Original Article

Detection of Y STR markers of male fetal dna in maternal circulation

Seema P. Nair, Sam Peter, V. V. Pillay, U. M. Remya, R. Krishnaprasad, B. Rajammal
DNA Typing Unit, Department of Analytical Toxicology, Amrita Institute of Medical Sciences, Kochi, Kerala - 682 026, India

BACKGROUND: Circulating fetal cells and cell free DNA in the maternal blood has been shown to help in prenatal diagnosis of genetic disorders without relying on invasive procedures leading to significant risk of pregnancy loss.
AIM: The current study was undertaken to detect the male fetal population using Y STR markers DYS 19, DYS 385 and DYS 392 and also to study the extent of persistence of fetal DNA in the mother following delivery.
MATERIALS AND METHODS: Blinded study was conducted on 50 mothers delivering male and female babies. Cellular and cell free DNA was extracted from maternal and fetal cord blood and amplified for Y STR markers by PCR.
RESULTS: The amplification sensitivity of Y specific STR, DYS19 was 100% (22/22) in the male fetal DNA samples. The incidence of other STRs, i.e., DYS385 and DYS392 were 91% (20/22) each. Analysis of results revealed that thirteen of the twenty six women had detectable male fetal DNA at the time of delivery. However fetal DNA was not detectable twenty four hours after delivery.
CONCLUSION: Preliminary results show that the separation of fetal cell-free DNA in the maternal circulation is a good low-cost approach for the future development of novel strategies to provide non-invasive techniques for early prenatal diagnosis.

Key Words: Y-STR, fetal DNA, prenatal diagnosis, DYS markers, cord blood

Introduction

During pregnancy, the fetal and maternal circulations are separated by the placental membrane. However, a variety of evidence has pointed towards the incompleteness of this barrier to cellular trafficking. Fetal nucleated cells have been demonstrated in maternal circulation and have been pursued as potential substrates for noninvasive prenatal diagnosis.[1-3] However, the difficulty in successfully detecting such fetal cells in maternal blood has been a major obstacle to the routine application of this concept. The finding of tumor-derived DNA in the plasma and serum of cancer patients has led to the use of plasma for molecular analysis.[4-6] Studies have also demonstrated the potential pathogenic effect of fetal cell persistence on the development of "autoimmune" diseases such as systemic sclerosis.[7,8] Circulating fetal DNA in the maternal blood has been shown to help predict some sex-linked diseases and fetal hemolytic disease resulting from Rh blood group incompatibility.[9,10] Prenatal diagnosis of genetic disorders has traditionally relied on invasive procedures such as amniocentesis and chorionic villus sampling (CVS), but these procedures carry a small but significant risk of pregnancy loss.[11-13]

Little is known about the parameters governing the level of circulating fetal DNA, except that it tends to increase as gestation progresses, especially toward the end of pregnancy. The clearance of fetal DNA from maternal plasma after delivery appears to occur in a rapid manner. The rate of clearance will also provide information as to the applicability of fetal DNA measurement in the study of the dynamic processes involved in the handling of circulating DNA during pregnancy.[14] The notable differences between fetal cell and cell-free DNA clearance suggest that the predominant cell populations involved may be distinct. For example, it is possible that the trophoblasts may be the predominant cell population involved in the liberation of fetal DNA into the cell-free fractions. Fetal erythroblasts, on the other hand, have been postulated to be the predominant fetal cell population found in maternal blood.[3] By the use of cell sorting and sensitive PCR assays, fetal hematopoietic progenitor cells have been shown to persist in some women, even decades

Address for Correspondence: Dr. Seema Nair P, DNA Typing Unit, Department of Analytical Toxicology, Amrita Institute of Medical Sciences, Kochi - 682 026, Kerala, India. E-mail: seemanair@aims.amrita.edu

after delivery.[15] The latter phenomenon has been associated with certain autoimmune disorders.[7,8]

Materials and Methods

Pregnant women (age 30-40) attending the Department of Obstetrics and Gynecology at the Amrita Institute of Medical Sciences, Cochin, Kerala were recruited for this study and informed consent was obtained prior to collection of samples. A total of 50 subjects comprising 26 mothers delivering male babies and 24 delivering female babies were recruited for the study.

Immediately upon delivery, the umbilical cord was double clamped and transected 5-7 cm from the umbilicus. The umbilical vein was catheterized aseptically and blood (2-4 ml) was collected into an EDTA vacutainer tube. Samples of maternal peripheral blood (2-4 ml) were collected from forearm into EDTA vacuettes at the time of delivery and 24 hours after. Blood was obtained by venepuncture, at a single time point, at the time of delivery. All blood samples were transported to the laboratory within an hour for immediate processing/ storage.

