UNIVERSITÀ
DEGLI STUDI
DI PADOVA

SEDE AMMINISTRATIVA: UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI SALUTE DELLA DONNA E DEL BAMBINO - SDB

SCUOLA DI DOTTORATO
in
MEDICINA DELLO SVILUPPO E SCIENZE DELLA PROGRAMMAZIONE

INDIRIZZO
EMATOONCOLOGIA, IMMUNOLOGIA E GENETICA

Ciclo XXV

NON-MEDICAL APPLICATIONS OF NON INVASIVE PRENATAL TESTING: ETHICAL ISSUES AND APPLICABILITIES

Direttore della Scuola: *Prof. Giuseppe Basso*
Coordinatore d'indirizzo: *Prof. Giuseppe Basso*
Supervisore: *Dott.ssa Geertruy te Kronnie*

Dottorando: *Paola Tasinato*

CONTENTS

ABSTRACT

The possibility of obtaining material for foetal molecular analysis without the need of invasive procedures has been a long wished improvement of practice in prenatal diagnostics. The demonstration of the presence of foetal cells and circulating foetal free-DNA in a sample of mother-to-be's blood promised that a non-invasive approach for prenatal diagnostics is near to becoming a reality.

The presence of foetal cells (albeit in low numbers) in maternal blood has been known since 1893, when Schomorl [1] described throphoblast cells in lung circulation of pregnant women who deceased from eclampsia. However, following the first observation numerous attempts to isolate these cells have proven disappointing. The main reasons for the lack of success with fetal cells isolation can be attributed to the very tiny proportion of fetal cells in the total maternal blood cell population. Moreover, isolation of fetal cells failed to demonstrate the origin of these cells using genetic profiling.

The presence of cell-free foetal circulating DNA sequences in the plasma of pregnant women was first described in 1997, when the Lo group [2] reported the presence of Y chromosome DNA sequences in pregnant circulation.

The demonstration of foetal genetic material in maternal circulation incited a new era of non-invasive prenatal diagnostics based on free foetal DNA, with the purpose of replacing the invasive approaches based on villi sampling, cordonocentesis and amniocentesis. Obtaining chorionic villi, amniocentesis or cord blood specimens is expensive and requires an invasive procedure that carries a small risk to the foetus and the possibility of adverse maternal effects, such as unnerving risk of miscarriage. Moreover, these tests are not provided until late in the first trimester thus implying a rate – limiting step in the provision of prenatal diagnosis: only pregnant women at the highest risk of having a foetus with a genetic syndrome or another disorder of major clinical significance have been eligible to these tests.

In this study we considered that genetic prenatal diagnosis could have forensic applications, in particular in cases of rape resulting in pregnancy. So, we explored the opportunity and ethical issues related to the introduction of non-invasive prenatal testing for non-medical applications, such as sex determination and

paternity testing. In particular, based on our laboratory analyses we achieved proof of principle of a non-invasive test for forensic purposes.

We obtain approval for our studies from the Ethics Committee of the University - Hospital of Padova (Protocol n. 2105 P / 2010), Italy. First of all we demonstrated the applicability of cell free DNA in human identification. Then, we enrolled pregnant women and their partner and investigated the STR profiles obtained from free DNA and nucleated red blood cells in peripheral blood of pregnant women that were compared to profiles of the putative father, in order to define paternity. The STR profiles in free DNA were investigated using consolidated methods, by performing multiplex PCR and sequencing, without succesfull results that we attributed to the low sensitivity of the method. This result pushed us to investigate the applicability of new high throughput technologies (e.g. next generation sequencing) in plasma DNA of mother's-to be. This new approach, of potential interest for forensic genetics, seems to be promizing thank to its high sensitivity.

The best results with profiling fetal DNA from maternal blood specimens were obtained from DNA profiling of nucleated red blood cells of embryonic origin when enrichment by Fluorescent Activated Cell Sorting (FACS) was performed. For enrichment a combination of monoclonal antibodies was employed: all amplified alleles amplified from sorted cells, others than those of the mother, matched the alleles of the putative father.

Our study, which is the first one to investigate DNA profiling analysis on nucleated fetal red blood cells for forensic genetics, demonstrated that non-invasive prenatal paternity testing could be developed as a tool for the identification of perpetrators of rape in case of resulting pregnancy. More efforts need to be spend to optimize the isolation of fetal cells. The proof of principle that non invasive prenatal paternity testing on DNA of rare fetal cells circulating in pregnant blood is feasible justifies further efforts in developing this new approach.

RIASSUNTO

La possibilità di ottenere materiale genetico di origine fetale per diagnosi prenatale senza l'ausilio di procedure invasive è sempre stato considerato un interesse condiviso in campo ostetrico ginecologico. Da quando è stata dimostrata la presenza di cellule fetali e di DNA plasmatico entrambi di origine fetale nel circolo periferico delle donne in gravidanza questa opportunità ha iniziato ad essere indagata al fine di poter essere proposta nella pratica clinica.

Che cellule fetali siano presenti, anche se in numero molto esiguo, nel circolo periferico delle donne gravide è noto fin dal 1893, quando il patologo tedesco Schomorl ha descritto la presenza di trofoblasti nel circolo polmonare di 14 donne decedute per eclampsia [1], ma i tentativi di isolamento sono sempre risultati fallimentari. I principali motivi del mancato successo sono da ricercare nella esiguità di questa quota cellulare e nella mancanza di dimostrazione dell'origine fetale attraverso tecniche di profiling genetico della stessa.

Nel 1997 il gruppo di Yo [2] ha dismostrato la presenza di frammenti di DNA libero di origine fetale nel sangue periferico delle donne gravide, attraverso l'amplificazione di sequenze del cromosoma Y nel plasma di queste donne.

Così, dalla dimostrazione della presenza di materiale genetico di origine fetale nel circolo materno è iniziata una nuova era della diagnosi prenatale non invasiva, particolarmente incentrata sulla frazione di acidi nucleici liberi. Infatti, il recupero dei villi e/o del liquido amniotico sono gravati da costi sostenuti e dai rischi legati all'invasività del prelievo sia per il prodotto del concepimento che per la donna, con un seppur basso ma esistente rischio di perdita della gravidanza stessa.

Inoltre, questi approcci invasivi sono eseguiti non prima del termine del primo trimestre di gravidanza o nel secondo: essi sono proposti solo alle donne con già rilevato rischio di sindromi genetiche malformative o con rischio aumentato in funzione dell'età materna.

Nel nostro studio abbiamo considerato che la diagnosi prenatale può avere anche applicazioni forensi, in particolare nei casi di gravidanza esitata dopo stupro. Abbiamo così analizzato l'opportunità e la valenza etica di introdurre tecniche di diagnosi prenatale non invasiva per scopi non squisitamente clinici, come la determinazione del sesso e della paternità.

Abbiamo ottenuto l'approvazione del Comitato Etico dell'Azienda Ospedaliero Universitaria di Padova (Protocollo n. 2105 P / 2010), attraverso la preliminare proposta di considerazioni bioetiche sullo studio.

Abbiamo, dunque, iniziato il nostro studio attraverso la dimostrazione dell'applicabilità della frazione libera del DNA, cosiddetto DNA plasmatico o DNA libero circolante, ai fini dell'identificazione personale in soggetti sani, comprese donne non gravide. Quindi, abbiamo arruolato donne in gravidanza afferenti al Servizio di Gestione della Gravidanza della Divisione Ostetrica dell'Azienda Ospedaliera di Padova e i loro partner in funzione di investigare i profili genetici degli *short tandem repeats* (STR) usati in genetica forense ai fini di identificazione personale, ottenuti dalla frazione di DNA libero e dagli eritroblasti fetali entrambi circolanti nel sangue periferico delle donne e di confrontarli con il profilo dei partner al fine di definirne e dimostrarne la paternità.

Nella frazione libera di DNA i consolidati metodi di amplificazione del DNA non hanno permesso di dimostrare la presenza di profilo aggiuntivo rispetto a quello materno. Questo ci ha spinto ad indagare l'applicabilità delle più recenti tecnologie come il sequenziamento di ultima generazione, cosiddetto *pyrosequenzing*, nel plasma delle future mamme. Questo approccio, di nuovo interesse nella disciplina della genetica forense, ci ha permesso di rilevare la presenza di sequenze fetali nel plasma materno.

Migliori risultati li abbiamo ottenuti isolando gli eritroblasti di origine fatale attraverso arricchimento con tecniche di *sorting* cellulare (*Fluorescent Activated Cell Sorting, FACS)*, con una combinazione di anticorpi monoclonali: tutti gli alleli amplificati diversi da quelli materni sono risultati essere coerenti con quelli del partner, ovvero del "presunto" padre, poi confermati alla nascita con quelli del bambino.

Il nostro studio, che rappresenta la prima apllicazione dell'impiego degli eritroblasti fetali in genetica forense, dimostra che la diagnosi di paternità non invasiva può rapprentare un valido mezzo nell'identificazione della figura dello stupratore in caso di successiva gravidanza. Altri studi dovranno comunque essere eseguiti al fine di ottimizzare l'isolamento delle cellule fetali.

References

[1] Schmo¨rl, G. (1893) Pathologisch-anatomische untersuchungen ueber Publereklapmsie. Vogel, Leipzig

[2] 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;16;350(9076):485-7

ABBREVIATIONS

CD: cluster differentiation

CE: capillary electrophoresis

cfDNA: cell free DNA

CVS: chorionic villi sampling

EBV: Epstein-Barr virus

emPCR: emulsion polymerase chain reaction

FACS: Fluorescence Activated Cell Sorting

FIVET: fertilization in vitro embryo transfer

EDTA: ethylenediamine tetra-acetic acid

ffDNA: free foetal DNA

F: newborn

FISH: in situ fluorescent hybridization

GS: Genome Sequencer

GWs: gestation weeks

LOH: loss of heterozygosity

M: mother

MACS: Magnetic Activated Cell Sorter

MID: multiplex identifier

NIPD: non-invasive prenatal diagnosis

NIPPT: non-invasive prenatal paternity testing

NRBC: nucleated red blood cells

P: presumptive biological father

PPi: pyrophosphate

US: ultra sound

SNPs: single nucleotide polymorphisms

STRs: short tandem repeats

INTRODUCTION

1. Invasive and non-invasive prenatal genetic diagnostics

Prenatal genetic diagnostics is a collection of procedures to assess the presence of abnormalities in the foetus.

Prenatal diagnosis can be achieved through either invasive methods or non-invasive methods. Invasive approaches consist in chorionic villi sampling (CVS), cordocentesis and amniocentesis. The major non-invasive methods include ultra sound (US), doppler, Tri-test and neck translucency. Although these are extremely useful in monitoring the pregnancy, they are entirely inappropriate for the diagnosis of many diseases of genetic origin (both chromosomal and monogenic), for which the only possibility of diagnosis consists in invasive sampling and molecular procedures.

Invasive prenatal diagnosis by amniocentesis and CVS started to be used in the late 60's and in the 80's respectively. Over the decades, these techniques have been extremely refined thanks to gynecologists's abilities and as a result of significant technological advances, thus minimizing the risk of miscarriage after withdrawal.

Unfortunately, the risk of miscarriage has not been completely cleared and is currently estimated at around 0.5-1%. For this reason, and because of high costs, only a small group of women is eligible to invasive prenatal diagnosis. In particular, women older than 35 years, since at this age the risk of having a child with chromosomal abnormalities increases dramatically, and the latter risk is quite comparable to the risk of a miscarriage risk related to the invasive procedures. Although the advanced maternal age is indicative of an increased risk of general chromosomal disorder, even women under the age of 35 may have affected children. However, in this risk group the costs and the risk invasive prenatal genetic testing do not counter balance the small risk of giving birth of a child with a chromosomal abnormality.

If it were possible to obtain foetal material to conduct genetic research without invading the uterus in any way, prenatal genetic diagnosis could be offered to all

pregnant women, regardless of their risk of having an affected child. For this reason interest in the development of techniques for a non-invasive prenatal genetic diagnosis is high.

These techniques are based on recovery of foetal genetic material from maternal blood, both in the form of foetal cells and in the form of free foetal nucleic acids.

2. Foetal cells in maternal blood

The discovery of the presence of foetal cells in the maternal circulation dates back to 1893 when the German pathologist Schmorl described the presence of trophoblast cells in the lung circulation of 14 pregnant women who died because of eclampsia [1] Later, in 1957 erythrocytes of foetal origin were isolated from the maternal circulation [2] and ten years later, thanks to the identification of metaphases with an XY karyotype, foetal leukocytes were detected in the peripheral blood of a pregnant women carrying a male foetus [3]. In particular, such finding delivered the first demonstration that trophoblast cells are not the only foetal cellular component found in maternal blood; however, the existence of foetal cells in maternal circulation remained controversial for many years.

Only the introduction of more sensitive analytical techniques, such as in situ fluorescent hybridization (FISH) and DNA amplification by PCR irrefutably demonstrated the presence of foetal cells in the maternal circulation.

Even if it is now widely recognized that foetal cells are present in maternal blood, data relating to foetal cell frequency are often discordant. Lack of concordance regarding the frequencies of foetal cells have been attributed to the difficulties of isolating these cells related to both their scarcity and fragility. It is very likely that cells are lost during the various steps of the isolation process, which is difficult to standardize.

Through the use of FISH, Krabchi and colleagues have reported the presence of about 2-6 foetal cells per milliliter of maternal blood [4]. These numbers are comparable to those obtained through quantitative real time PCR assays, which is around 1-4 foetal cells per milliliter of maternal blood [5]. However many authors

failed to confirm these data, isolating lower numbers of cells or even isolating no cell at all [6 - 8].

Numerous studies have shown an increase in number of foetal cells in the maternal circulation during complications of pregnancy, such as diabetes and preeclampsia [9 - 11] and in cases of foetuses with aneuploidy, suggesting the use of foetal cells in pregnancy monitoring.

In particular, Bianchi and colleagues demonstrated an increase in the number of foetal cells in blood of pregnant women carrying a foetus with Down syndrome [12]. Also other aneuploidies, including those affecting the sex chromosomes, have been shown to be characterized by an increase of foetal cells in maternal circulation [13].

Presence of relatively high numbers of foetal cells in the circulation of a pregnant woman can be explained by a defective placentation, as found in the presence of preeclampsia and aneuploidy, resulting in a altered placental barrier, with consequent increase in the transfer of foetal cells to the maternal circulation [14]. Other factors influencing the concentration of foetal cells in maternal blood were found to be related to foetal gestational age [15], multiple pregnancies, foetal maternal blood group incompatibility and the previous use of invasive procedures to the uterus [16].

Numerous approaches have been evaluated to isolate and analyze foetal cells. They include centrifugation on a density gradient, the use of immunomagnetic beads, antibody conjugation, single cell micromanipulation, Fluorescence Activated Cell Sorting (FACS) and Magnetic Activated Cell Sorting (MACS). FACS and MACS are mainly based on the recognition of foetal cells through antigen-antibody complexing, wherein antibodies to surface markers of the foetal cells are respectively conjugated to fluorochromes or magnetic microbeads. FACS sorting is also based on using physical parameters of the foetal cell such as specific volume and size.

Based on the use of antibodies, the success of FACS and MACS for recognizing foetal cells depends on the specificity of the antibody. The most commonly used antibody is the anti CD71, which recognizes the transferrin surface membrane receptor expressed on almost all nucleated foetal blood cells in the first trimester [17]. Its expression decreases with gestational age but increases in foetuses with

chromosomal aneuploidy. It is also expressed on the surface of a subpopulation of maternal cells, constituting its main disadvantage. Other antibodies used are the anti CD36 antibody (against the receptor thrombospondin), anti glycophorin A, antigens against blood group I / i and antibodies FB3-2, 2-6B / 6 and H3-3: none of these antibodies in itself represents the optimal antibody capable of isolation of foetal cells. Antibodies against the embryonic and foetal globin are also employed. The ς-globin and the ε-globin, although embryo specific markers, are expressed only for a short gestational period. The ς-globin decreases drastically from the sixth to seventh week of gestation and the ε-globin from the twelfth week is present only in half of the nucleated blood cells of the embryo [18]. With regard to foetal globin, the γ-globin has the advantage of being produced by foetal erythrocytes for an extended period of pregnancy, even if its use results in a greater contamination by maternal cells [19].

So, up to day, except for the embryonic globin, no specific antigen for the foetal cells has yet been found, so it remains difficult to isolate foetal cells without maternal contamination.

The more frequently adopted strategy to isolate foetal cells is the use of a combination of more than one of the above reported procedures. The problem of the rarity of foetal cells could be overcome by culturing of the foetal cells to obtain a sufficient number of cells for genetic analysis.

Also in vitro expansion of trophoblast cells and foetal leukocytes has been proposed [20 - 21], albeit without satisfactory results.

Three main types of foetal cells that have been isolated in the maternal circulation are: *trophoblast cells*, *leukocytes* and *erythroblasts*.

The cell type of choice should have three requirements:

- be predominant in the maternal circulation during the first trimester of pregnancy;

- have a limited half-life in maternal blood;

- possess morphological characteristics distinguishable from the maternal blood cells.

Trophoblast cells have a morphology which allows unequivocal identification under the microscope. They are released into the maternal blood during the first trimester of pregnancy; however, trophoblast cells are not easily isolated in normal

pregnancies because they are quickly captured from pulmunary circulation [22]. Moreover, they present more than one nucleus, limiting their usefulness for cytogenetic diagnosis.

