNA in maternal plasma is, in general, shorter than maternal DNA (Rong et al. 2012). Failures in identification in loci D18S51 and D21S11 could also have been caused by problems associated with the amplification process or the occurrence of “false negative” results in the DNA multiplication process.

Table 1 Comparison of percentage fetal DNA in loci analysis using Willcoxon Signed Rank test

	p value					
	D13S317	D18S51	D21S11	vWA	TH01	amelogenin
Success of Paternity Test using STR analysis	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*

*was significant statistically ($p < 0.01$)

These were possibly caused by the quality of circulating cell-free fetal DNA from the foetus or of fetal origin in the maternal plasma. Hence, the research samples in loci D18S51 and D21S11 were not successfully identified.

This research provided promising results for the use of circulating cell-free fetal DNA as a specimen of paternity examination in loci vWA, TH01, D13S317, D18S51, D21S11 and gender examination using amelogenin genes with non-invasive techniques. The study conducted by Wegner et al. (2009) was not able to successfully amplify autosomal fetal alleles from maternal plasma. Amelogenin was the only locus that was reliably amplified by the use of AmpFLSTR. Amelogenin revealed only fetal gender, while the amplification of other autosomal loci was sporadic and insufficient for reliable paternity testing (Wagner et al. 2009). The study reported here confirmed the success of this research as being relatively high: approximately 100% for loci of vWA, TH01, D13S317, 100% for amelogenin genes, 90% for loci D18251 and D21S11, giving an average success rate of 96%. This rate is relatively high considering the circulating cell-free fetal DNA. The results of this study are in accordance with those of a study conducted by Jiang et al. (Jiang et al. 2016) which proved that maternal plasma DNA sequencing-based technology is feasible and accurate in determining paternity, possibly providing an alternative

Table 2 Comparison in success analysis of loci length above and below 200 bp using Willcoxon Signed Rank test

p value	
locus < 200 bp	0.000*
locus > 200 bp	0.000*

*was significant statistically ($p < 0.01$)

for forensic application in the future. This method could also be a unique means of detecting DNA loci with an average length of 100 bp (Rong et al. 2012).

According to a previous study conducted by Rong et al. (Rong et al. 2012), the Loci of vWA, TH01, D13S317, D18S51, D21S11 and gender examination using amelogenin feature relatively long-base pairs which facilitate targeted locus examination. Locus D13S317 has a length of 169–201 bp, D18S51 is 290–266 bp in length, D21S11 is 203–259 bp in length, VWA is 127–167 bp in length, TH01 is 179–203 in length, while amelogenin genes are 106–112 bp in length. Long-base pair loci can be employed as paternity examination loci using cell-free fetal DNA as the examination material, although fetal cfDNA has reportedly also played this role for at least a decade (Ryan et al. 2013).

Paternity tests can be carried out using circulating cell-free fetal DNA (Ryan et al. 2013). There are three main reasons why cell-free DNA can be used for the purposes of paternity tests. The first is that circulating cell-free fetal DNA is derived from a foetus circulating in maternal blood even though at low concentration levels. Cell-free fetal DNA is essentially the DNA molecules outside the maternal DNA derived from the foetus whose use in DNA paternity tests has been sanctioned. Circulating cell-free fetal DNA inherits half of the maternal genetic sequences, thereby enabling it to be distinguished from the mother's DNA (Wright 2009). This study could prove that there is allele conformity at loci D13S317, D18S51, D21S11, VWA, TH01 between fetal alleles and those derived from a mother and father in accordance with Mendel's hereditary law.

Regarding the amount of human DNA, there are several different opinions about the minimum amount required for DNA analysis in forensic science. The minimum required amount for DNA typing in forensics ranges from 0.1 to 50 ng (Mandrekar et al. 2001). Another study showed that the DNA templates recommended for use in DNA typing contain 100–1000 ng (Kline et al. 2003). Meanwhile, a further piece of research confirmed that the minimum amounts of DNA used in forensic science are 50 ng and 20 ng respectively (Notosoehardjo 1999). STR method-based DNA testing could produce reliable results at minimum DNA concentrations of 0.5–2.5 ng (Kline et al. 2003). Although there are different opinions about the minimum amount of DNA that can be used in DNA analysis, in principle, the required amount in DNA forensic analysis depends on the necessity for and type of examination conducted. The Restriction Fragment Length Polymorphism (RFLP)-based forensic DNA examination requires relatively large amounts of about 100–1000 ng (Kline et al. 2003). The large amount of DNA used for RFLP tests is far from fresh, but the large amount of DNA is aimed to increase the likelihood of success in the management of DNA profiling. The STR test only requires a

minimum DNA concentration ranging from 0.25–2 ng (Andelinović et al. 2005).

In addition, the amount or concentration of DNA for PCR-based DNA examination needs to be adequate, while its degraded condition should be at a minimum. Seriously degraded DNA has the potential to prevent primer attachment or aneling on the target DNA. Therefore, the quality of DNA represents a fundamental factor in the successful use of the PCR method (Yamada et al. 2002). The sensitivity of PCR is dependent on the function of the DNA's cycle, amount and integrity. Low DNA levels in forensic investigations may sample up to a certain level that will not significantly affect the success of DNA profiling in forensic science, especially in STR tests. This is due to the amount of DNA required for the successful conducting of STR tests tending to be the lowest compared to other DNA-based tests. Consequently, the risk of failure of DNA amplification with this technique is relatively small (Chung et al. 2004).

Conclusion

The fraction of DNA derived from circulating cell-free fetal DNA is low in quantity. Nevertheless, the circulating cell-free fetal DNA or cffDNA can be used as a DNA specimen by STR-PCR using loci D13S317, D18S51 and D21S11, VWA, TH01 and amelogenin genes for the purposes of paternity detection.

Abbreviations

bp: Base pair(s); cffDNA: Circulating cell-free fetal DNA; cfDNA: Cell free DNA; CODIS: Combined DNA Index System; DNA: Deoxyribonucleic Acid; FBI: Federal Bureau of Investigation; PCR: Polymerase Chain Reactions; PCR-STR: Polymerase Chain Reactions-Short Tandem Repeat; RFLP: Restriction Fragment Length Polymorphism; STR: Short Tandem Repeat

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Authors' contributions

Conception and design of the study: AS, DR, IN. Acquisition of data: AS, DR, IN. Drafting the manuscript: NK, APN, MDAA. Revising the manuscript critically for important intellectual content: AS, NK, APN, MSA. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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