Genomic DNA was extracted from maternal and cord blood samples, according to the "salting out modified extraction procedure" of Miller *et al.*[16] Extracted DNA was suspended in 1x TE buffer [0.060g of Tris, 0.0186 g of EDTA; pH 7.5] and then treated with RNase (10 mg/ml). Quantitative and qualitative analysis of extracted DNA was done by DNA/RNA Quantifier (Gene Quant Pro). Extracted DNA was stored at -20°C.

The Y chromosome-specific STR markers selected for the study were DYS19, DYS385 and DYS392. These were amplified in a multiplex PCR setting (Thermal Cycler, Eppendorf, USA) for the detection of fetal DNA from umbilical cord and maternal blood.

PCR was done by using the following primers:

DYS19-F5'-CTATGAGTTTCTGTTATAGT3';

R5'-ATGGCATGTAGTGAGGACA3',

DYS392-F5'-TCATTAATCTAGCTTTTAAAAACAA3';

R5' -AGACCCAGTTGATGCAATGT 3'

DYS385 - F5 '- AGCATGGGTGACAGAGCTA 3',

R5'-GGGATGCTAGGTAAAGCTG3'. All PCR amplifications were performed in a total reaction volume of 12.5 µl with extracted DNA. The total reaction volume consisted of DD Water 7.5 µl, 1.25 µl of 10x Taq buffer, 1 µl (3 mM) of $MgCl_2$, 0.5 µl of dNTP (10 mM), 0.5 µl (20 pmol) each of forward and reverse primers, 0.25 µl Taq (5 U/µl) and 1 µl of DNA (5 ng/µl). Amplification was done for 5 minutes at 96°C for initial denaturation of DNA and polymerase activation, followed by 35 cycles at 95°C for 30 seconds, 59.1°C for 60 seconds and 70°C for 60 seconds, with final extension at 72°C for 10 minutes. Final holding temperature was 4°C. The amplified product was separated by electrophoresis in a 2% agarose gel containing ethidium bromide (10 mg/ml), at 40 mA constant current and 50V for 1 h. The amplicons were observed on UV Transilluminator (Genei, Bangalore).

Ethics

Approval for the study was obtained from the Scientific Review Committee (SRC) and Institutional Ethics Committee (IEC) of the Institute. Informed consent was obtained from the patient/relatives prior to collection of samples.

Results and Discussion

This is a preliminary study designed to screen the Y specific STRs DYS19, DYS385 and DYS392 in male fetal DNA samples [Figure 1]. The DYS markers selected in the study are commonly used markers in the Indian Population.[17-19] In our study, 50 cord blood samples were collected during delivery, among which, 24 were from

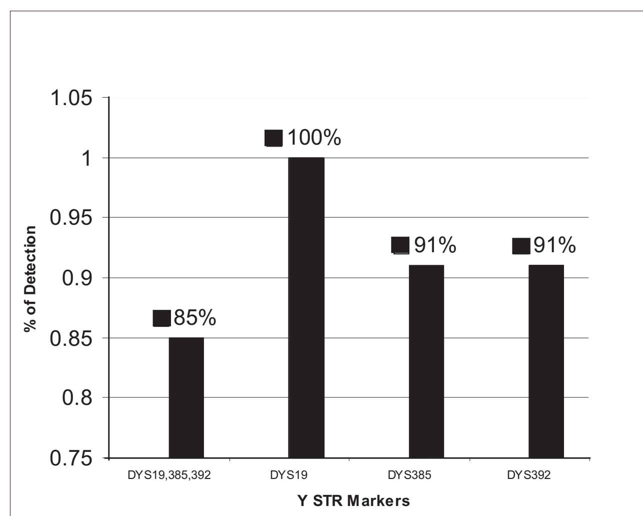

Figure 1: Detection of Y STR Markers in Male cord blood DNA

This PDF is available for free download from a site hosted by Medknow Publications (www.medknow.com).

female fetuses and 26 from male fetuses. The DNA from female cord blood was used as negative control for the study and male donor blood DNA as positive controls. Analysis of results revealed that Y specific STRs were amplified in 85% (22/26) of the male fetal cord blood. Specific STRs for Y chromosome were not amplified in any of the cord blood samples of female fetuses. Several studies have reported the use of DYS19, 392 and 385 in STR analysis of male DNA in the Indian population but no such studies have been reported in the Kerala Population.[17-19] Recent work done by Deng *et al.*[20] have also reported screening for Y-specific STR markers DYS393, DYS460, DYS392, DYS389, DYS456 and DYS458 in fetal DNA.