Leukocytes are the first type of foetal cells isolated with success from maternal blood by Herzenberg and co-workers [23] performing a fluorescence separation based on the maternal and foetal HLA antigens differences: this approach is not universally applicated, because it needs information on the paternal HLA antigens and thus assumes known paternity. Further, even if leukocytes have the potential to proliferate in vitro, their use for genetic testing is not recommended because they may persist in the maternal circulation up to 27 years after delivery [24]. This microchimerism might cause a diagnostic error, as there is a risk that selected leukocytes do not belong to the current pregnancy.

Foetal erythroblasts or nucleated red blood cells (NRBCs) are cells with a single nucleus and are relatively well differentiated. They also have a short life-span compared to foetal lymphocytes given their limited proliferative capacity [25], making it unlikely that they persist throughout pregnancy, thus eliminating the risk of microchimerisms. These characteristics make NRBCs particularly suitable for non-invasive prenatal diagnostic testing.

In 1957, Kleihauer et al. demonstrated the presence of erythroblasts circulating in maternal blood [26]. In 1964, Clayton et al. observed NRBCs more frequently under rhesus incompatibility or following amniocentesis and pregnancy termination [27]. NRBCs are one of the first hematopoietic cells produced during foetal development and these cells are abundantly present in the foetal circulation during the early developmental period [28]. At the interface between foetal and maternal tissues transfer of erythrocytes including NRBCs into the maternal circulation predominates over that of other cell types including leukocytes and trophoblasts.

In 1990, Bianchi et al. described how to enrich NRBCs by FACS using a monoclonal antibody against the transferrin receptor (CD71), which is highly expressed on erythoblasts [29]. Other investigators confirmed these observations using a variety of monoclonal antibodies and cell enrichment techniques [30 - 31]. These successful results demonstrate the potential of using NRBCs for non-invasive prenatal diagnosis.

However, the isolation of foetal cells from the maternal circulation presents considerable challenges, given their limited numbers. Foetal cells are estimated to range from 1 to 5000 cells in maternal blood [32]. Hamada et al. used FISH on mononuclear cells isolated by density gradient separation from maternal blood to identify Y chromosome-bearing cells: they screened as many as 144,000 nuclei to find one single foetal cell containing DNA that hybridized to the Y chromosome probe [33].

An increased frequency of foetal cells with gestational age was observed. Bianchi et al. examined the number of foetal-cell DNA equivalents present in maternal blood by PCR amplification of a Y chromosome-specific sequence and found approximately one foetal cell per 1 mL of maternal blood [34].

Thus, although the presence of foetal NRBCs in maternal blood is well established, and NRBCs are considered the best target for non-invasive prenatal diagnosis, their detection remains problematic. Moreover, undoubtedly, some NRBCs are of maternal origin [35]. De Graff et al. used foetal hemoglobin to differentiate maternal from foetal NRBCs, but 20% of all foetal hemoglobin positive NRBCs were still of maternal origin [36]. These results imply that the origin of each cell needs to be confirmed for reliable clinical use when performing non-invasive prenatal diagnosis through analysis of cells recovered from maternal blood.

In vitro expansion of foetal cells could help to solve this issue. If selective induction of proliferation occurs in vitro, foetal genetic material could be amplified. Lo et al. were the first to culture foetal erythroid progenitors from the peripheral blood of pregnant women [37]. However, these results have not been consistently validated in other laboratories, and thus far selective amplification of foetal over maternal hemopoietic progenitors has not been successful [38].

Foetal mesenchymal stem cells have also been identified in maternal blood [39]: they appear early, starting from the seventh gestation week (GW), with characteristic morphology and immunophenotype. Unfortunately, however, the attempts to isolate the latter cells have shown that also these cells are extremely rare, excluding the possibility of their use in non-invasive prenatal diagnosis [40].

Although by now numerous cases of non-invasive prenatal diagnosis have been reported using various types of foetal cells in relatively small population of

pregnant women, no results of studies on larger series have been published that justify the optimism of smaller studies.

Concerning foetal cells persistence in maternal circulation, all observations to date suggest their persistence in maternal blood. The latter would imply that this approach could theoretically lead to misdiagnosis in cases of pluriparity.

3. Cell free foetal DNA

3.1 Cell free DNA

Cell-free DNA (cfDNA) is defined as DNA occurring in the extracellular compartment.

In 1948, Mandel and Metais discovered the presence of circulating nucleic acids in plasma and serum [41]. Studies on circulating cell free DNA or plasma DNA, were at first focused on autoimmune diseases [42]. Thirty years after the cfDNA discovery, Leon et al. demonstrated elevated cfDNA levels in cancer patients when compared with healthy subjects [43]. Milestones on cfDNA are highlighted in Table 1.

YEAR	EVENT
1948	Discovery of cfDNA in blood [44]
1965	Oncogenesis and cfDNA [45]
1966- 1973	Detection of high levels in patients with rheumatoid arthritis, systemic lupus erythematosus, leukemia, and other diseases [46 - 49]
1972- 1975	Procedures of determining cfDNA in normal plasma samples [50]
1977	Evidence of increased levels of cfDNA in cancer patients depending on tumor stage and treatment [51]
1989	Characteristics of cfDNA and tumor DNA in cancer patients [52]
1994-1999	Tumor-related genetic alterations in circulating DNA [53 - 59]
1997	Foetal DNA in plasma of pregnant women [60]
1998	Description of plasma DNA chimerism after transplantation [61]
2000-2010	Circulating DNA in diagnosis and prognosis of numerous diseases (tumors, trauma, heart infarction, stroke etc.) [62 - 68]
2010	Oncogenic transformation of cultured cells by circulating DNA in plasma [69].

Table 1: Milestones in the research of cfDNA

3.1.1 Cell free DNA in cancer

Cancer-derived oncogenic mutations in plasma DNA has been demonstrated only in 1994, when Vasioukhin and co-workers investigated point mutations of N-RAS in cfDNA of patients affected by myelodysplastic syndrome or acute myelogenous leukemia [70].

Being loss of heterozygosity (LOH) a common observation in tumor cells, the phenomena was also investigated in cfDNa of tumor patients. The LOH was found in the plasma of cancer patients, contributing to evidence of tumor derived DNA in the circulation. LOH patterns have been associated with particular cancer types such as breast [71], lung [72], kidney cancer [73], and melanoma [74]. In particular, serum LOH detection has been considered to be a good marker in predicting severity, disease outcome and therapeutic response in melanoma and breast cancer [75], suggesting the possibility of early stage cancer screening by LOH detection in plasma [76].

As a result of these findings, the interest of many researchers is directed to search for other sequence specific fragments of cfDNA in plasma and serum.

One of the areas of rapid progress is the detection of viral DNA in the plasma of patients suffering from virus-associated tumors. An intimate relationship of circulating Epstein-Barr virus (EBV) DNA has been shown with nasopharyngeal carcinoma [77], lymphomas [78] and gastric cancer [79].

Also human papilloma-virus (HPV) DNA in patients suffering from cervical cancer has been investigated [80], showing that detection of HPV DNA in plasma is associated with disease recurrence and distant metastasis.

3.1.2 Cell free DNA in organ transplants

The discovery of tumor-derived plasma DNA prompted researchers to search for donor-derived DNA in plasma of transplant recipients. In plasma of recipients donor derived DNA has been successfully identified in cases of kidney [81], liver and bone marrow transplantations [82]. However, it is still unclear whether this kind of “plasma DNA chimerism” has any direct biological role in enhancing graft acceptance, such as been suggested for “cellular chimerism”.

3.1.3 Cell free foetal DNA

Recognizing the pseudo-malignant nature of the placenta, Lo et al. searched for and were able to identify the presence of foetal DNA in the plasma of pregnant women in 1997 [83]. These researchers discovered the existence of free foetal DNA (ffDNA) in maternal plasma detecting Y chromosome specific DNA sequences in women bearing male foetuses: Y-specific sequences were detected in 80% and 70% of samples of plasma and serum respectively of women with a male foetus.

These same researchers have also demonstrated bidirectional traffic of foetal cells at the foetal - maternal interface during pregnancy [84]. These evidences suggested that maternal blood might be a useful source of material for non-invasive prenatal diagnosis [85]. The vast majority of free-DNA in maternal plasma is of maternal origin (maternal free DNA, mfDNA), accounting for around 95% of total free-DNA in plasma [85]. This aspect considerably reduces the efficacy and specificity of conventional or real time allele-specific PCR approaches for the detection of foetal loci.

However, recently it has been shown that ffDNA molecules in maternal plasma are smaller than mfDNA [86], offering possibilities for enrichment of foetal DNA that should improve the detection of foetal loci.

The mechanism of foetal DNA release has not yet been elucidated. One possibility is that foetal DNA is released from dying cell, but the identity of the cell types predominantly involved in such DNA release is not definitely resolved: in anembryonic pregnancies Alberry et al. reported a trophoblastic origin [87].

The heated debate on the origin of foetal DNA in maternal circulation is ongoing. The hypothesis that achieved most consensus suggested a placental origin, due to massive apoptosis of syncytiotrophoblasts constantly being replaced on the surface of the placenta [88]. There are three evidences in favour of this hypotesis:

1. absence of ffDNA in placental mosaicism [89];
2. presence of ffDNA after childbirth with placenta retention [90];
3. detection of foetal sequences with specific methylation patterns of the placenta [91].

It was also suggested that ffDNA originated from apoptotic foetal cells in maternal circulation. Although theoretically possible, the scarcity of foetal cells does not correspond to the amounts of ffDNA reported.

Finally, one might hypothesize a direct transfer of DNA from the embryo-foetal compartment to the maternal circulation. The latter hypothesis found support from findings of ffDNA in the amniotic liquid samples [92].

Concerning clearance, plasma foetal DNA has a more rapid clearance kinetics compared to that of foetal nucleated cells; further more, although most of the cells are cleared within weeks from the maternal circulation, it has been shown that sub populations persist for up to after delivery [93]. Foetal DNA is cleared rapidly from maternal plasma, with a half-life of minutes [94]. The notable differences between foetal cell- and free- DNA clearance suggest that the predominant cell populations involved in these two phenomena may be distinct. It has been suggested that the trophoblasts may be the predominant cell population involved in the liberation of foetal DNA into the cell-free fraction. Foetal erythroblasts, on the other hand, have been postulated to be the predominant foetal cell population found in maternal blood [95].

Concerning the amount, total cell free DNA is not constant: it varies considerably both in the circulation of healthy individuals and in that of healthy pregnant women. More over it varies throughout pregnancy. Although there might be a tendency of greater fluctuations to occur around 25 weeks of gestation (2.4- to 4.5-fold) compared to other moments during gestation, there is no clear increase in variation toward the end of pregnancy [96].

4. State of the art of non-invasive prenatal paternity testing througt STRs analysis

To our knowledge no laboratory is currently performing a complete paternity testing from maternal blood according to accuracy, precision and reproducibility of results as required in forensic DNA testing.

By analyzing free foetal DNA from maternal plasma the only locus that reliably amplified with AmpFLSTR Identifiler kit (Applied Biosystems®) is amelogenin,

which revealed only foetal gender, while the amplification of other autosomal loci is only sporadic and is not sufficient for reliable paternity testing. More success is obtained with AmpFLSTR Yfiler kit (Applied Biosystems®), which, in case of male foetuses, successfully amplifies between 6 and 16 Y foetal loci [97].

Vecchione et al. employed simultaneously 10 X-STR loci and the amelogenin gene in the same multiplex QF PCR [98] on free foetal DNA of maternal plasma: a mean of 2.67 ± 1.28 X-STR markers per sample (range 1-5) of paternally inherited foetal alleles was detected in pregnant women carrying a female foetus.

Concerning the foetal cells in maternal plasma [99] isolation of foetal DNA is reliable in non-pathological condition from nucleated erythrocytes whereas until now no paternity testing has been performed on foetal cells circulating in maternal blood.

References

[1] Schmorl G. Pathologisch-anatomische untersuchungen ueber Publereklapmsie. 1893 Vogel, Leipzig

[2] Kleihauer E, Braun H, Betke K. Demonstration von foetalem hämoglobin in den erythrocyten eines blutausstrichs. Klein. Wochenschr 1957;15: 637

[3] Walknowska J, Conte FA, Grumback MM. Pratical and implications of foetal/maternal lymphocyte transfer. Lancet 1969;1:1119-22

[4] Krabchi K, Gros-Louis F, Yan J, Bronsard M, Masse J, Forest JC, Drouin R. Quantification of all foetal nucleated cells in maternal blood between the 18th and 22nd weeks of pregnancy using molecular cytogenetic techniques. Clin Genet. 2001;60: 145-50

[5] Ariga H, Ohto H, Busch MP, Imamura S, Watson R, Reed W, Lee TH. Kinetics of foetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. Transfusion 2001;41: 1524-30

[6] Gänshirt Ahlert D, Burschyk M, Garritsen HSP. Magnetic cell sorting and tha transferrin receptor as potential means of prenatal diagnosis from maternal blood. Am J Obstet Gynecol 1992;166: 1350-55.

[7] Durrant L, McDowall K, Holmes R, Liu D. Non-invasive prenatal diagnosis by isolation of both trophoblasts and foetal nucleated red blood cells from the peripheral blood of pregnant women. Br J Obstet Gynaecol. 1996;103: 219-22

[8] Mavrou A, Kouvidi E, Antsaklis A, Souka A, Kitsiou Tzeli S, Kolialexi A. Identification of nucleated red blood cells in maternal circulation: A second step in screening for foetal aneuploidies and pregnancy complications. Prenat Diagn. 2007;27: 150-3

[9] Bianchi DW. Prenatal diagnosis by analysis of foetal cells inmaternal blood. J Pediatr. 1995;127: 847-56

[10] Holzgreve W, Ghezzi F, DiNaro E, Ganshirt D, Maymon E, Hahn S. Feto-maternal cell traffic is disturbed in preeclampsia. Obstetrics and Gynecology 1998;91: 669–72

[11] Troeger C, Holzgreve W, Ladewig A, Zhong XY, Hahn S. Examination of maternal plasma erythropoietin and activin A concentrations with regard to

circulatory erythroblast levels in normal and preeclamptic pregnancies. Foetal Diagn Ther. 2006; 21: 156-60

[12] Bianchi DW, Simpson JL, Jackson LG, Elias S, Holzgreve W, Evans MI, Dukes KA, Sullivan LM, Klinger KW, Bischoff FZ, Hahn S, Johnson KL, Lewis D, Wapner RJ, de la Cruz F. Foetal gender and aneuploidy detection using foetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Foetal Cell Isolation Study. Prenat Diagn. 2002;22: 609-15

[13] Krabchi K, Gadji M, Forest JC, Drouin R. (2006) Quantification of all foetal nucleated cells in maternal blood in different cases of aneuploidies. Clin Genet. 69: 145-54

[14] Al-Mufti R, Hambley H, Albaiges G, Lees C, Nicolaides KH. Increased foetal erythroblasts in women who subsequently develop pre-eclampsia. Hum Reprod. 2000;15: 1624-8

[15] Guetta E, Simchen MJ, Mammon-Daviko K, Gordon D, Aviram-Goldring A, Rauchbach N, Barkai G. Analysis of foetal blood cells in the maternal circulation: challenges, ongoing efforts, and potential solutions. Stem Cells Dev. 2004;13: 93-9

[16] Jansen, MW, Brandenburg H, Wildschut HI, Martens AC, Hagenaars AM, Wladimoroff JW, in't Veld PA. The effect of chorionic villus sampling on the number of foetal cells isolated from maternal blood and on maternal serum alphafetoprotein levels. Prenat Diagn 1997;17: 953–9

[17] Zheng YL, Zhen DK, De Maria M, Berry SM, Wapner RJ, Evans MI, Copeland D, Williams JM, Bianchi DW. Search for the optimal foetal cell antibody: results of immunophenotyping studies using flow cytometry. Hum Genet. 1997;100: 35-42

[18] Mesker WE, Ouwerkerk-Van Velzen MCM, Oosterwijk JC, Bernini LF, Golbus MS, Kanhai HHH, Van Ommen GJB, Tanke HJ. Two-color immunocytochemical staining of gamma (g) and epsilon (var-epsilon) type hemoglobin in foetal red cells. Prenat Diagn. 1998;18: 1131–37

[19] Zheng YL, De Maria M, Zhen DK, Vadnais TJ, Bianchi DW. Flow sorting of foetal erythroblast using intracytoplasmic antifoetal haemoglobin: preliminary observations on matrnal samples. Prenat Diagn 1995;15: 897-99

[20] Eridani S, Mazza U, Massaro P, La Targia ML, Maiolo AT, Mosca A. Cytokine effect on ex vivo expansion of haemopoietic stem cells from different human sources. Biotherapy 1998;11: 291-6

[21] Jansen MW, Korver-Hakkennes K, van Leenen D, Brandenburg H, Wildschut HI, Wladimiroff JW, Ploemacher RE. How useful is the in vitro expansion of foetal CD34+ progenitor cells from maternal blood samples for diagnostic purposes? Prenat Diagn 2000;20: 725-31

[22] Sargent IL, Johansen M, Chau S, Redma CWG. Clinical experience: isolating trophoblasts from maternal blood. Annals of the New York Academy of Sciences 1994;731: 154–61

[23] Herzenberg LA, Bianchi DW, Schröder J, Cann HM, Iverson GM. Foetal cells in the blood of pregnant women: detection and enrichment by fluorescent activated cell sorting. Proc Natl Acad Sci USA 1979;76: 1453-1455

[24] Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, De Maria M. Male foetal progenitor cells persist in maternal blood for as long as 27 years post-partum. Proc Natl Acad Sci USA 1996;93: 705-8

[25] Pearson HA. Life-span of the foetal red blood cell. J Pediatr 1967;70:166–71

[26] Kleihauer E, Braun H, Betke K. Demonstration of foetal hemoglobin in erythrocytes of a blood smear. Klin Wochenschr 1957;35:637–8]

[27] Clayton EM Jr, Feldhaus WD, Whitacre FE. Foetal erythrocytes in the maternal circulation of pregnant women. Obstet Gynecol 1964;23:915–9

[28] Ganshirt D, Garritsen H, Miny P, Holzgreve W. Foetal cells in maternal circulation throughout gestation. Lancet 1994;343:1038–9

[29] Bianchi DW, Flint AF, Pizzimenti MF, Knoll JH, Latt SA. Isolation of foetal DNA from nucleated erythrocytes in maternal blood. Proc Natl Acad Sci USA 1990;87:3279–83

[30] Price JO, Elias S, Wachtel SS, et al. Prenatal diagnosis with foetal cells isolated from maternal blood by multiparameter flow cytometry. Am J Obstet Gynecol 1991;165:1731–7

[31] Wachtel S, Elias S, Price J, et al. Foetal cells in the maternal circulation: isolation by multiparameter flow cytometry and confirmation by polymerase chain reaction. Hum Reprod 1991;6:1466–9

[32] Ganshirt-Ahlert D, Pohlschmidt M, Gal A, Miny P, Horst J, Holzgreve W. Ratio of foetal to maternal DNA is less than 1 in 5000 at different gestational ages in maternal blood. Clin Genet 1990;38:38–43

[33] Hamada H, Arinami T, Kubo T, Hamaguchi H, Iwasaki H. Foetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. Hum Genet 1993;91: 427–32

[34] Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW, Shuber AP. PCR quantitation of foetal cells in maternal blood in normal and aneuploid pregnancies. Am J Hum Genet 1997;61:822–9

[35] Slunga-Tallberg A, el-Rifai W, Keinanen M, et al. Maternal origin of transferrin receptor positive cells in venous blood of pregnant women. Clin Genet 1996;49:196–9

[36] de Graaf IM, Jakobs ME, Leschot NJ, Ravkin I, Goldbard S, Hoovers JM. Enrichment, identification and analysis of foetal cells from maternal blood: evaluation of a prenatal diagnosis system. Prenat Diagn 1999;19:648–52

[37] Lo YM, Morey AL, Wainscoat JS, Fleming KA. Culture of foetal erythroid cells from maternal peripheral blood. Lancet 1994;344:264–5

[38] Bohmer RM, Johnson KL, Bianchi DW. Foetal and maternal progenitor cells in co-culture respond equally to erythropoietin. Prenat Diagn 2001;21:818–23

[39] Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester foetal blood, liver, and bone marrow. Blood. 2001;98: 2396-402

[40] O'Donoghue K, Choolani M, Chan J, de la Fuente J, Kumar S, Campagnoli C, Bennett PR, Roberts IA, Fisk NM. Identification of foetal mesenchymal stem cells in maternal blood: implications for non-invasive prenatal diagnosis. 2003;Mol Hum Reprod. **9**: 497-502

[41] Mandel P, Métais P. Les acides nucléiques du plasma sanguin chez l'homme. C. R. Acad. Sci. Paris 1948;142: 241-3

[42] Leon SA, Ehrlich GE, Shapiro B, Labbate VA. Free DNA in the serum of rheumatoid arthritis patients. J Rheumatol 1977; 4: 139-43

[43] Leon S, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977; 37: 646-50

[44] Mandel P, Metais P. Les acides nucleiques du plasma sanguin chez l'homme. C R Seances Soc Biol Fil 1948;142:241–3

[45] Bendich A, Wilczok T, Borenfreund E. Circulating DNA as a possible factor in oncogenesis. Science 1965;148:374–6

[46] Tan EM, Schur PH, Carr RI, Kunkel HG. Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. J Clin Invest 1966;45:1732–40

[47] Kofler D, Agnello V, Winchester R, Kunkel HG. The occurrence of single-stranded DNA in the serum of patients with systemic lupus erythematosus and other diseases. J Clin Invest 1973;52:198–204

[48] Perlin E, Moquin RB. Serum DNA levels in patients with malignant disease. Am J Clin Pathol 1972;58:601–2

[49] Steinman CR. Free DNA in serum and plasma from normal adults. J Clin Invest 1975;56:512–5

[50] Kamm RC, Smith AG. Nucleic acid concentrations in normal human plasma. Clin Chem 1972;18:519–22

[51] Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977;37:646–50

[52] Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology 1989;46:318–22

[53] Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M. Pointmutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol 1994;86:774–9

[54] Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ, Yao SL. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomark Prev 1994;3:67–71

[55] Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 1996;2:1035–7

[56] Chen XQ, Stroun M, Magnenat JL, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 1996;2:1033–5

[57] Anker P, Lefort F, Vasioukhin V, et al. K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. Gastroenterology 1997;112:1114–20

[58] Chiang PW, Beer DG, Wei WL, Orringer MB, Kurnit DM. Detection of erbB-2 amplifications in tumors and sera from esophageal carcinoma patients. Clin Cancer Res 1999;5:1381–6

[59] Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res 1999;59:67–70

[60] Lo YM, Corbetta N, Chamberlain PF, et al. Presence of foetal DNA in maternal plasma and serum. Lancet 1997;350:485–7

[61] Lo YM, TeinMS, Pang CC, Yeung CK, Tong KL, HjelmNM. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. Lancet 1998;351:1329–30

[62] Rainer TH, Wong LK, Lam W, et al. Prognostic use of circulating plasma nucleic acid concentrations in patients with acute stroke. Clin Chem 2003;49:562–9

[63] Chang CP, Chia RH, Wu TL, Tsao KC, Sun CF, Wu JT. Elevated cell-free serum DNA detected in patients with myocardial infarction. Clin Chim Acta 2003;327:95–101

[64] Hu L, Chen G, Yu H, Qiu X. Clinicopathological signi!cance of RASSF1A reduced expression and hypermethylation in hepatocellular carcinoma. Hepatol Int 2010;4:423–32

[65] Cabral RE, Caldeira-de-Araujo A, Cabral-Neto JB, Costa Carvalho MD. Analysis of GSTM1 and GSTT1 polymorphisms in circulating plasma DNA of lung cancer patients. Mol Cell Biochem 2010;338:263–9

[66] Arnalich F, Menendez M, Lagos V, et al. Prognostic value of cell-free plasma DNA in patients with cardiac arrest outside the hospital: an observational cohort study. Crit Care 2010;14:R47

[67] Liggett T, Melnikov A, Yi QL, et al. Differential methylation of cell-free circulating DNA among patients with pancreatic cancer versus chronic pancreatitis. Cancer 2010;116:1674–80

[68] Kamat AA, Baldwin M, Urbauer D, et al. Plasma cell-free DNA in ovarian cancer: an independent prognostic biomarker. Cancer 2010;116:1918–25

[69] Garcia-Olmo DC, Dominguez C, Garcia-Arranz M, et al. Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. Cancer Res 2010;70:560–7

[70] Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol. 1994 Apr;86(4):774-9

[71] Silva JM, Dominguez G, Garcia JM, Gonzalez R, Villanueva MJ, Navarro F, Provencio M, San Martin S, España P, Bonilla F. Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. Cancer Res. 1999 Jul 1;59(13):3251-6

[72] Sozzi G, Musso K, Ratcliffe C, Goldstraw P, Pierotti MA, Pastorino U. Detection of microsatellite alterations in plasma DNA of non-small cell lung cancer patients: a prospect for early diagnosis. Clin Cancer Res. 1999 Oct;5(10):2689-92

[73] Goessl C, Heicappell R, Münker R, Anker P, Stroun M, Krause H, Müller M, Miller K. Cancer Res. Microsatellite analysis of plasma DNA from patients with clear cell renal carcinoma. 1998 Oct 15;58(20):4728-32

[74] Fujiwara Y, Chi DD, Wang H, Keleman P, Morton DL, Turner R, Hoon DS. Plasma DNA microsatellites as tumor-specific markers and indicators of tumor progression in melanoma patients. Cancer Res. 1999 Apr 1;59(7):1567-71

[75] Stroun M, Anker P, Lyautey J, Lederrey C, Maurice PA. Isolation and characterization of DNA from the plasma of cancer patients. Eur J Cancer Clin Oncol 1987;23:707–12

[76] Schwarzenbach H, Müller V, Stahmann N, Pantel K. Detection and characterization of circulating microsatellite-DNA in blood of patients with breast cancer. Ann N Y Acad Sci. 2004;1022:25-32

[77] Lo YM, Chan LY, Lo KW, Leung SF, Zhang J, Chan AT, Lee JC, Hjelm NM, Johnson PJ, Huang DP. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res. 1999 Mar 15;59(6):1188-91

[78] Lei KI, Chan LY, Chan WY, Johnson PJ, Lo YM. Quantitative analysis of circulating cell-free Epstein-Barr virus (EBV) DNA levels in patients with EBV-associated lymphoid malignancies. Br J Haematol. 2000 Oct; 111(1):239-46

[79] Lo YM, Chan WY, Ng EK, Chan LY, Lai PB, Tam JS, Chung SC. Circulating Epstein-Barr virus DNA in the serum of patients with gastric carcinoma. Clin Cancer Res. 2001 Jul;7(7):1856-9

[80] Widschwendter A, Blassnig A, Wiedemair A, Müller-Holzner E, Müller HM, Marth C. Human papillomavirus DNA in sera of cervical cancer patients as tumor marker. Cancer Lett. 2003 Dec 30;202(2):231-9

[81] Lo YM, Tein MS, Pang CC, Yeung CK, Tong KL, Hjelm NM. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. Lancet. 1998 May 2;351(9112):1329-30

[82] 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 Mar;48(3):421-7

[83] Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. Lancet. Presence of foetal DNA in maternal plasma and serum.1997 Aug 16;350(9076):485-7

[84] Lo YM, Lo ESF, Watson N, Noakes L, Sargent Il, Thilaganthan B, Wainscot Js Two-Way Cell Traffic Between Mother And foetus: Biologic And Clinical Implications. Blood 1996; 88:4390– 5

[85] Lo YM, Tein MSC, Lau TK, Haines CJ, Leung TN, Poon PMK, Et Al. Quantitative Analysis Of Foetal Dna In Maternal Plasma And Serum: Implications For Noninvasive Prenatal Diagnosis. Am J Hum Genet 1998; 62:768–75

[86] Chan KC, Zhang J, Hui AB, Wong N et Al. Size distributions of maternal and foetal DNA in maternal plasma. Clin. Chem. 2004; 50, 88–92

[87] Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N, Soothill PW. Free foetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. Prenat Diagn 2007; 27: 415–8

[88] Hahn S, Ruppertz B, Holzgreve W. Foetal cells and cell free foetal nucleic acids in maternal blood: new tools to study abnormal placentation? Placenta. 2005; 26: 515-26

[89] Flori E, Doray B, Gautier E, Kohler M, Ernault P, Flori J, Costa JM. Circulating cell-free foetal DNA in maternal serum appears to originate from cyto- and syncytio-trophoblastic cells. Case report. Hum Reprod. 2004;19: 723-4

[90] Jimbo M, Sekizawa A, Sugito Y, Matsuoka R, Ichizuka K, Saito H, Okai T. Placenta increta: Postpartum monitoring of plasma cell-free foetal DNA. Clin Chem. 2003; 49: 1540-1

[91] Chan KC, Ding C, Gerovassili A, Yeung SW, Chiu RW, Leung TN, Lau TK, Chim SS, Chung GT, Nicolaides KH, Lo YM. Hypermethylated RASSF1A in maternal plasma: A universal foetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. Clin Chem. 2006;52: 2211-8

[92] Bianchi DW, LeShane ES, Cowan JM. Large amounts of cell-free foetal DNA are present in amniotic fluid. Clin Chem. 2001;47: 1867-9

[93] Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, Demaria MA. Male Foetal Progenitor Cells Persist In Maternal Blood For As Long As 27 Years Postpartum. Proc Natl Acad Sci U S A 1996; 93:705–8

[94] Lo YM, Zhang J, Leung TN, Lau TK, Chang A, Hjelm NM. Rapid Clearance of foetal DNA from maternal plasma. Am J Hum Genet 1999;64:218–24

[95] Bianchi DW. Foetal Cells In The Maternal Circulation: Feasibility For Prenatal Diagnosis. Review. Br J Haematol 1999; 105:574–83

[96] Zhong X Y, Bu¨ Rk M R., Troeger C, Kang A, Holzgreve W, Hahn S. Fluctuation of maternal and foetal free extracellular circulatory DNA in maternal plasma. Obstetrics & Gynecology 2000; 96: 991-6

[97] Wagner J, Dzijan S, Marjanović D, Lauc G. Non-invasive prenatal paternity testing from maternal blood. Int J Legal Med. 2009;123(1):75-9

[98] Vecchione G, Tomaiuolo M, Sarno M, Colaizzo D, Petraroli R, Matteo M, Greco P, Grandone E, Margaglione M. Foetal sex identification in maternal plasma

by means of short tandem repeats on chromosome X. Ann N Y Acad Sci. 2008 Aug;1137:148-56

[99] Ganshirt D, Garritsen H, Miny P, HolzgreveW. Foetal cells in maternal circulation throughout gestation. Lancet 1994;343(8904):1038-9

AIMS OF THE RESEARCH PROJECT

The purpose of this study is to investigate non medical apllications of non invasive prenatal diagnosis and in particular to detective the paternally inherited polymorphism STRs, in a non-invasive approach, using free foetal DNA and rare foetal cells from maternal blood at different gestational ages to perform a non-invasive prenatal paternity testing.

Kinship and parental testing are usually performed by forensic laboratories using commercial kits for STR multiplex analysis. In these tests DNA is extracted from oral swabs or blood samples, but also banked pathological tissues are used for this purpose.

Now a day, for prenatal paternity testing, in particular in case of rape resulting in pregnancy, the analysis could be performed on amniotic fluid or chorionic villi: such samples can be obtained only after the 13^{th} gestational week (GW) and sampling is characterized by invasiveness, with a small but existing risk for both pregnant women and foetuses, as well as stressful for the women, with evident drawbacks. For these reasons the invasive approaches are not acceptable in non-medical purposes such as prenatal paternity testing for forensic aims.

A convenient non-invasive prenatal diagnostic approach has long been sought.

A preliminary ethical evaluation has been performed and proposed.

Our interest in ffDNA and NRBC analysis arises from the consideration that they could offer the possibility to perform non-invasive prenatal paternity testing, a usefull tool in forensic medicine in cases of rape resulting pregnancies.

We proposed a preliminary ethical evaluation.

Our interest in ffDNA and NRBC analysis arises from the consideration that they could offer the possibility to perform non-invasive prenatal paternity testing, a usefull tool in forensic medicine in cases of rape-related pregnancies.

As first approach we considered the opportunity of perform DNA typing in circulating cell free nucleic acids in healthy subjects, in order to propose the use of cfDNA as a tool for forensic analysis.

Then, after approval by the Ethics Committee of the University – Hospital of Padova (No. 2105P) we enrolled a series of couples expecting a baby, with the aim to verify whether foetal polymorphic STR loci covered by NGM™ kit can also be successfully amplified from maternal blood and used for paternity testing.

CHAPTER 1

Non medical applications of non invasive prenatal diagnosis: ethical issues

Paola Tasinato[1], Massimo Montisci[2], Geertruy te Kronnie[1] and Giuseppe Basso

1. Department of Pediatrics, Laboratory of Haematology – Oncology, University of Padova, Italy

2. Department of Enviromental Medicine and Public Health, Legal Medicine, University of Padova, Italy

FORENSIC SCIENCE INTERNATIONAL: GENETICS SUPPLEMENT 2011; SERIES 3:E554-E555

Abstract

Non-invasive prenatal diagnosis (NIPD) is becoming increasingly important and its application in prenatal diagnosis is reaching consensus in the scientific research community. We discuss the opportunities and ethics of non-invasive prenatal testing for non-medical purposes, including forensic genetics. A number of ethical issues arise from non-medical applications of NIPD, such as sex determination and paternity testing in earlier gestational age and subsequent offspring selection. NIPD provides a source of information about the genetic make-up of the foetus, avoiding the small but significant risk of pregnancy loss related to invasive testing such as amniocentesis or chorionic villi sampling. NIPD is characterized by: safety, early detection and easy sampling. These features of NIPD increase the opportunity of prenatal testing also for non-medical reasons. Even if NIPD can be qualified as a good practice prenatal diagnosis tout court remains a topic of ethical judgements. The non-medical use of NIPD will benefit from an informed and open debate involving both pregnant women and physicians.

Introduction

The lack of risk of NIPD both for pregnant women and foetuses enhances the opportunity to use this technology for non-medical applications. The most evident non-medical issues addressed by NIPD are sex determination and paternity testing concerning potential pregnancy termination and illegal abortion [1].

Invasive – non-invasive access to foetal material

The invasiveness of foetal sampling through amniocentesis or chorionic villi sampling (CVS) carries a small but significant risk of pregnancy loss. CVS and amniocentesis cause miscarriage in around 0.3–2% of cases with a slightly higher risk for CVS [2–5]. Even if wide consensus supports the application of CVS and amniocentesis for prenatal diagnosis of important diseases a convenient non-invasive prenatal diagnostic approach that could eliminate the risk of pregnancy loss has longbeen sought. During pregnancy, the placental membranes separate the foetal and maternal circulations. However, a number of evidences points toward the incompleteness of this barrier for cellular [6] and free foetal DNA (ffDNA) trafficking [7]. Detection of ffDNA in samples of pregnant woman can be exploit for NIPD.