The amplification sensitivity of Y specific STR, DYS19 was 100% (22/22) in the male fetal DNA samples. The incidence of other STRs, i.e. DYS385 and DYS392 were 91% (20/22) each. This shows that among the three STRs studied, the highest incidence was observed in DYS19. The absence of detection of Y STRs in the four negative samples, could be because of the low quantity and purity of the DNA isolated from clotted cord blood samples. It is well known that the quantity and purity of DNA are two important factors, which determine the percentage of detection.

It has been shown that in addition to the presence of fetal cells in maternal blood, cell-free fetal DNA is also present in maternal circulation.[9] The finding of circulating fetal DNA has been confirmed by other groups of investigators also.[21,22] Fetal DNA has been demonstrated to be present in high concentrations in maternal plasma.[14]

These data prompted us to investigate as to whether the fetal DNA is present in maternal blood at the time of delivery. Thus, maternal peripheral blood samples were collected at the time of delivery along with the cord blood. The cell free fetal DNA concentration in maternal blood was determined by conventional multiplex PCR assay. Analysis of results revealed that thirteen of the twenty six women had detectable cell-free male fetal DNA at the time of delivery. This means that, Y STRs were amplified in 50% (13/26) of the maternal blood samples. These data confirmed the findings of earlier investigators about the incidence of fetal DNA in maternal circulation.[9,21] The fact that the remaining thirteen subjects did not have detectable circulating fetal DNA suggests that fetal DNA is cleared very rapidly from maternal circulation. Figure 2 shows the presence of Y STR amplicons in DNA isolated from maternal blood. The positive controls are Y STR amplifications in blood DNA from male donors. The test sample is DNA isolated from maternal peripheral blood collected at the time of delivery.

To investigate the clearance of fetal DNA from maternal circulation, we also analyzed maternal blood samples collected a day after the delivery. Absence of any amplicons revealed the complete clearance of circulating fetal DNA in all subjects. Previous work done by Lo *et al.*[23] has shown that the clearance of fetal DNA from maternal plasma after delivery will occur in a rapid manner, with a half-life of minutes. Earlier work involving the injection of exogenous DNA into the circulation of laboratory animals showed the rapid elimination kinetics of fetal DNA from maternal circulation.[24,25] The exact mechanism by which fetal DNA is liberated into the maternal plasma is unknown, as is the mode of removal from the circulation, although it has been suggested that liver, spleen or kidney may be implicated in the clearance.[23]

In this study we used conventional multiplex PCR assay for the detection of cell-free fetal DNA in maternal blood. The frequency of non-specific amplifications and relatively less sensitivity indicates the requirement for more efficient methods of detection of cell free fetal DNA. Advanced techniques such as Fluorescence assisted

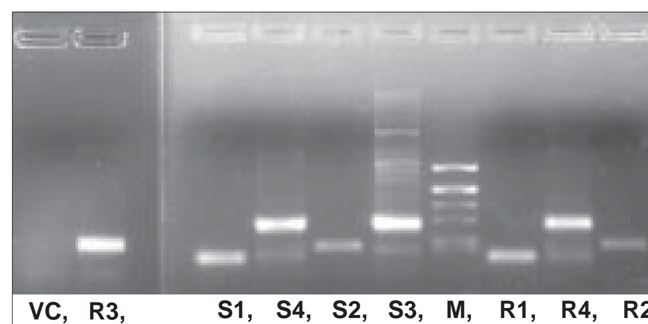

Figure 2: Y-STR Analysis of Fetal DNA from Maternal Blood

Lane 1: VC -Negative control -Female DNA; Lane 2 and 5: R3 and S2 - Positive control -male DNA (DYS392); Lane 3: S1 - Positive control -male DNA (DYS19); Lane 4: S4 - Positive control -male DNA (Multiplex PCR - for DYS19, 392 and 385). Lane 6: S3 - Positive control -male DNA (DYS385); Lane 7: M - Ladder -pBR322/AluI [908, 659, 656, 521, 403, 281, 257, 226, 100, 90]; Lane 8: R1 - Test Sample - DYS19 amplicon; Lane 9: R4 -DYS385; Lane 10: R2 -DYS392

This PDF is available for free download from a site hosted by Medknow Publications (www.medknow.com).

Cell Sorter (FACS) to sort the circulating fetal cells in the maternal blood and Real-time PCR might give a better rate of detection of cell free fetal DNA. In summary, the presence of fetal cell-free DNA in the maternal circulation is a good low-cost approach for the future development of novel strategies to provide non-invasive techniques for early prenatal diagnosis, as compared to methods like fluorescence *in situ* hybridization and Karyotyping.