Medical use of NIPD through ff-DNA

To date NIPD through ffDNA is used for a number of clinical applications involving both the foetus and the pregnant woman, including for the foetus sex determination, single gene disorder detection and detection of aneuploidy and for the pregnant woman detection of elevated concentrations of ffDNA predictive for preeclampsia, hyperemesis gravidarum and pre-term delivery or other pregnancy-related disorders such as Rhesus incompatibility.

Non-medical use of NIPD: state of the art

The two major non-clinical applications of NIPD are sex determination and paternity testing. Foetal gender determination was the first studied application of NIPD [8, 9]. Several Y chromosome specific sequences have been investigated for sex determination using real-time polymerase chain reaction (PCR) [10 – 12] in

particular the SRY gene [13, 14] and the multicopy DYS14 sequence located within the TSPY [15]. Female foetuses have been directly identified through detection of paternally inherited X chromosome specific short tandem repeats (STRs) in pregnant blood [16].

Concerning prenatal paternity testing for forensic purposes, to our knowledge no groups are currently performing complete paternity testing on maternal plasma according to guidelines as required in forensic DNA testing.

The only locus that reliably amplified with AmpFLSTR Identifiler kit (Applied Biosystems) is Amelogenin revealing only foetal gender, whereas the amplification of the autosomal loci is only sporadically found. More success is obtained with AmpFLSTR Yfiler kit (Applied Biosystems), which, in case of male foetuses, successfully amplifies between 6 and 16 foetal loci [17].

NIPD in general

Generally speaking NIPD offers opportunities with specific ethical qualities. Firstly, safety that makes NIPD a more suitable approach also for women in non-risk pregnancies; secondly early detection which means longer time for parent decision-making about the progress of pregnancy. Last but not least NIPD is characterized by easy sampling using a simple blood drawing: this in itself favourable feature introduces the risk of a poorly informed choice by the pregnant woman, resulting in a lack of true consent. All these features together (safety; early detection and easy sampling) ease of access of non-invasive prenatal testing also for non-medical reasons.

Sex selection

Sex determination is the more suitable application of cff DNA: it can be clinically relevant in cases of sex-linked diseases of the foetus, but it can also be used for non-clinical motivated sex selection.

In the last years questions are arising within the ethical debate regarding to use of these new reproductive technologies for selecting the sex of one's child before implantation [18]. According to Italian law (L. 40/2004) the selection of embryos on the basis of sex is not permitted, but for the interruption of a pregnancy after

embryo transfer – in case of in vitro fertilization – (before the 90th day according to Italian law L. 194/1978) it is permitted for personal maternal reasons, and could include sex selection. The latter choice needs to be valued as a reproductive autonomy of the pregnant woman.

The main concern is the selection of male foetuses following to the preference in some societies of giving birth to a boy over a girl such as in China [19] and in India [20]: this discrimination of women constitutes an injustice that should be prevented [21]. Moreover, large-scale execution of male selection could undermine a public good reinforcing a bias in the ratio between men and women [22].

Prenatal paternity testing

Although to date non-invasive prenatal paternity testing (NIPPT) is not yet performed with accuracy and reliability required in forensic kinship parentage testing improvement of technologies are expected to make quality-certified NIPPT available in the near future.

We can identify three major non-medical applications of NIPPT including: surveying the perpetrator in pregnancy as the result of a rape, unsure paternity in case of women with multiple partners, unsure paternity in case of suspect of erroneous embryo transfer.

In the first case, paternity testing is neither questioned nor disputed. It serves forensic requirements and allows interruption of pregnancy if required by the pregnant woman, even if this might raise religious objections.

Concerning ambiguous paternity in case of women with multiple partners some additional considerations need to be made. Refusing to perform paternity testing in these cases is clearly moralistic and unprofessional: such inference in the life of women reflects an illicit sentence of licentious life, which is not the assignment of a physician. The denial of access to NIPPT could have other ethically non-acceptable implications for the woman involved. A pregnant woman in case of ambiguous paternity could decide to terminate the pregnancy anyhow, or on the other hand decide to continue pregnancy with remaining uncertainty about the kinship. Another questionable implication of this position favours the expansion of services or laboratories that provide NIPPT without legal, deontological or ethical guarantees. Paternity testing typically postnatal paternity testing is performed by private or and

academic laboratories, after direct contact with the applicants. Even if also paternal consent in postnatal paternity testing is required a number of laboratories offer via the wwweb paternity testing without any guarantee regarding consent and privacy [23]. This practice is likely to extend to prenatal paternity testing in a near future. A long this line NIPPT will become available through the wwweb and with the risk of lesion of privacy and consent rights with consequent denial of autonomy and responsibility of involved subjects.

For these reasons we retain that empirical research into pregnant women's attitudes, needs and preferences are necessary for the debate and decision-making process.

REFERENCES

[1] Smith RP, Lombaard H, Soothill PW. The obstetrician's view: ethical and societal implications of non-invasive prenatal diagnosis, Prenat. Diagn. 2006;26:631–634

[2] Mujezinovic F, Alfirevic Z. Procedure-related compilations of amniocentesis and chorionic villous sampling: a systematic review, Obstet. Gynecol. 2007;110:687–694

[3] Nanal R, Kyle P, Soothill PW. A classification of pregnancy losses after invasive prenatal diagnostic procedures: an approach to allow comparison of units with a different case mixm, Prenat. Diagn. 2003;23; 488–492

[4] Bui TH, Meiner V. State of the art in prenatal diagnosis, in: M. Leuzinger-Bohleber, E.-M. Engels, J. Tsiantis (Eds.). The Janus Face of Prenatal Diagnostics. A European Study Bridging Ethics, Psycholanalysis, and Medicine, Karnac Books, London, 2008, 61–86

[5] Saller DN, Canick JA. Current methods of prenatal screening for Down Syndrome and other foetal abnormalities, Clin. Obstet. Gynecol. 2008;51: 24–36

[6] Lo Y, Lo E, Watson N, Noakes L, Sargent II, Thilaganthan B, Wainscot JS. Twoway cell traffic between mother and foetus: biologic and clinical implication, Blood 1996;88: 4390–5

[7] Lo Y, Corbetta N, Chamberlain PF, Rai V, Sargent II, Redman CVG, Wainscoat JS. Presence of foetal DNA in maternal plasma and serum, Lancet 350 (1997) 485–7

[8] Lo Y, Tein M, Lau TK, Haines CJ, Leung TN, Poon P et al. Quantitative analysis of foetal DNA in maternal plasma and serum: implications for non-invasive prenatal diagnosis. Am. J. Hum. Genet. 1998;62: 768–75

[9] Sekizawa A, Kondo T, Iwasaki M, Watanabe A, Jimbo AM, Saito H, Okai T. Accuracy of foetal gender determination by analysis of DNA in maternal plasma, Clin. Chem. 2001;47: 1856–8

[10] Zhong XY, Laivouri H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W, Hahn S. Elevation of both maternal and foetal extracellular circulating deoxyribonucleic acid concentration in the plasma of pregnant women carrying foetus with preeclampsia, Am. J. Obstet. Gynecol. 2001;84: 414–9

[11] Zimmermann B, El-Sheikhah A, Nicolaides K, Holzgreve W, Hahn S. Optimized real-time quantitative PCR measurement of male foetal DNA in maternal plasma, Clin. Chem. 2005;51:1598–1604

[12] Gerovassili A, Garner C, Nicolaides KH, Lay Thein S, Rees DC. Free foetal DNA in maternal circulation: a potential prognostic marker for chromosomal abnormalities? Prenat. Diagn. 2007;27: 104–10

[13] Lo Y, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, Poon PM, Redman CW, Wainscoat JS. Prenatal diagnosis of foetal RhD status by molecular analysis of maternal plasma, N. Engl. J. Med. 1998;339: 1734–8

[14] Hromadnikova I, Houbova B, Hridelova D, Voslarova S, Kofer J, Komrska K, Habart D. Replicate real-time PCR testing of DNA in maternal plasma increases the sensitivity of non-invasive foetal sex determination, Prenat. Diagn. 2003;23: 235–8

[15] Picchiassi E, Coata G, Fanetti A, Centra M, Pennacchi L, Di Renzo GC. The best approach for early prediction of foetal gender by using free foetal DNA from maternal plasma, Prenat. Diagn. 2008;28: 525–30

[16] Tang NL, Leung TN, Zhang J, Lau TK, Lo YM. Detection of foetal-derived paternally inherited X-chromosome polymorphisms in maternal plasma, Clin. Chem. 1999;45: 2033–5

[17] Wagner J, Dzijan S, Marjanovic´ D, Lauc G. Non-invasive prenatal paternity testing from maternal blood, Int. J. Legal Med. 2009;123: 75–9

[18] Hall A, Bostanci A, CF Wright. Non-invasive prenatal diagnosis using cell free foetal DNA technology: applications and implications, Public Health Genomics 2010;13: 246–55

[19] Liao SM. The ethics of using genetic engineering for sex selection, J. Med. Ethics 2005;31: 116–8

[20] Chan CL, Blyth E, Chan CH. Attitudes to and practices regarding sex selection in China, Prenat. Diagn. 2006;26: 610–3

[21] George SM. Millions of missing girls: from foetal sexing to high technology sex selection in India, Prenat. Diagn. 2006;26: 604-9

[22] Toebes B. Sex selection under international human rights law, Med Law Int. 2008;9: 197–225

[23] Pennings G, de Wert G. Evolving ethics in medically assisted reproduction. Hum. Reprod. Update 2003;9: 397–404

CHAPTER 2

Use of plasma DNA in human identification

Paola Tasinato[1], Elena Seganfreddo[1], Massimo Montisci[2], Geertruy te Kronnie[1] and Giuseppe Basso[1]

1. Department of Women's and Children's Health, University of Padova, Italy

2. Department of Enviromental Medicine and Public Health, Section of Legal Medicine, University of Padova, Italy

JOURNAL OF FORENSIC SCIENCES

SUBMITTED

Abstract

Circulating cell free nucleic acids have been detected in peripheral blood specimens of healthy subjects as well as in patients. In recent years applications that analyze circulating cell free DNA (ccfDNA) have increased in particular for non-invasive diagnosis such as early tumor detection and non-invasive prenatal diagnosis.

With the aim to test the applicability of ccfDNA analysis in human identification, we verified short tandem repeats (STRs) PCR amplification in extracellular circulating DNA. We report complete concordance between amplified alleles from the cell free fractions and the matched DNA samples from whole blood, demonstrating that ccfDNA can be exploited for identification purposes.

In forensic sciences cell free blood samples are not used for human profiling. Here we propose that plasma can be considered a useful matrix in cases, such as unambiguous sample tracking in diagnostic routine, in the coming age of non invasive genetic prenatal diagnosis or in contested cases of toxicological analysis.

Introduction

Studies of circulating nucleic acids promise exciting developments in molecular diagnostics in the years to come.

Literature reported only two attempts investigating human identification through plasma DNA: a case report investigating three short tandem repeats (STRs) without validation for forensic purposes [1] and a more recent study [2] that however did not report results on plasma samples, even if plasma samples were mentioned in the introduction and material and methods paragraphs.

So, until now, to our knowledge no data have been published of successful studies that investigated human identification using an established panel of polymorphic STR loci starting from samples of cell free plasma that were also validated on matched cellular samples.

We embarked on a study aiming to determine whether extracellular DNA (i.e. cell free DNA, cfDNA) present in plasma can be used to support human genetic identification by STR analysis.

Materials and methods

1. Ethics

The study was approved by the ethics committee of the University - Hospital, Padova, Italy (No. 2105P). Consent was obtained from all subjects. The study protocol was conform the guidelines of the "World Medical Association Declaration of Helsinki—Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo, Japan, 2004.

2. Samples collection

A flexible 18 G or 20 G, 1¼ Teflon catheter (Angiocath, Becton Dickinson, UT) was inserted in a superficial arm vein to obtain 2 ml of venous blood in EDTA from 10 men and 10 non-pregnant women (all healthly volunteers). Specimens were numbered for anonymous work-up. Age ranged from 26 to 33 years and women included in the study disclosed no previous pregnancies.

1 ml of whole blood was centrifuged at 4°C for 10 min 1000 g (Heraeus Fresco 21 Centrifuge, Thermo Electron Corporation, Karlsruhe, DE) within 1 hours from the blood drawing. The clear supernatant obtained after centrifugation was transferred to a new 2,5 ml micro tube, re-centrifuged at 4°C for 15 minutes at 2,000x g to eliminate platelets in the plasma sample. There after all cells were removed and approximately 600ul of plasma was obtained. Three 15ul aliquots of each plasma sample were microscopically inspected and absence of cells was confirmed.

TE buffer (10 mM Tris, 0,1 mM EDTA, pH 8,0) was used as blank of extraction every 5th samples.

3. DNA preparation and quantification

ccfDNA was extracted in two replicates from 200 ul of plasma and in four negative controls (TE buffer) using a QIAamp Mini blood kit (Quiagen, Valencia, CA) according to the manufacturer's protocol DNA purification from blood or body fluids (Spin protocol). The final elution volume was 100 ul. The eluted DNA were combined, obtaining 200 ul of eluted DNA for each subject. CcfDNA obtained was stored at -20°C until further use.

DNA Quantification was performed by real-time PCR (RT-PCR) using the relative quantification with a standard curve method. Albumin (gene) was used as reference (FW 5' TGAAACATACGTTCCCAAAGAGTTT '3, RW 5' CTCTTCTCAGAAAGTGTGCATAT '3 probe FAM-TAMRA 5' TGCTGAAACATTCACCTTCCATGCAC '3). The Albumin amplicon sequence measured 78 bp and was monitored on an ABI7900 sequence detection system using SDS Software v 1.2..

As profile control, 100 ul of DNA from 200 ul for each whole blood sample was obtained with the same extraction protocol.

4. DNA profiling

Genetic human identification analysis was performed by simultaneous PCR amplification of fifteen DNA STR autosomal loci and a sex determination marker, amelogenin, using the commercial kit AmpFl STR NGM® (AB). Autosomal STR loci (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433,

TH01, FGA, D10S1248, D22S1045, D2S441, D1S1656 & D12S391) and the specific sex STR locus were amplified from the extracted ccfDNA and DNA: the 29-cycle amplification was executed according to the original manufacturer's protocol in a 25ul final reaction volume, consisting of 10 ul SGM plus reaction mix, 5ul NGM primer set and 10ul of ccfDNA at concentrations ranging from 0,08 to 0,1 ng/ul as measured by RT-PCR or 10ul of DNA at 0.1ng/ul concentration.

The positive controls were represented by the human Control DNA 007 (0,1 ng/ul) provided by the manufacturer's kit AmpFl STR NGM®. The negative controls were represented by the TE Buffer samples previously extracted.

All amplifications were performed on DNA thermal cyclers GeneAmp PCR System 9700 (AB).

Amplified fragments were resolved on a ABI Prism 310 Genetic Analyzer (AB) and genotyping was carried out using GenemapperID v3.2 software (AB).

Electrophoresis was run using a 50 cm capillary and POP-4 polymer (AB) by mixing 1,5 ul of the PCR product with 24,25 ul Hi-DiTM Formamide (AB) and 0,75 ul of GeneScan 500® LIZ Size Standard (AB). The mix was denatured at 95°C for 3 minutes, snap-cooled, and kept in ice water for 3 minutes until used for electrophoresis. For each run the identity of each allele was determined by comparison to the allelic ladder processed in parallel to samples.

To distinguish allele peaks from technical artefacts and background noise a threshold was set at 50 relative fluorescence units (RFUs) [3, 4].

For all cases, the STR profile generated from ccfDNA was compared to the matching STR profile generated by DNA obtained from whole blood.

Samples presenting artifactual peaks (i.e. stutter, pull-up from another dye) were re-amplified and reanalyzed to obtain high quality reads for allele genotyping.

Results and discussion

Cell free nucleic acids (cfNA) is defined as extracellular DNA occurring in blood [5]. It is normally occurring in healthy people as well as in patients suffering from cancer [6] or other destructive diseases or physiological conditions such as pregnancy [7].

In 1947, when the presence of circulating cell free nucleic acids in human plasma and serum was first described this raised little interest [8]. Nowadays, with the increasing demand for non-invasive tests for molecular markers, cfDNA analysis is considered an important opportunity [9]. The progress in diagnostic applications of ccfDNA has been reported for uses ranging from pathological to physiological events, from cancer biomarkers, prognostic marker in trauma patients, and transplant follow-up to genetic prenatal diagnosis. In particular, non-invasive prenatal diagnosis (NIPD) through cell-free foetal DNA (ffDNA) is becoming increasingly important and its applications in prenatal diagnosis is reaching consensus in the scientific research community also for non medical applications, such as forensic ones [10].

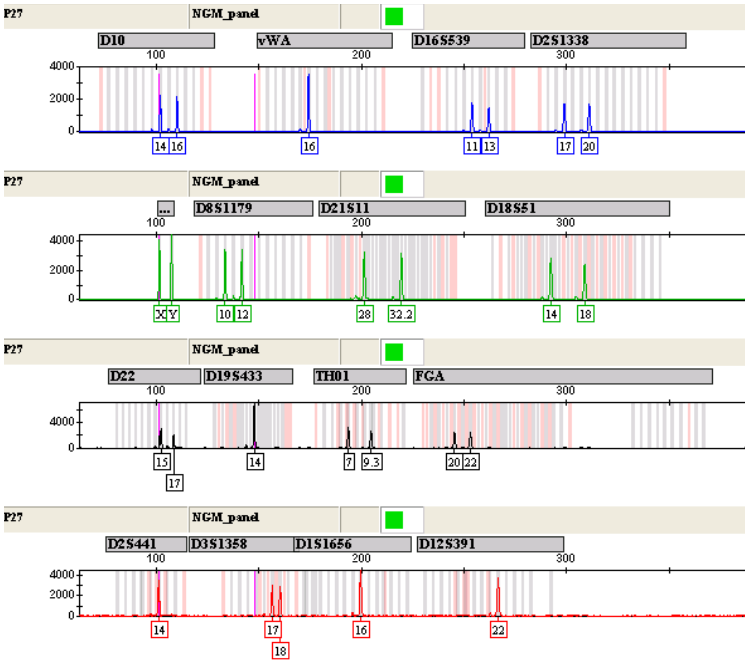
Current evidences suggest that in healthy subjects cellular apoptosis, in the largest part of haematopoietic cells [11-12] contributes to cfDNA found in plasma and serum. However, in sick subjects additional cell origins and mechanisms of release seem to play a role [13] such as lyses of circulating tumour cells [14] or even active release from lymphocytes [15].

It has been shown that cfDNA has lower molecular weights than DNA derived from cellular blood or tissues: cfDNA of cancer origin is present in fragments which are multiples of 180 bp by gel electrophoresis [16], while in the circulation of pregnant women 86% of DNA of fetal origin is smaller than 201 bp [17].

The Qiagen DNA extraction method resulted in 1,1 ± 0,55 ng/ul cfDNA and of 78 ± 13 ng/ul DNA from whole blood using RT PCR measurements. All blank extracted samples failed to produce any amplification.

The STR amplification performed in order to obtain the profiles from cfDNA showed good signal strength, low background, and accurate allele identification. We reported that each of the amplified STR alleles from free DNA matched to the profiles obtained from genomic double stranded DNA from whole blood (Figure 1). Only differences in Relative Fluorescence Units (RFUs) were observed, being lower in cfDNA specimens in all cases.

a



b

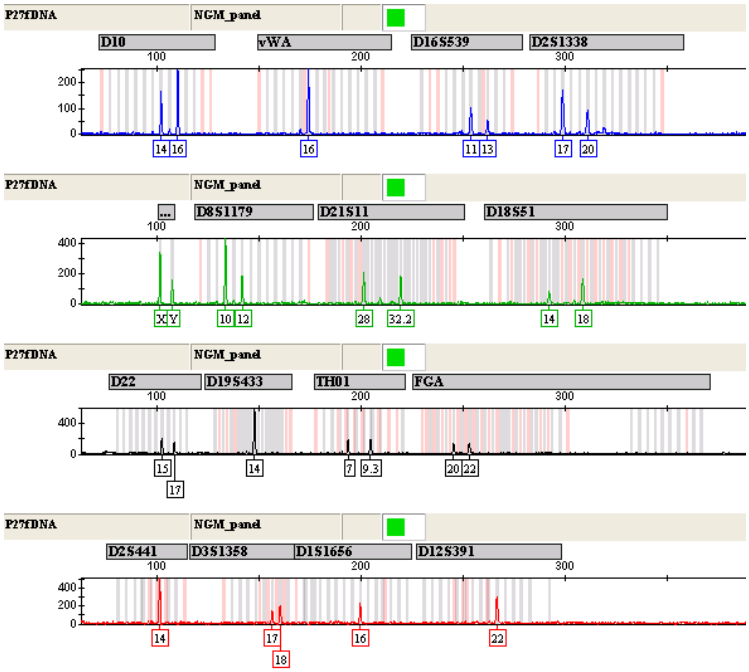


Figure 1. STRs profiles of DNA from whole blood (a) and of cfDNA of the same subject (b)

The reduced height of the RFUs in the profiles from cfDNA was attributed to diminished performance of the multiplex PCR due to a naturally occurring DNA fragmentation and or degradation in plasma, with reduced primer sites for amplification.

In the past, due to low concentrations in specimens of healthy persons cfDNA could not be detected with the limited analytical sensitivity of the methods at that time [18]. This is the reason of the erroneous conclusion that cfDNA was associated with pathological conditions only. The development and availability of new techniques improved sensitivity and resulted in detection also in healthy subjects [19].

We chose to use the Qiagen method of DNA extraction specific for plasma, and in particular we took care to process blood samples within 1 hour of drawing: it is known that increasing concentrations of cfDNA are associated with longer time of blood storage at room temperature, particular when plasma is separated after 24 or 48 hours of drawing [20].

In conclusion, even if use of ccfDNA analysis is increasing, to the best of our knowledge no data are available concerning successful performance of STR amplification for identification profiling using cell free plasma as the matrix for DNA extraction.

We report, that ccfDNA from plasma is present in sufficient quantity and quality to perform human forensic STR profiling: we demonstrated the possibility of attributing identity to equivocal individual plasma samples.

We propose that plasma can be considered a useful matrix in cases of forensic interest where human identification is requested. We think of the following situations: (1) in case of a misattributed plasma sample in the era of a diagnostic test from plasma, especially in oncology, (2) on plasma samples routinely used in monitoring infectious diseases, and (3) in contested cases of toxicological analysis on plasma of forensic interest.

References

[1] Tamaki Y, Fukuda M, Kishida T. STR typing of plasma DNA in a deficiency case of disputed maternity against a patient dying in the hospital. Nihon Hoigaku Zasshi 1996;50(6):427-9

[2] Glock B, Reisacher RBK, Rennhofer SO, D Troscher, Dauber EM, Mayr WR. Evaluation of Powerplex 16 for typing of degraded DNA samples. International Congress Series 1239 (2003) 609-11

[3] Mulero JJ, Chang CW, Lagacé RE, Wang DY, Bas JL, McMahon TP, LK Hennessy. Development and validation of the AmpFlSTR MiniFiler PCR Amplification Kit: a MiniSTR multiplex for the analysis of degraded and/or PCR inhibited DNA. J Forensic Sci 2008;53(4):838-52

[4] Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. J Forensic Sci 2000;112(1):17-40

[5] Jung K, Fleischhacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker--a critical appraisal of the literature. Clin Chim Acta. 2010;411(21-22):1611-24

[6] Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol 1994; 86: 774–9

[7] Lo YM, Corbetta N, Chamberlain PF, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997; 350: 485–7

[8] Mandel P, Métais P. Les acides nucléiques du plasma sanguin chez l'homme. C. R. Acad. Sci. Paris 1948;142: 241-3

[9] Tsang JC, Lo YM. Circulating nucleic acids in plasma/serum. Pathology. 2007; 39(2):197-207

[10] Tasinato P, Montisci M, te Kronnie G, Basso G. Non-medical applications of non-invasive prenatal diagnosis: Ethical issue, Forensic Science International: Genetics Supplement Series 2011;3:e554–5

[11] Lui YY, Chik KW, Chiu RWK, Ho CY, Lam CW, Lo YMD. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem 2002;48:421–27

[12] Jahr S, Hentze H, Englisch H, Hardt D, Fackelmayer FO, Hesch,RD et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61:1659 – 65

[13] Ziegler A, Zangemeister-Wittke U, Stahel RA. Circulating DNA: a new diagnostic gold mine? Cancer Treatment Reviews 2002;28:255–71

[14] Sorenson GD. Detection of mutated KRAS2 sequences as tumor markers in plasma/serum of patients with gastrointestinal cancer. Clin Cancer Res 2000;6:2129–37

[15] Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. Clin Chim Acta 2001;313:139–42

[16] Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch HR, Knippers R. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61:1659–65

[17] Chan KC, Zhang J, Hui AB, Wong N, Lau TK, Leung TN, Lo KW, Huang DW, Lo YM. Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem 2004;50:88–92

[18] Kamm RC, Smith AG. Nucleic acid concentrations in normal human plasma. Clin Chem 1972; 8:519-22

[19] Jung K, Fleischhacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker - a critical appraisal of the literature. Clin Chim Acta 2010;411(21-22):1611-24

[20] Albano MS, Scaradavou A, Stevens CE, Rubistein P. Extracellular DNA in cord blood plasma and applications in cord blood banking for sample identification. Transfusion 2009;49:1685-91

CHAPTER 3

Circulating free foetal DNA in pregnant circulation: STRs amplification for non-invasive prenatal paternity testing

Paola Tasinato[1], Elena Seganfreddo[1], Massimo Montisci[2], Santo Davide Ferrara[2], Giuseppe Basso[1] and Geertruy te Kronnie[1]

1. Department of Women's and Children's Health, University of Padova, Italy

2. Department of Molecular Medicine, Section of Legal Medicine, University of Padova, Italy

Abstract

Molecular analysis of plasma DNA during human pregnancies has led to the discovery that maternal plasma contains both foetal and maternal DNA. Non-invasive prenatal diagnosis is changing the landscape of prenatal testing: tests performed on free foetal DNA are already proven for foetal gender, rhesus D blood type and some Mendelian conditions (achondroplasia).

The purpose of this study is to perform non-invasive prenatal paternity testing monitoring paternally inherited polymorphism STRs of forensic use on free foetal DNA from pregnant peripheral blood.

Non-invasive approaches for paternity testing for forensic purposes are of increasing importance, such as in cases of rape resulting in pregnancy.

Introduction

Circulating cell free nucleic acids have been detected in peripheral blood specimens of healthy subjects as well as in patients.

Previously we demonstrated the applicability of cfDNA analysis in human identification, verifying short tandem repeats (STRs) PCR amplification in extracellular circulating DNA for human identification [1].

Kinship and parental tests are usually performed by forensic laboratories using commercial kits for STR multiplex analysis. In these tests DNA is extracted from oral swabs or blood samples, but also banked pathological tissues may be used for this purpose [2].

For prenatal paternity testing, in particular in case of rape resulting in pregnancy, the analysis could be performed on amniotic fluid or chorionic villi: however, (1) such samples can be obtained only after the 13^{th} gestational week (GW) and (2) sampling is characterized by invasiveness, with a small but existing risk for both the pregnant woman and her foetus, as well as being stressful for the woman, with evident drawbacks.

As these procedures carry a small but significant risk of pregnancy loss, a convenient non-invasive prenatal diagnostic approach has long been sought.

With the aim to verify whether plasma DNA of pregnant origin can be used as matrix for prenatal paternity testing by amplification of STR loci used in forensic human identification we analyzed plasma DNA of 26 pregnant women and compared their STR profiles with the STR profiles of putative biological fathers of their children.

Materials and methods

Ethics

The study has been approved by the Ethics Committee of the University - Hospital, Padova, Italy (No. 2105P) and consent to participate in the study was obtained from all subjects. The study protocol was conform the guidelines of the "World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo, Japan, 2004.

In order to obtain written consent from the couple pregnant women received counselling after which her approval of informed consent in all instances followed by that of her partner. During the preliminary counselling sessions it has been explained that the results of this research project would not become available neither to the pregnant woman nor to the couple.

Samples collection

Inclusion criteria: singleton pregnancy as determined by US examination and known gestational age.

Exclusion criteria: fertilization in vitro embryo transfer (FIVET) pregnancy, previous or current hematologic or lymphatic diseases, foetal congenital anomalies, vaginal bleeding during pregnancy.

Gestational age was in all cases determined by US before GW 15. The samples from the pregnant women were collected at 3 gestational time windows when the women come to the clinic for the US control:

- range from 6 weeks to 13,
- range from 14 weeks to 27,
- range from 28 weeks to 40.

Maternal blood will be used immediately after sampling. Post delivery cord blood or excess material from chorionic villi will be used for the preparation of foetal genomic DNA. All these materials will be stored at -70°C prior to DNA isolation.

Plasma was obtained from peripheral blood of 26 pregnant women at different gestational ages. Specimens were numbered for anonymous work-up.

A volume of 2 ml of whole blood was centrifuged at 4°C for 10 min at 1000x g (Heraeus Fresco 21 Centrifuge, Thermo Electron Corporation, Karlsruhe, DE) within one hour from drawing. The clear supernatant obtained after centrifugation was transferred to a new 2,5 ml micro tube, re-centrifuged at 4°C for 15 minutes at 2,000x g to eliminate platelets in the plasma sample. There after all cells were removed and approximately 600ul of plasma was obtained. Three 15ul aliquots of each plasma sample were microscopically inspected and absence of cells was confirmed.

TE buffer (10 mM Tris, 0,1 mM EDTA, pH 8,0) was used as blank of extraction every 5th samples. 1 mL of whole blood was drawn from the putative fathers.

DNA preparation and quantification

cfDNA was extracted in two replicates from 200 ul of plasma with four negative controls (TE buffer) using a QIAamp Mini blood kit (Quiagen, Valencia, CA) according to the manufacturer's protocol *DNA purification from blood or body fluids (Spin protocol)*. The final elution volume was 100 ul. The eluted DNA samples from the same starting sample were combined, obtaining 200 ul of eluted DNA from each subject. cfDNA was stored at -20°C until further use.

DNA Quantification was performed by real-time PCR (RT-PCR) using the relative quantification with a standard curve method. Albumin (gene) was used as reference and monitored on an ABI7900 sequence detection system using SDS Software v 1.2..

As control profile of pregnant women, their partner and baby, 100 ul of DNA from 200 ul for whole blood sample or cord blood was obtained following the same extraction protocol.

DNA profiling

Genetic human identification analysis was performed by simultaneous PCR amplification of fifteen DNA STR autosomal loci and a sex determination marker, Amelogenin, covered by the commercial kit AmpFl STR NGM® (AB). Autosomal STR loci (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, FGA, D10S1248, D22S1045, D2S441, D1S1656 & D12S391) and the sex specific STR locus were amplified from the extracted ccfDNA and DNA: the 29-cycle amplification was executed according to the original manufacturer's protocol in a final reaction volume of 25 uL, composed of 10 ul SGM plus reaction mix, 5ul NGM primer set and 10ul of ccfDNA at concentrations ranging from 0,08 to 0,1 ng/ul as measured by RT-PCR or 10ul of DNA at 0.1ng/ul concentration. Amplified fragments were resolved on an ABI Prism 310 Genetic Analyzer (AB) and genotyping was carried out using GenemapperID v3.2 software (AB). To distinguish allele peaks from technical artefacts and background noise a threshold was set at 50 relative fluorescence units (RFUs) [3, 4].

For all cases, the STR profile generated from ccfDNA was compared to the matching STR profile generated by DNA obtained from cord blood of the newborn and whole blood of the putative father.

Results and discussion

Five percent of sexual assault crimes results in rape related pregnancy [5]. Up to day, prenatal paternity testing that could represent a guilt test, was only available from genetic analysis of material obtained through invasive sampling of chorionic villi and amniocentesis. The latter are both associated with a small but existing risk for both mother and child, that cannot justify the forensic aim of the exams [6].

To our knowledge previous attempts based on cell-free foetal DNA demonstrated suppressed amplification of short tandem repeats (STR) of foetal alleles due to predominance of cell-free maternal DNA in these samples [7]. Here we performed an attempt to amplifying foetal alleles from pregnant women using the commercial kit AmpFl STR NGM® (AB), different form the protocols used by others. The above kit employs shorter STR amplicons, which is expected to improve amplification of ff-DNA fragments. Recently, it has been shown that ff-DNA fragments in maternal plasma are smaller than mf-DNA [8].

We extracted DNA from 200 ul of 55 maternal plasma samples and successfully amplified all maternal STR loci covered by the commercial kit used and validated in forensic casework. The literature reports [7] that Y-chromosome specific amelogenin in cases of male foetuses was amplified only sporadically. For the amplicon sequence we were not able to reproduce the results previously published by Wagner et al. with the AmpFLSTR Identifier kit.

We consider two possible reasons for the failed amplification: first, the excess of plasma DNA of maternal origin suppresses the primer binding to the foetal plasma DNA, and second, the smaller length of the foetal plasma DNA can reduce the probability of primer binding.

Conclusions

Classical methods based on capillary electrophoresis that are used in forensic genetics for human identification through polymorphic STRs loci amplification can not be considered a useful approach to investigate foetal alleles on maternal plasma specimens during pregnancy because of primer binding suppression due to the excess of the pregnant allele fractions and due to the smaller size of the foetal fraction. Our results demonstrated that using smaller amplicon sizes there was no improvement of the multiplex reaction.

Finally, we retain that free foetal DNA analysis for forensic purposes needs to be investigated using other approaches.

References

[1] Tasinato P, Seganfreddo E, Montisci M, Ferrara SD, Basso G, te Kronnie G. Use of Plasma DNA in human identification. J For Sci, submitted 2012

[2] Pelotti S, Ceccardi S, Alù M, Lugaresi F, Trane R, Falconi M, Bini C, Cicognani A. Cancerous tissues in forensic genetic analysis. Genet Test. 2007 Winter;11(4):397-400

[3] Mulero JJ, Chang CW, Lagacé RE, Wang DY, Bas JL, McMahon TP, LK Hennessy. Development and validation of the AmpFlSTR MiniFiler PCR Amplification Kit: a MiniSTR multiplex for the analysis of degraded and/or PCR inhibited DNA. J Forensic Sci 2008;53(4):838-52

[4] Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. J Forensic Sci 2000;112(1):17-40

[5] Holmes MM, Resnick HS, Kilpatrick DG, Best cl. Rape-related pregnancy" Estimates and descriptive characteristics from a national sample of women. Am J Obstet Gynecol 1996; 175: 320-4

[6] Tasinato P, Montisci M, te Kronnie G, Basso G. Non-medical applications of non-invasive prenatal diagnosis: Ethical issue, Forensic Science International: Genetics Supplement Series 2011;3:e554–5

[7] Wagner J, Dzigan S, Marjanovic D, Lauc G. Non-invasive prenatal paternity testing from maternal blood. Int J Legal Med 2009;123:75-9

[8] Chan, K. C., Zhang, J., Hui, A. B., Wong, N. Et Al. Size distributions of maternal and foetal DNA in maternal plasma. Clin. Chem. 2004; 50, 88–92

CHAPTER 4

A deep sequencing investigation of VWA, D16S1156, AME and D8S1179 on cell free DNA of a mother's to be blood

Paola Tasinato, Silvia Bresolin, Giuseppe Basso and Geertruy te Kronnie

Department of Women's and Children's Health,
University of Padova, Italy

Abstract

The profiling of short tandem repeat (STR) loci is currently used in forensic genetics for identification purposes.