Acknowledgment

The authors wish to thank Dr. M.S. Rao, Director of the Directorate of Forensic Science, New Delhi for arranging funds for the study. Thanks are also due to the following: Dr E.M Elango, Scientist-in-Charge, Molecular Biology Lab, AIMS for extending the laboratory facilities for successful implementation of the study; Dr. S.K. Shukla, Director, Central Forensic Science Laboratory, Hyderabad, Dr. D.M Vasudevan, Principal, AIMS School of Medicine and Dr. A.K.K. Unni, Professor and HOD, Biotechnology, AIMS for their advice and guidance.

References

1. Lo YM, Patel P, Wainscoat JS, Sampietro M, Gillmer M, Fleming KA. Prenatal sex determination by DNA amplification from maternal peripheral blood. Lancet 1989;2:1363-5.
2. Walknowska J, Conte FA, Grumbach MM. Practical and theoretical implications of fetal/maternal lymphocyte transfer. Lancet 1969;1:1119-22.
3. Bianchi DW. Fetal cells in the maternal circulation: Feasibility for prenatal diagnosis. Br J Haematol 1999;105:574-83.
4. Anker P, Lefort F, Vasioukhin V, Lyautey J, Lederrey C, Chen XQ, *et al.* K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. Gastroenterology 1997;112:1114-20.
5. Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J, *et al.* Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 1996;2:1033-5.
6. Wong IH, Lo YM, Zhang J, Liew CT, Ng MH, Wong N, *et al.* Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res 1999;59:71-3.
7. Nelson JL, Furst DE, Maloney S, Gooley T, Evans PC, Smith A, *et al.* Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. Lancet 1998;351:559-62.
8. Artlett CM, Smith JB, Jimenez SA. Identification of fetal DNA and cells in skin lesions from women with systemic sclerosis. N Engl J Med 1998;338:1186-91.
9. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485-7.
10. Bianchi DW. Fetal DNA in maternal plasma: The plot thickens and the placental barrier thins. Am J Hum Genet 1998;62:763-4.
11. Chen CP, Chern SR, Wang W. Fetal DNA in maternal plasma: The prenatal detection of a paternally inherited fetal aneuploidy. Prenat Diagn 2000;20:353-7.
12. Zhong XY, Holzgreve W, Hahn S. Detection of fetal Rhesus D and sex using fetal DNA from maternal plasma by multiplex polymerase chain reaction. BJOG 2000;107:766-9.
13. Lo YM, Tein MS, Lau TK. Quantitative analysis of fetal DNA in maternal plasma and serum: Implications for non invasive prenatal diagnosis. Am J Hum Genet 1998;62:768-75.
14. Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, *et al.* Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. N Engl J Med 1998;339:1734-8.
15. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci USA 1996;93:705-8.
16. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.
17. Kashyap VK, Sanghamitra Sahoo, Sitalaximi T, Trivedi R. Deletions in the Y-derived amelogenin gene fragment in the Indian population. BMC Med Genet 2006;7:37.
18. Kayser M, Caglia A, Corach D, Fretwell N, Gehrig C, Graziosi G, *et al.* Evaluation of Y-chromosomal STRs: A multicenter study. Int J legal Med 1997,110:125-33.
19. Redd AJ, Clifford SL, Stoneking M. Multiplex DNA typing of Short-tandem-repeat loci on the Y-Chromosome. Biol Chem 1997, 378:923-7.
20. Deng Z, Wu G, Li Q, Zhang X, Liang Y, Li D, *et al.* Noninvasive genotyping of 9 Y-chromosome specific STR loci using circulatory fetal DNA in maternal plasma by multiplex PCR. Prenat Diag 2006;26:362-8.
21. Smid M, Lagona F, de Benassuti L, Ferrari A, Ferrari M, Cremonesi L. Evaluation of different approaches for fetal DNA analysis from maternal plasma and nucleated blood cells. Clin Chem 1999;45:1570-2.
22. Houfflin-Debarge V, O'Donnell H, Overton T, Bennett PR, Fisk NM. High sensitivity of fetal DNA in plasma compared to serum and nucleated cells using unnested PCR in maternal blood. Fetal Diagn Ther 2000;15:102-7.
23. 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.
24. Emlen W, Mannik M. Kinetics and mechanisms for removal of circulating single-stranded DNA in mice. J Exp Med 1978;147:684-99.
25. Tsumita T, Iwanaga M. Fate of injected deoxyribonucleic acid in mice. Nature 1963;198:1088-9.

Source of Support: Nil, **Conflict of Interest:** None declared.

This PDF is available for free download from a site hosted by Medknow Publications (www.medknow.com).