We recently demonstrated the possibility to perform DNA profiling for identification purposes on plasma DNA also named cell free DNA.

Our attempt to investigate foetal STR profiles from plasma DNA of pregnant women, carrying also a small proportion of cell free foetal DNA, with traditional STRs amplification and fragments length separation by capillary electrophoresis (CE) failled. Recently, it has been demonstrated that pyrosequencing has exceeding capacity of data generation and discriminatory power [1].

Here we embarked on a journey of Next Generation Sequencing (NGS) on a DNA specimen from plasma of a pregnant woman. This new approach represents an STR profiling method based on the use of Roche Genome Sequencer Junior 454 Life Science Titanium simultaneously sequencing four STR loci of forensic interest. We found that 454 STR sequence data out put is composed of maternal and foetal STR sequences.

Introduction

Forensic DNA profiling is mainly based on the analysis of STR loci. They are typed by DNA fragment analysis using PCR-based forensic kits and multicolor fluorescent CE [2]. STRs investigated are correlated to a well-defined allelic ladder repeat number; however, occasional variant alleles are observed: so-called "off-ladder" variant alleles. In any case, analysis of the lengths of the PCR amplicons does not reveal information on small fractions of contaminating DNA or sequence variations, overlooking important sub-information.

Recently, for research purposes sequencing methods based on clonal amplification by emulsion polymerase chain reaction (emPCR) followed by pyrosequencing have been investigated [3].

The pyrosequencing technology is based on detection of pyrophosphate (PPi) released as a result of dNTP incorporation. Nucleotides are added stepwise and if incorporated, PPi is released and used as a substrate for ATP sulfurylase forming ATP. Light is emitted when luciferase uses ATP to convert luciferin to its oxi-derivative. Any excess of dNTPs is degraded by apyrase prior to addition of a new nucleotide. The out-put sequence is shown in a pyrogram where peak heights are proportional to the number of incorporated nucleotides [4]. Pyrosequencing has been used for a variety of applications, mutation detection of disease associated genes above all.

Here, we sequenced for the first time four STR loci (Amelogenin, VWA, D8S1179 and D1S1656) using the Genome Sequencer (GS) Junior 454 platform on plasma DNA of a pregnant woman and DNA from blood of her partner. We examined the feasibility of applying Roche GS 454 to STR typing for the purpose of forensic genetic investigation by directly comparing sequence data generated on the GS 454 platform to conventional CE-based methods. We successfully detected sequences of foetal's alleles in the maternal plasma DNA, confirmed by classical STR profiling of the newborn after delivery.

Materials and methods

PCR amplification and capillary electrophoresis

The DNA of a pregnant woman and the DNA of her partner had been profiled in a 25 ul reaction volume using the commercial STR kit for forensic identification profiling (AmpFlSTR® NGM kit, Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol.

At delivery, a sample of cord blood had been profiled as well.

DNA specimens were extracted using the QIAamp Mini blood kit (Quiagen, Valencia, CA) according to the manufacturer's protocol *DNA purification from blood or body fluids (Spin protocol)*.

The PCR products were visualized on an AB Prism 310 Genetic Analyzer (Applied Biosystems). CE profiles of parents and newborn are shown in Table 1.

Locus	**M**	**P**	**F**
D22S1045	15	15	15
D19S433	12/16	12/14	14/16
TH01	9/9,3	8/9	8/9
FGA	20/21	20	20
D2S441	11	10/12	10/11
D3S1358	16/17	17/18	16/17
D1S1656	13/17,3	12/16	12/13
D12S391	16/19	15/18	18/19
D10S1248	14/18	16	16/18
VWA	14/18	16/19	14/16
D16S539	11/12	11	11
D2S1338	17/20	17/25	17
D8S1179	9/16	11/15	9/15
D21S11	28/31	29/31,2	28/31.2
D18S51	13/17	14/17	14/17
Amelogenin	XX	XY	XY

Table 1: CE profiles of mother's to be (M), presumptive biological father (P) and newborn (F). the four STRs indicated in grey were used for the deep sequencing approach.

The allele calls determined by the AmpFlSTR NGM Amplification kit were used for comparison with the pyrosequencing data.

GS 454 sequencing

The plasma DNA of the pregnant woman and the DNA of her partner were sequenced for 4 STR loci (Amelogenin, VWA, D8S1179 and D1S1656) on 454 GS.

The plasma DNA specimen of the pregnant woman and the DNA from whole blood of her partner were extracted within the first hour from withdrawal using the QIAamp Mini blood kit (Quiagen, Valencia, CA) according to the manufacturer's protocol *DNA purification from blood or body fluids (Spin protocol)*. Amplicon libraries were prepared using PCR amplification with specific fusion primers using the FastStart High Fidelity PCR System, dNTP Pack (Roche) following manufacterer's instructions [Amplicon Library Preparation Method Manual GS Junior Titanium Series (March 2012)].

The following primer sets for template specific sequences were used to design the fusion primers for library preparation as indicated at

http://www.cstl.nist.gov/strbase/multiplx.htm:

AME FW : 5' CCCTGGGCTCTGTAAAGAATAGTG 3'

AME RW : GCTTAAACTGGGAAGCTG

VWA FW : CCCTAGTGGATGATAAGAATAATCAGTATG

VWA RW : GACAGATGATAAATACATAGGATGGATGG

D8S1179 FW TTTTTGTATTTCATGTGTACATTCG

D8S1179 RW : CGTATCCCATTGCGTGAATA

D1S1656 FW: GTGTTGCTCAAGGGTCAACT

D1S1656 RW : GAGAAATAGAATCACTAGGGAACC

All samples were PCR amplified using designed fusion primers composed of three parts fused together:

- 5'-portion of 25-mer called adaptator, for the binding to the DNA Capture Beads (Lib-A) and for annealing the Emulsion PCR (emPCR) amplification primers and the sequencing primer;

- 3'-portion to anneal with the specific sequence of interest;

- Multiplex Identifier (MID) used to "barcode" the samples, that allow the 2 samples to be pooled and sequenced together.

EmPCR (Lib-A kit) and subsequent bead-enrichment and sequencing steps followed manufacturer's instructions [GS Junior Titanium Series emPCR Method Manual (March 2012) and GS Junior Titanium Series Sequencing Method Manual (November 2012), respectively].

Data sorting

The sequence reads were first sorted by MID tag into the original 2 libraries using linux command.

The .sff files generated by GS Junior 454 were converted in FASTA format. Furthermore, we used BLASTClust to cluster sequences with a stringency identity of 100%. Using a Perl script we counted the number of sequences grouped in each cluster to estimate the coverage of identical sequences.

Sequences were aligned with a reference and STRs were identified. To align reads with the reference we used Amplicon Variant Analysis Sofware and Mapper Software (Roche).

Results and discussion

A total of 110815 sequence reads were successfully aligned with the references. A median of 15512 reads were obtained per amplicon in each of the two samples. No reads were obtained for STR D8S1179 in the mother's to be sample.

Amelogenin STR locus

For the sample of the putative father, in the Amelogenin locus next to 50% of allele X and Y were obtained (5712 and 5962 reads, respectively), confirming the robustness of the technology. For the sample of pregnant woman, only X allele sequences were obtained. Y allele sequences were not found. Even if the Y allele of

the foetus was expected, deep sequencing did not show the corresponding sequence. Amelogenin amplification on nucleated red blood cells of foetal origin (see Chapter 5) failed, demonstrating a low performance of the Amelogenin sex marker.

D1S1656 locus

The sample of the putative father showed a next to equal proportion between allele 12 and allele 16, as also called by CE. The deep sequence analysis showed that the CE called allele 12, with sequence motif $[TAGA]_{12}$ $[TG]_5$, has indeed a different composition in bases: it presents an overlapping size to the CE 12th (133bp length) but the deep sequencing analysis revealed its base composition in $[TAGA]_{11}[TAGG][TG]_5$, described as the 12' allele.

The mother's to be plasma DNA sample had three alleles at the D1S1656 locus:

-7423 sequences corresponding to allele 13, also present at the mother's CE profile (Table 1)

-10529 sequences corresponding to allele 17.3, also present at the mother's CE profile (Table 1)

-7017 sequences corresponding to allele 12', present in the deep sequencing profile of the putative father and by deduction there of belonging to foetal DNA.

Note that CE data of the newborn showed the D1S1656 locus profile 12-13, where the allele 12 corresponded to the allele 12 of the mother's partner CE profile.

The obtained data did not show the aspected proportion 95:5 of pregnant to foetus cfDNA fragments, as discussed by Lo and collegues in 1998 [5].

VWA locus

The sample of the putative father showed a next to equal proportion of sequences corresponding to allele 16 and allele 19, as reported on CE analysis, confirming the expected proportion between the two alleles.

The mother's to be sample is triallelic for this locus, confirming the CE profiling for the two maternal alleles, with the addition of a small proportion of a thirth allele:

-7512 sequences of allele 14'

-3852 sequences of allele 18'

-53 sequences correspondig to allele 16, also present in the profiling of the putative father and of the male newborn, by deduction there of belonging to foetal DNA.

Also in this case the relative quantity of abtained sequences seems to rebut the proposed proportion of mother's to be and foetus cfDNA [5].

D8S1179 locus

The sample of the putative father presented about 50% of allele 11, and 43% of allele 15, confirming the CE profiling. In addition, the pyrosequencing analysis revealeded that allele 15 is equal in length to CE allele 15th, but presents a different sequence due to the presence of 2 single nucleotide polymorphisms (SNPs) (Figure 1). The published sequence of the 15th allele is:

$$\text{TCTA TCTG [TCTA]}_{13}$$

while the GS 454 sequence obtained is

$$\text{TCTA TCTA TCTG [TCTA]}_{12}$$

SNPs indicated in red.

The identification of the SNPs variants increases the identification and discriminatory power in case of alleles with the same length.

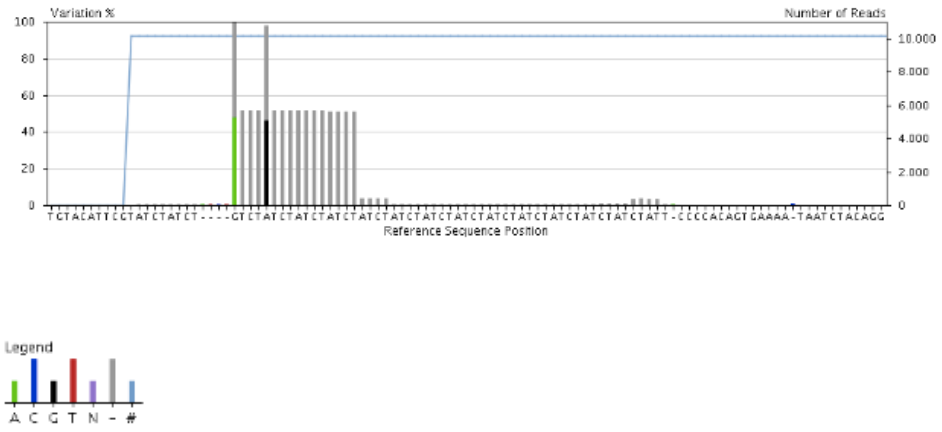


Figure 1: Frequencies distribution of variant reads of putative father's D8S1179 locus. Bars indicate relative quantities of specific variations aligned with the reference. In grey, bars with "deletion" of tetraplets corresponding to allele 11 (percentage of variations: 50%), in green and black, bars with SNPs calling compared to reference corresponding to allele 15 (green G>A conversion and black A>G conversion).

In the sample of the pregnant woman no reads corresponding to alleles D8S1179 were obtained. Due to the lack of amplification of this locus we cannot draw conclusions about the performance of 454 GS for this locus. We speculated that the absence of reads for locus D8S1179 could be due to the low quality of the specific DNA fragments.

Our preliminary 454 data were promising in terms of total data recovery. In the presumptive biologic father's DNA we obtain equal distribution of reads. In mother's to be plasma DNA we obtained an imbalanced reads ratios among the four loci, with no reads for the D8S1179 locus and none for the Y reads.

We considered that plasma DNA is highly degradated and fragmented, and, moreover, that foetal fragments are present in very small percentages among the pregnant plasma.

It has recently been demonstrated that the deep sequencing approach and data analysis produced reliable results, comparable to the CE approach [6]. In fact, PCR-based fragment analysis and CE provide amplicon lengths, that are used to estimate the number of STR repeat units to determine allele identity, but do not allow a complete base composition resolution. STR base composition could be investigated only with sequencing approaches and provided additional information.

In forensic context up to now presentation of data obtained with next generation sequencing technology based on clonal amplification by emPCR followed by pyrosequencing have focused on the discriminating power of detecting loci with overlapping amplicon size ranges [1]. Moreover, even if sequence data for STR can also be generated using the classical Sanger method, this method is not used in routine practice in forensic genetics, because of the extremely laborious workup. The simplicity of simultaneously typing multiple samples for a number of STR loci amplicons and resolution of its basepair composition underlines the benefits of next generation sequencing for forensic applications.

With our preliminary results, even if further optimization of the multiplex amplification is necessary to correct the imbalance, we demonstrated for the first time that foetal STR profiles could be investigated with next generation deep sequencing on plasma DNA of pregnant women.

The sequence data and resulting aligned reads were more than sufficient to reveal sequence variation among sample. SNPs were observed, introducing an opportunity for discrimination of alleles not available with commercial kits.

We retain that this approach has great promise and should be developed for application in forensic genetics. It seems to be promising in STR allele discrimination also in contaminated samples thanks to the extra-value of distinguishing also DNA base substitutions and sequence variations which would not have been otherwise identified using conventional STR typing.

STR sequencing on the 454 platform could represent a good approach to perform non-invasive prenatal paternity testing on cf DNA in rape-related pregnancies.

References

[1] Scheible M, Loreille O, Just R, Irwin J. Short tandem repeat sequencing on the 454 platform. Forensic Science International: Genetics Supplement Series, 2001;3:e357–e358

[2] Butler JM, Buel E, Crivellente F, McCord BR. Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis. Electrophoresis 2004;*25*:1397-1412

[3] Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, et al. Genome sequencing in microfabricated high-density pico-litre reactors. Nature 2005; 437: 376-80

[4] Divne AM, Edlund H, Allen M. Forensic analysis of autosomal STR markers using Pyrosequencing. Forensic Sci Int Genet. 2010;4:122-9

[5] Lo YM, Tein MSC, Lau TK, Haines CJ, Leung TN, Poon PMK, Et Al. Quantitative Analysis Of Foetal Dna In Maternal Plasma And Serum: Implications For Noninvasive Prenatal Diagnosis. Am J Hum Genet 1998; 62:768–75

[6] Fordyce SL, Ávila-Arcos1 MC, Rockenbauer E, Børsting C, Frank-Hansen R, Petersen FT, Willerslev E, Hansen AJ, Morling N, Gilbert MTP. High-throughput sequencing of core STR loci for forensic genetic investigations using the Roche Genome Sequencer FLX platform. BioTechniques, 2011; 51:127–33

CHAPTER 5

Nucleated red blood cells of embryonic and foetal origin: a new tool in forensic genetics

Tasinato Paola[1], Seganfreddo Elena[1], Gervasi Maria Teresa[2], Frasson Chiara[1], Montisci Massimo[3], Ferrara Santo Davide[3], te Kronnie Geertruy[1], Basso Giuseppe[1]

1. Department of Women's and Children's Health, University of Padova, Italy

2. Section of Obstetrics and Gynecology, Azienda Ospedaliera of Padova, Italy

3. Department of Molecular Medicine, Section of Legal Medicine, University of Padova, Italy

Abstract

Introduction and aim

Currently, prenatal genetic analysis is limited to invasive approaches on amniotic fluid or chorionic villi. A convenient non-invasive prenatal diagnostic approach has long been sought. Genetic prenatal diagnosis can have forensic applications, in particular in cases of rape-related pregnancy. We report preliminary results of a non-invasive prenatal paternity test on circulating foetal cells of peripheral blood of pregnant women.

Materials and methods

Pregnant blood samples were taken at various time points during pregnancy. Fluorescent Activated Cell Sorting (FACS) using a combination of monoclonal antibodies was used to enrich nucleated red blood cells of embryonic and foetal origin. After delivery a sample of cord blood was taken as newborn control. DNA was extracted from blood of the parental couple; nucleated red blood cells of foetal origin, and of newborn and genetic profiles were obtained and analyzed.

Results and discussion

In one case comparison of the genetic profiles showed that all amplified foetal alleles from nucleated red blood cell of embryonic origin matched the alleles of their putative fathers. We carried out a non-invasive prenatal paternity testing on DNA of rare foetal cells circulating in pregnant blood. This is the first report on nucleated foetal red blood cell analysis for forensic purpose.

Introduction

Prenatal genetic testing can be performed only on embryonic or foetal material. Such material can be obtained only invasively on amniotic fluid and chorionic villi samples after the 13th gestational week (GW) or on foetal blood samples after 20th GW. From pregnant women's blood specimens there is ample evidence of circulating fragmented free foetal DNA [1] that would permit early non-invasive prenatal sex determination also for medical applications [2].

Above all, invasive sampling is characterized by a small but existing risk for both pregnant women and their foetus [3 – 5], as well as being stressful for women, with evident drawbacks. As these procedures carry a small but significant risk of pregnancy loss, a convenient non-invasive prenatal diagnostic approach has long been sought, but rarity of foetal cells, however, has made the development of such approaches very challenging.

However, non-invasive prenatal diagnosis (NIPD) is becoming increasingly important and its application in prenatal diagnosis is reaching consensus in the scientific research community. In addition non-invasive prenatal testing for non-medical purposes, including forensic genetics is gaining attention. In relation to non-invasive prenatal testing we discussed ethical issues arising from non-medical applications of NIPD, such as sex determination and paternity testing in earlier gestational age and subsequent offspring selection [6]. Nucleated red blood cells of foetal origin circulating in pregnant blood can represent an alternative candidate source of foetal DNA: NRBCs contain a true representation of the foetal genotype [7].

Thus, aiming to verify whether foetal polymorphic STR loci used for forensic analysis can be amplified from nucleated red blood cell isolated from pregnant blood to confirm the foetal cell identity, we performed genetic STR profiling on NRBCs obtained with Fluorescence Activated Cell Sorting from maternal blood. We performed also paternity testing comparing DNA isolated in sorted cells to those of the putative fathers. We retain that the foetal origin of the NRBCs needs to be fully confirmed prior to any genetic analysis for prenatal testing.

Materials and methods

Ethics

The study obtained approval of the Ethics Committee of the University - Hospital of Padova (Protocol n. 2105 P), Italy. Consent has been obtained from all subjects of the couples enrolled. The study protocol has been qualified conform the ethical guidelines of the "World Medical Association Declaration of Helsinki—Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo, Japan, 2004.

Enrollment of the couples

Twentysix couples of pregnant women and their partners were enrolled in the study. A total of 55 blood samples of pregnant women and 26 blood samples of their partners were collected (sampling period of gestation was between 6 gestation weeks –GWs- and 36 GWs + 6 days) attending the Section of Obstetrics and Gynecology Azienda Ospedaliera of Padova (Italy) between 2011 and 2012, under information and consent at first of the pregnant women and after that of the putative fathers with the specification that no results of paternity testing would be communicated to the couple

At each obstetric monitoring seven milliliter of pregnant woman peripheral venous blood was collected, while one blood spot of the putative fathers was collected at the beginning of the recruitment. All samples from the pregnant women were collected into ethylenediamine tetra-acetic acid (EDTA) tubes, all taken before any invasive procedure (amniocentesis or corial villi sampling) and processed within an hour of collection. After the delivery 200 microliter of cord blood were taken.

Enrichment of the foetal nucleated red blood cells from pregnant peripheral blood

A double discontinuous density gradient of Ficoll was used in order to enrich the foetal nucleated red blood cells through a syringe and spinal needle apparatus according to D'Souza et al. [8] with the following differences: five millilitres of 1,073 g/ml solution of Ficol Plaque were poured through the syringe into a 50 ml Polypropylene Conical Tubes (Falcon®) followed by 5 ml of the 1,085 g/ml of

Ficol Plaque solution; then 14 ml of 1:1 solution of maternal blood and 0.9% NaCl was poured through the syring layering the blood solution onto the gradient. The tube was centrifuged at 3,000 g for 30 min (Megafuge 1.0R, Heraeus, Hannau, Germany) without brake. The cells at the interphase of the two gradients (Figure 1) were collected and washed once with 10 ml of Running Buffer (PBS, 0,5% BSA, 0,5% EDTA).

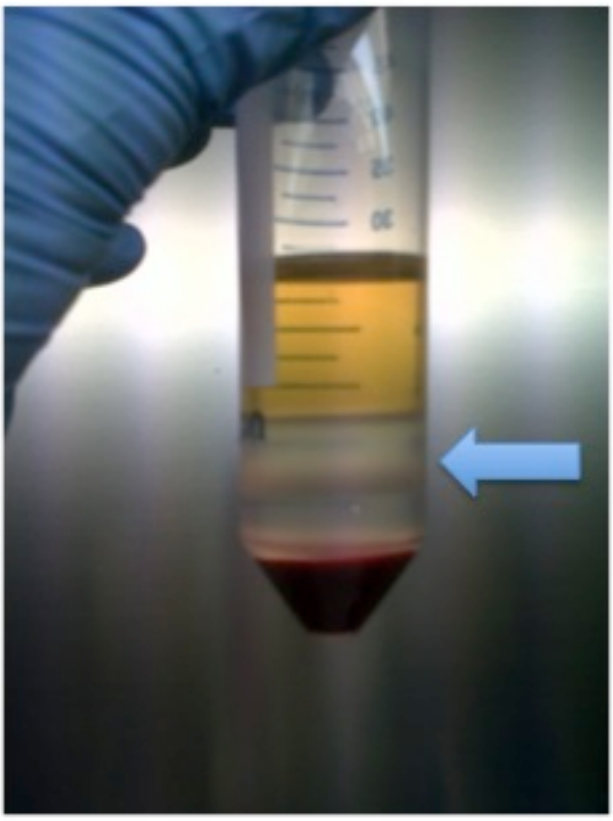


Figure 1. The cell layer at the interface (arrow) contains the enriched fraction of NRBCs.

Permeabilization and staining procedures

Following enrichment, the cells were permeabilized and stained with monoclonal antibodies according to the Fix & Perm® protocol (Invitrogen, USA). The staining protocol required the preliminary division into 3 aliquots of 100 μl of cell solution:

1. aliquot was not stained using the blank cells for instrument calibration;

2. aliquot was stained with the isotype controls 5 μl IgG –Phycoerythrin (PE) (Cat: CYT-IC004F, Cytognos, Salamanca, Spain) and 5 μl IgG 2b-Fluorescein

isothiocyanate (FITC) (Cat: CYT-IC004F, Cytognos, Salamanca, Spain) also used for instrument calibration;

3. the third aliquot was stained with a set of three antibodies: 5 μl CD45-peridinin chlorophyll protein (PerCP) (Clone: 2D1, Cat: 347464, BD Pharmingen, San Diego CA), 10 μl CD71-PE (Clone: M-A712, Cat: 555537, BD Pharmingen, SanDiego, CA) and 10 μl anti-HbF-FITC (Clone:2D12, Ct:552829, BD Pharmingen, SanDiego, CA). The cells were stained for 20 min at room temperature in the dark.

Sorting of the cells

The BD-FACSAria III flow cytometer, equipped with three lasers (488, APCand UV) (Becton Dickinson, San Jose CA, USA) was used for cell sorting. Prior to acquisition, the blank cells and the isotype controls were used to subtract auto-fluorescence. Firstly the CD45 negative cells were selected and from these dual positive CD71 and anti-HbF cells were sorted in 20 μl sheath fluid i.e. running buffer.

DNA preparation and profiling

DNA was extracted from 200ul of blood of the parental couple and of cord blood of the newborn using a QIAamp blood kit (Quiagen, Valencia, CA). DNA from sorted nucleated red blood cell of foetal origin was obtained using Kapa Express Extract (KapaBiosystem, Boston, US) with the following changes: extraction volume of 20ul, instead of 100ul (1), precipitation with ethanol and sodium acetate (500 ml EtOH,20 ml NaAc 3M, pH 5.2 20), and two EtOH 70% washing, final elution in 10 ul of DNAse free water.

Genetic human identification analysis was performed by simultaneous PCR amplification of fifteen DNA STR autosomal loci and a sex determination marker, Amelogenin, using the commercial kit AmpFl STR NGM® (Applied Biosystems), in 12,5 ul of total volume. Autosomal STR loci (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, FGA, D10S1248, D22S1045, D2S441, D1S1656 & D12S391) and the sex-determining STR locus (Amelogenin) were amplified from the extracted DNA: the 29-cycle amplification was executed according to the original manufacturer's protocol in a 25ul final

reaction volume, consisting of 10 ul SGM plus reaction mix, 5ul NGM primer set and 10ul of DNA at concentrations of 0,1 ng/µl as quantified by RT-PCR. Human Control DNA 007 (0,1 ng/µl) provided by the manufacturer's kit AmpFl STR NGM® served as control DNA. The negative controls were represented by the TE Buffer samples previously extracted. All amplifications were performed on DNA thermal cyclers (GeneAmp PCR System 9700, Applied Biosystems). Amplified fragments were resolved on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) and genotyping was carried out using GenemapperID v3.2 software (Applied Biosystems). Electrophoresis was run using a 50 cm capillary and POP-4 polymer (Applied Biosystems) by mixing 1,5 ul of the PCR product with 24,25 ul Hi-Di^{TM} Formamide (Applied Biosystems) and 0,75 ul of GeneScan 500® LIZ Size Standard (Applied Biosystems). The mix was denatured at 95°C for 3 minutes, snap-cooled, and kept in ice water for 3 minutes until used for electrophoresis. The identity of each allele was determined by comparison to the allelic ladder processed in parallel to samples for each run. Allele peaks were measured as relative fluorescence units (RFUs) and interpreted when peak height was greater than (or equal to) 50 RFUs. STR profiles generated by using DNA from NRBCs were compared to STR profile generated by DNA of cord blood of the newborn.

Results

During the sampling period ranging from 6 GW to 36 GW+ 6 days the number of samples obtained per pregnant women varied from one to 3 samples per woman. Three women (M3, M5, M20) withdrew consent to genetic analysis and profiling comparison with the partner half way the study participation period. One woman lost her pregnancy at 12 GW (M22). Four women moved to other obstetric units and delivered in other delivery centres (M2, M19, M21, M24), giving up participation to the research. Eleven women delivered in the emergency room during night and no cord blood samples were collected (M4, M6, M11, M12, M13, M14, M19, M24, M26, M27, M28, M29). Cord blood of 6 newborns was collected.

The amount of negative CD45 and double positive CD71 e HbF events sorted varied along the pregnancy. Starting from the 8th GW in one case we obtain 300

events; before this gestation age no events was obtained in any of the samples. From 300 sorted events, no DNA amplification or profile was obtained. We observed an inter-individual variability and an intra-individual variability in the numbers of sorted events (Table 1).

Trimester	Identification No. of case	Percentage of NRBCs from enriched cells (%)	No. of NRBCs isolated
First (0-13 GWs)	M6.1	0.1	5570 (HbF-)
	M11.1	-	-
	M13.1	0.02	458 (HbF-)
	M15.1	0.001	425
	M15.2	0.015	1000
	M17.1	0.005	300
	M17.2	0.006	137
	M19.1	0.007	499
	M22.1	-	-
	M24.1	0.003	403
	M25.1	0.038	249
	M26.1	0.027	313
	M27.1	0.015	515
	M27.2	0.039	305
	M28.1	0.1	5000 (HbF-)
	M28.2	0.01	21
	M29.1	-	-
Second (14-27 GWs)	M3.1	0.6	14187 (HbF-)
	M4.1	0.1	14440 (HbF-)
	M4.2	0.01	6200 (HbF-)
	M4.3	0.001	42
	M5.1	0.01	1931 (HbF-)
	M6.2	0.1	2692 (HbF-)
	M6.3	0.014	1737
	M8.1	0.035	103
	M8.2	0.038	94
	M9.1	0.012	54
	M10.1	0.04	807 (HbF-)
	M10.2	0.15	1546 (HbF-)
	M11.2	0.007	120
	M11.5	0.01	7780 (HbF-)
	M12.1	1.5	79000 (HbF-)
	M12.2	0.2	1797
	M13.2	0.028	797
	M13.3	0.016	95
	M14.1	0.007	410
	M14.2	0.025	490
	M17.3	0.010	1162
	M19.2	0.003	73
	M20.1	-	-
	M21.1	0.004	50
	M21.2	1.1	11400 (HbF-)
	M24.2	0.022	2972
	M29.2	1.2	155000 (HbF-)
Third (28-40 GWs)	M8.3	0.021	485
	M9.2	0.018	202
	M9.3	0.12	2513 (HbF-)
	M10.3	0.011	143
	M14.3	1.1	50340 (HbF-)
	M15.3	1.4	85000 (HbF-)
	M23.1	0.011	584
	M23.2	0.005	850
	M25.2	0.5	34400 (HbF-)
	M26.2	0.8	36000 (HbF-)
	M27.3	1.2	21000 (HbF-)

Table 1. Fetal NRBCs isolated using FACS and CD 45 negative gate, CD 71 and Anti-HbF dual positive over on three gestational period. HbF- is equal to No HbF positive cells.

During the first trimester the amount of NRBCs in maternal circulation varies between 0,003 % to 0,039 % of the total cell population and from 21 to 1000 cells by counting the events at the sorter. We noted that in all samples (55) but six HbF positive cells were present. In cases lacking HbF we sorted CD45 negative cells contemporary with CD71 positive cells raising the total number of events to a maximum 5000 events, and in all cases when a STR profile was obtained it indicated only maternal alleles.

In the second trimester, from the third sample of the subject M17 (M17.3, 15 + 4 GWs) we obtained valid STR amplification from 1162 sorted events with alleles matching with those of the putative father and with the ones of the new born cord blood at delivery. The profile obtained from sorted NRBCs was a mixture of the pregnant woman and the foetus she was carrying (Figure 1). Unfortunately only one peak for the sex determination marker Amelogenin was obtained instead of two which were expected for the male born (Figure 2).

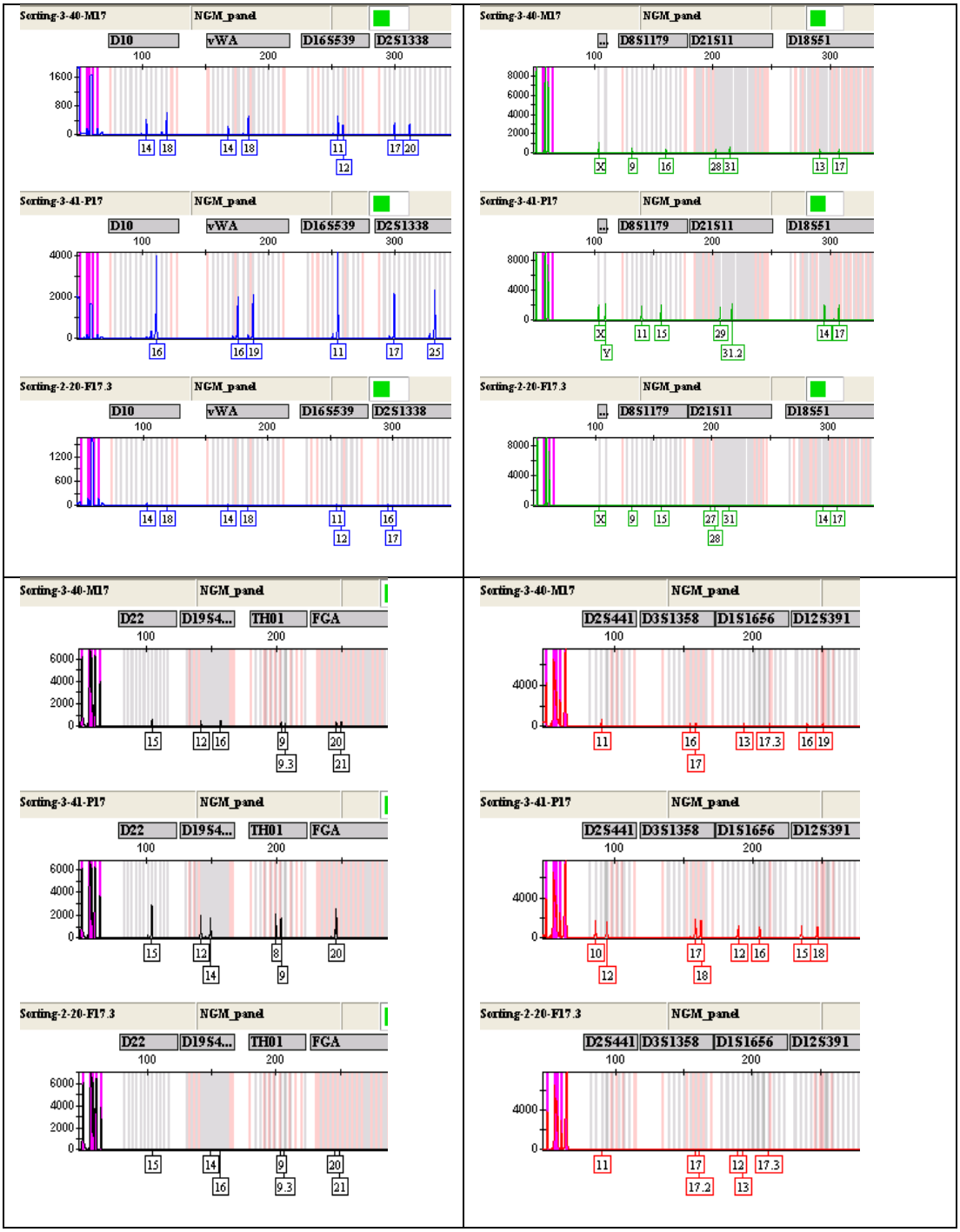


Figure 1. Comparison between profiles of mother (M17), upper electropherogram, her partner (P17), middle, and the profile obtained from CD45 negative, CD 71 and HbF dual positive cells sorted after enrichment from pregnant peripheral blood at 15 + 4 GW (1062 sorted events).

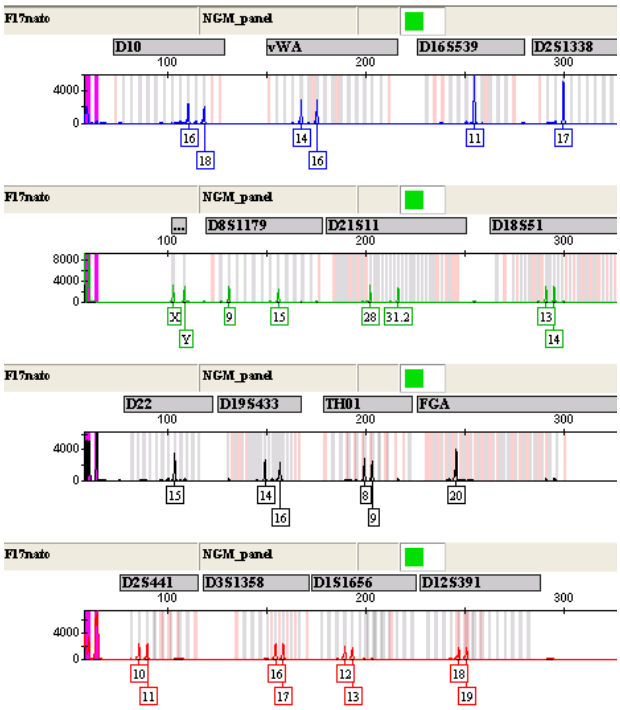


Figure 2. STR profile from cord blood of F17.

Discussion

Foetal-maternal trafficking results in the presence of foetal cells and circulating free foetal DNA in the maternal circulation throughout pregnancy. In the last years efforts have been made to isolate foetal genetic material from pregnant blood, especially foetal nucleated cells with genomic DNA of the foetus. NRBCs represent a potential source of foetal genetic material for non-invasive prenatal diagnosis [9]. However the isolation of an adequate numbers of a pure foetal cells has up to now shown to be very challenging.

Nucleated red blood cells are immature erythrocytes present in the foetal circulation, in foetal placental vessels and also in the maternal circulation [10 - 11].

Invasive prenatal sampling, such as amniocentesis, chorionic villi sampling or foetal blood sampling, are currently the only ways to perform prenatal genetic testing and these sampling methods pose a not negligible risk both for the pregnant and the foetus. Moreover these risks cannot be accepted for non-medical requests of genetic

testing, such as prenatal paternity testing or sex determination. In particular, for forensic purposes invasive genetic testing of foetuses is not permitted because of the minimal but existing risk of pregnancy loss, lacking clinical benefits neither for the mother nor for the foetus [12]. So, aiming to demonstrate foetal origin of circulating cells in order to permit prenatal genetic diagnosis we attempted to amplify foetal alleles of paternal origin from nucleated red blood cells of foetal origin circulating in maternal blood during pregnancy.

To confirm the profile we compared it with that of the putative father, performing a non-invasive prenatal paternity testing. The comparison was also extended to the samples of the newborn at delivery and the previous finding was confirmed.

Previous studies reported attempts to amplify validated STRs for paternity testing in pregnant plasma, but successful amplification of all autosomal foetal alleles of paternal origin has not been shown. Only the Amelogenin locus with sporadic amplification of other loci has been shown in these studies [13].

We isolated NRBCs by FACS after preliminary enrichment with a double density gradient and then by selection of CD71 (transferrin receptor) –positive and HbF (foetal hemoglobin) in the CD45 negative gate, leaving only erythroid cells and platelets [14 – 15]. The partial foetus profile obtained from 1162 sorted events from the CD45- CD71+ and HbF+ gate of enriched cells demonstrated for the first time the unquestionable origin of foetal cells. Autosomal loci of foetal origin amplified sporadically: we did recognize that the sorted events included also maternal cells, as demonstrated by the profile including maternal alleles not present in the cord blood of the newborn. The limitation in this sorting protocol arises from the lack of a specific antibody that would allow to isolate a pure foetal cell population. We had chosen a combination of monoclonal antibodies according to D'Souza et al. [8]. The poor results obtained in our study can be attributed to (1) the small amount of DNA obtained from NRBCs and (2) the presence of contaminating maternal DNA due to lack of high specificity of isolated erythroblasts of foetal origin.

Conclusions

Even if there is not doubt that foetal cells subsist in the maternal circulation, features that distinguish these foetal cells from the maternal ones need to be explored further in order to provide a reliable tool of non-invasive prenatal

diagnosis. NRBCs fingerprinting needs to be deeper investigated prior to validation of non-invasive prenatal genetic testing. This approach represents also a new tool in forensic genetics, when invasive sampling is not ethically accepted: par example in cases of paternity testing in pregnancies resulting from rape or in wrongly attributed samples of *in vitro* fertilization.

References

[1] Lo YM, Corbetta N, Chamberlain PF, Rai V, Sarqent IL, Redman CW, et al. Presence of foetal DNA in maternal plasma and serum. Lancet. 1997;350:485-7

[2] Devaney SA, Palomaki GE, Scott JA, Bianchi DW. JAMA. Non-invasive foetal sex determination using cell-free foetal DNA: a systematic review and meta-analysis. 2011 Aug 10;306(6):627-36

[3] Tabor A, Philip J, Madsen M et al. Randomised controlled trial of genetic amniocentesis in 4606 low-risk women. Lancet. 1986; 1: 1287–93.

[4] Daffos F, Capella-Pavlovsky M, Forestier F. Foetal blood sampling during pregnancy with use of a needle guided by ultrasound: a study of 606 consecutive cases. Am J Obstet Gynecol 1985; 153: 655–60

[5] Buscaglia M, Ghisoni L, Bellotti M et al. Percutaneous umbilical blood sampling: indication changes and procedure loss rate in a nine years' experience. Foetal Diagn Ther 1996; 11: 106–13

[6] Tasinato P, Montisci M, te Kronnie G, Basso G. Non-medical applications of non-invasive prenatal diagnosis: Ethical issues. Forensic Science International: Genetics Supplement Series. 2011;3: e554–e555

[7] Choolani M. The promise of foetal cells in maternal blood. Best Practice & Research Clinical Obstetrics and Gynecology (2012)

[8] D'Souza E, Anjaksha G, Roshan C. A comparison of the choice of monoclonal antibodies for recovery of foetal cells from maternal blood using FACS for non invasive prenatal diagnosis of hemoglobinopathies. Cytometry Part B 76B (2009) 175-80

[9] Avent ND, Plummer ZE, Madgett TE. Post-genomics studies and their application to non-invasive prenatal diagnosis. Seminars in foetal & Neonatal Medicine 2008;13:91-8

[10] Mavrou A, Colialexi A, Tsangaris GT. Foetal cells in maternal blood: isolation by magnetic cell sorting and confirmation by immunophenotyping and FISH. In Vivo 1998;12: 195-200

[11] Rolfo A, Maconi M, Cardaropoli S, Biolcati M. Nucleated red blood cells in term foetuses: reference values using an automated analyser. Neonatology 2007;92: 205-8

[12] Primorac D, Schanfield MS. Apllication of forensic DNA testing in the legal system. Croat Med J 2000;64: 218-24

[13] Wagner J, Dzijan S, Marjanović D, Gordan L. Non-invasive prenatal paternity testing from maternal blood. Int J Legal Med 2009;123: 75-9

[14] Iveron GM, Bianchi DW, Cann HM. Detection and isolation of foetal cells from maternal blood using the fluorescence activated cell sorter (FACS). Prenat Diagnosis 1981;1: 61-73

[15] Bianchi DW, Flint AF, Pizzimenti MF et al. Proc Natl Acad Sci USA 1990;87:3279-83

CONCLUSIONS

Rape-related pregnancy occurs with significant frequency. It is a cause of many unwanted pregnancies and is closely linked with family and domestic violence, but also extra-domestic violence plays an important role in sexual assault and subsequent unwanted pregnancy. Great attention and efforts should be aimed at prevention but also identification of unwanted pregnancies that result from sexual victimization need more consideration. The identification of rape-related pregnancy is required for the persecution of the perpetrator by the law.

In 1996 Holmes et al. reported that 5% of women who are raped become pregnant, which resulted in an estimated 32.000 rape related pregnancies in the United States annually [1].

Prenatal paternity determination can be performed only by invasive procedures, such as sampling of chorionic villi, amniocentesis or cordocentesis that carry a small but existing risk both for the pregnant woman and for the foetus.

The existing risk of invasive procedures is not acceptable for forensic requirement, such as the identification of the perpetrator in case of rape-related pregnancy, excluding the possibility to perform a risky procedure for justice benefits.

Since significant progress in molecular biology have brought non-invasive prenatal diagnosis (NIPD) to a point where it is now in common use, a non-invasive approach also for non medical applications of paternity testing came into sight.

Generally speaking NIPD offers opportunities with specific ethical qualities; firstly, safety; secondly early detection and last but not least NIPD is characterized by easy sampling using a simple blood drawing. All these features together ease of access of non-invasive prenatal testing also for non-medical causes such as sex determination and non-invasive prenatal paternity testing (NIPPT).

Sex determination can be clinically relevant in cases of sex-linked diseases of the foetus, but it can also be used for non-clinical motivated sex selection. Questions are arising within the ethical debate regarding to use of new reproductive technologies for selecting the sex of one's child before implantation [2]. According to Italian law (L. 40/2004) the selection of embryos on the basis of sex is not permitted, but for the interruption of a pregnancy after embryo transfer – in case of in vitro fertilization – (before the 90th day according to Italian law L 194/1978) it is

permitted for maternal reasons, and could include sex selection. The latter choice needs to be valued as a reproductive autonomy of the mother's to be.

Male foetuses sex selection follows the preference in some societies of giving birth to a boy over a girl: this discrimination of women constitutes an injustice that should be prevented [3]. Moreover, large-scale execution of male selection could undermine a public good reinforcing a bias in the ratio between men and women [4].

Concerning NIPPT, three major non-medical applications can be considered: surveying the perpetrator in pregnancy as the result of a rape (a), unsure paternity in case of women with multiple partners (b), unsure paternity in case of suspect of erroneous embryo transfer (c).

In the first case, paternity testing is neither questioned nor disputed. It serves forensic requirements and allows interruption of pregnancy if required by the pregnant woman, even if this might raise religious objections.

Concerning ambiguous paternity, some additional considerations need to be made. Refusing to perform paternity testing in these cases is moralistic and unprofessional: such inference in the life of women reflects an illicit sentence of licentious life, which is not the assignment of a physician. The denial of access to NIPPT could have other ethically non-acceptable implications for the woman involved. A pregnant woman in case of ambiguous paternity could decide to terminate the pregnancy anyhow, or on the other hand decide to continue pregnancy with remaining uncertainty about the kinship. Another questionable implication of this position favours the expansion of services or laboratories that provide paternity testing without legal, deontological or ethical guarantees. Paternity testing typically postnatal paternity testing is performed by private or and academic laboratories, after direct contact with the applicants. Even if also paternal consent in postnatal paternity testing is required a number of laboratories offer via the wwweb paternity testing without any guarantee regarding consent and privacy [5]. This practice is likely to extend to prenatal paternity testing in a near future. A long this line NIPPT will become available through the wwweb with the risk of lesion of privacy and consent rights of involved subjects.

For these reasons we retain that empirical research into pregnant women's attitudes, needs and preferences are necessary for the debate and decision-making process.

The non-invasive approach in prenatal diagnostics is feasible by the presence of cells and DNA fragments of foetal origin in the maternal circulation both in the whole blood and plasma fraction.

With the aim to investigate the possibility of performing a NIPPT for forensic purposes we obtained the approval of the Ethics Committee of the University - Hospital of Padova (Protocol n. 2105 P / 2010), Italy.

Our first approach was on free foetal DNA. In order to investigate the possibility to perform a human identification test on so called plasma DNA (or circulating cell free DNA), we firstly demonstrated the feasibility of fingerprint profiling based on STRs of common use on DNA extracted from cell free plasma: complete STR profiles where obtained from all 'cell free' plasma samples of volunteers enrolled in this preliminary approach. We noted that comparing the fingerprint profiles from whole blood and corresponding plasma, lower RFUs in cfDNA specimens were obtained in all cases even if the same quantity of DNA was explored. We attributed this result to the diminished performance of the multiplex PCR due to a naturally occurring DNA fragmentation and or degradation in plasma, with reduced available primer sites for amplification.

When we investigated the cell free DNA of the pregnant women's plasma at different gestational ages aiming to enable non-invasive paternity testing on free foetal DNA, we did not obtain STR allele amplification other than that of the pregnant women. Amplification of foetal alleles were apparently suppressed by the presence of maternal cell free DNA, even if we performed our test with AmpFlSTR NGM® (AB), a sophisticated multiplex PCR, validated for degraded samples. We opt for these methods, being recently shown that ffDNA molecules in maternal plasma are smaller than mfDNA [6]. The next-generation chemistry of the used kit is considered capable of producing more discriminating DNA profiles on a wider number of samples. Such a chemistry would facilitate data sharing initiatives through the inclusion of new, highly discriminating, shorter STR loci to maximize performance on degraded and challenging samples, such as plasma DNA is to be considered.

Two possible causes can explain the failure of foetal allele amplification: first, the excess of plasma DNA of maternal origin suppresses the primer binding to the

foetal plasma DNA, and second, the smaller length of the foetal plasma DNA reduces the probability of primer binding. Also sex marker Amelogenin failed the amplification in case of pregnant women carryng a male foetus, indicating that other specific Y chromosome markers are to be preferred, instead of the proposed Amelogenin, differing in female and male for 6 base pairs only.

Our attempts to investigate foetal STR profiles from plasma DNA of pregnant women, carrying also a small proportion of free foetal DNA continued with, the recently introduced technology in molecular genetics, the next generation deep sequencing (NGS).

NGS is a sequencing technology of recent introduction in molecular genetics. SNPs, repeat structure variations and low quantities of genomic material that go undetected on CE are recently discovered with NGG [7].

Is has been demonstrated that pyrosequencing has a deep capacity of data generation and discriminatory power [8]. The peak information from CE results contains a value for the size of the peak, while the deep sequencing processed data gives the ratios of the various sequences and reveals base resolution information.

We applied the single molecule sequencing, commonly referred to as Next Generation Sequencing on DNA a pregnant woman and her partner. We used a second-generation sequencing platform - Roche Genome Sequencer 454 Life Science Titanium - for four STR loci of forensic interest.

We tested the capacity of data generation and discriminatory power to detect also sequences of foetal origin. Comparing the NGS data with the CE data we showed that foetal DNA alleles were present in mother's to plasma. Unfortunately, Amelogenin of Y chromosome origin was not sequenced (being the foetus a male). For two autosomal allele we not only demostrated the foetal presence, but also the repeat structure variations corresponding to those of paternal origin. Imbalance of foetal and maternal allele ratio was detected, and more efforts have to be employed to understand this phenomenum.

Even if further optimization of the multiplex amplification is necessary, we demonstrated for the first time that foetal STR profiles could be investigated with next generation sequencing on pregnant plasma DNA.

The recovered data and resulting alligned reads were more than sufficient to reveal sequence variation among samples. The additional discriminatory power provided

by SNPs otherwise indiscernible and the possibility to multiplex many markers with overlapping size ranges highlight the benefits of foetal STR sequencing on the 454 platform, opening the possibility to explore genetic features also for genetic prenatal non-invasive diagnosis.

The family we explored with the 454 NGS was the one in which STR fingerprint on enriched erythroblast cells of presumptive foetal origin circulating on pregnant whole blood gave amplification of STR alleles different from the maternal ones and corresponding to those in the cord blood at delivery, also matching with those of the putative father. Anyway the sporadic amplification is not sufficient for reliable paternity testing.

Finally, the results obtained in this research project, can be summarized as follows:

- genetic non-invasive prenatal diagnosis present specific features that allow ethically feasible applications also for non medical purposes;
- between non medical applications, NIPPT represents the more usefull methods, above all in forensic genetics, such as the investigation of perpetretors in case of rape-related pregnancy and paternity in case of suspect of erroneous embryo transfer;
- free foetal DNA in pregnant plasma DNA represents a promising matrix if investigated with next generation sequencing, even if optimization of the multiplex amplification is necessary;
- nucleated red blood cells, even are considered the more useful foetal cells for non invasive prenatal diagnosis, are challenging in detection, and difficult to be explored by multiplex PCR.

References

[1] Holmes MM, Resnick HS, Kilpatrick DG, Best CL. Rape-related pregnancy: estimates and descriptive characteristics from a national sample of women. Am J Obstet Gynecol 1996;175:320-4

[2] A. Hall, A. Bostanci, C.F. Wright, Non-invasive prenatal diagnosis using cell free foetal DNA technology: applications and implications, Public Health Genomics 13 (2010) 246–55

[3] S.M. George, Millions of missing girls: from foetal sexing to high technology sex selection in India, Prenat. Diagn. 26 (2006) 604–9

[4] Toebes B. Sex selection under international human rights law. Med Law Int. 2008;9:197–225

[5] Pennings G, de Wert G. Evolving ethics in medically assisted reproduction. Hum. Reprod. Update 2003;9: 397–404

[6] Chan, K. C., Zhang, J., Hui, A. B., Wong, N. Et Al. Size distributions of maternal and fetal DNA in maternal plasma. Clin. Chem. 2004; 50: 88–92

[7] Fordyce SL, Ávila-Arcos1 MC, Rockenbauer E, Børsting C, Frank-Hansen R, Petersen FT, e Willerslev E, Hansen AJ, Morling N, Gilbert MTP. High-throughput sequencing of core STR loci for forensic genetic investigations using the Roche Genome Sequencer FLX platform. BioTechniques, 2011;51:127–133

[8] Scheible M, Loreille O, Just R, Irwin J. Short tandem repeat sequencing on the 454 platform. Forensic Science International: Genetics Supplement Series, 2001;3:e357–e358