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Real-Time Quantitative PCR for Detection Cell Free Fetal DNA

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1. Introduction

Basically, diagnosis inherited diseases in gestation is very important. But amplication of the progress in early stage of pregnance is the serious and great aim for rapid application of risk assesment for both and fetus. Fetal genetic tissues collected through techniques such as amniocentesis and chorionic villus sampling (CVS) for prenatal diagnosis of fetal genetic diseases. These procedures are associated with a risk of fetal loss (1%) and two week needed for cultivation (Chiu et al., 2010). Rapid methods for prenatal diagnosis of fetal chromosomal aneuploidies have been developed. This method is multicolor fluorescence in situ hybridization (FISH). FISH is very reliable but requires intact cells and it can only be used on fresh samples (Klinger et al.,1992) The other method is quantitative fluorescent polymerase chain reaction PCR anaylsis (real-time PCR) (Lo et al., 1997). Real-time PCR is a powerful tool for quantifying gene expression combining both high sensitivity and specificity with efficient signal detection (Kubista, 2008). A major advantage of real-time PCR is that it can be used to determine the amount of initial temlates (Heidi et al., 1996). In addition, by making use of a closed whereby the samples are analyzed directly in PCR reaction vessel during the amplification. Furthermore, the assay is less prone contamination (Zimmerman, 2006). Real-time PCR has found wide spread applicability in the analysis of gene expression measurement and cell-free nucleic acids in body fluids. The assay is readily amenable to automation, and by making use of the current real-time PCR 96-384 well formats. The discovery of cff DNA in maternal plasma in 1997 has opened non-invasive prenatal diagnosis (NIPD). This research area is a rapidly developing and dynamic field. NIPD using cell-free fetal DNA (and RNA) is likely to become increasingly available within the next few years. There have been a lot of reported applications, including fetal rhesus-D genotyping (Bombard et al.,2011), fetal sexing for X-linked disorders (Costa et al., 2002), paternally inherited genetic diseases, and pregnancy-associated conditions such as preeclampsia (Zhong et al., 2001). Diagnosing by using free DNA a variety of limitations due to some of the features of this resource. CffDNA analyses would be applicable only when the involved target sequence is in the fetus but absent from mother, such as paternally

inherited disorders or "de novo" mutations (Gonzales et al., 2005). In this chapter, we focused on NIPD with real-time PCR via the use of cell-free fetal DNA

2. The cell free fetal DNA

The cffDNA circulate freely in the peripheral blood during pregnancy and derived from the placenta, the fetal hematopoietic system, or the amniotic fluid, it appears to be transferred as a naked molecule or very quickly assumes that form (Robert et al., 2003). The cffDNA is most likely of placental origin (Bianchi&Lo, 2001). It rapidly disappears from the maternal circulation folowing chilbirth. Unlike fetal cells, which can persist for decades, cffNA are rapidly cleared from the maternal circulation and are undetectable 2 hours after delivery. PHG foundation group was summarized of key properties of fetal/placental elements in maternal blood, specifically cell-free fetal nucleic acids and intact fetal cells (table 1). Fetal DNA molecules are amount to just 3–6 % of the total DNA circulating in the maternal plasma (Lo et.al., 1998a). High levels of cffDNA in maternal plasma is associated with several pregnancy related disorders (Holzgre et al., 2001). The size of cffDNA have confirmed that it is of average size 300 bp or smaller than maternal free DNA (Chan et al., 2004). Fetal DNA can be detected in maternal plasma between the fifth to seventh week gestation (Gonzalez et al., 2005). The technical problem that cffDNA makes up a low proportion in maternal plasma in high background of maternal DNA.

Properties	cffNA	Fetal Cells
Earliest detection	4 weeks	~ 7 weeks
Proportion	5-10%	0.0001 - 0.01%
Persistence in maternal blood	< 24 hours	> 27 years
Relevant physical properties	Short fragments	Dense nucleus

Table 1. Differences cell-free fetal nucleic acids and intact fetal cells (phg foundation, 2009)

2.1 Isolation of cell fetal free DNA from maternal plasma

The discovery of cffDNA in maternal plasma of pregnant women in 1997 by Lo et al (Lo et al., 1998b), many laboratories have shown that several protocols for isolating this material. The nucleic acid extraction procedure is most important for the detection of fetal nucleic acids in maternal plasma. To separate plasma from whole blood, parameters including multiple centrifugations at different speed, filtration to rmove contaminating intact or apoptotic cells, and removal of protein impurities have been explored (Jorgez et. al., 2006).The critical point with techniques used is the avoidance of contamination from other blood components. The protocols for isolation of plasma were optimized to reduce cell lysis. The standard protocol for isolating plasma, which is commanly, involves two centrifugation steps. First step is high speed. This step is removal of cellular material from plasma layer. Store plasma samples in smaller aliquots (500 μL). The second step, different commercial kits have been used to isolate cell free fetal DNA. The free fetal DNA particles in maternal blood are promising as an important step for diagnosis of numerical and structural

chromosome anomalies belonging to fetus without invasive procedures. The main problem is that extremely low concentration of fetal DNA is present in maternal plasma. Circulating fetal DNA concentration increases with progression of pregnancy and disappears from maternal plasma rapidly after delivery , with median half-life of 16.3 min (Lo et al., 1999, Smid et al., 2003). However, methods have been optimized for the extraction of fetal DNA from maternal plasma. Although extracted DNA is generally stable for long periods, levels of fetal DNA have been shown to decrease over long storage periods. Our laboratories use of the QIAampDSP virus kit (QIAGEN). To extract DNA, 600 µL of plasma from maternal sample for method.

3. Clinical applications of real-time PCR for non-invasive prenatal diagnosis

For many years, scientisist has focused seperation of fetal cells and fetal DNA in maternal blood. The discovery of circulating cell-free fetal nucleic acids in maternal plasma has opened up new possibilities for non invasive prenatal diagnosis. Cff DNA is detectable in maternal blood as early as 5-7 weeks of gestation (Honda et al., 2002; Galbiati et al., 2005). CffDNA present has been used succesfully for non-invasive diagnosis of the fetal sex and fetal Rhd genotype in Rh negative women (Chen et al., 2004). Recent advances in molecular methods enable other applications of fetal DNA purified from maternal plasma samples (Vlkova et al., 2010). Studies put more emphasis on this area (Purvosunu et al., 2008). The sensivity of the studies that have been recently carried out by the devoleped PCR techniques has been increased, but the greatest difficulty encountered is the isolation of pure and high scale fetal DNA. By doing this isolation, the need of invasive procedures, such as amniocentesis will largely be eliminated (Hahn et al., 2008).

3.1 Fetal RHD status

Hemolytic disease of newborn (HDN) is a clinic phenomenon, which occurs during pregnancy due to the RhD alloimmunization between Rh (-) pregnant woman, who has become sensitive with RhD antigens, and her Rh (+) fetus. Unnecessary application of Anti D Ig can be prevented for pregnant women who carry Rh – fetus, also this results in a better follow-up of pregnant women who carry Rh+ fetus with the detection of RhD genotype of fetus in maternal plasma at the first trimester of pregnancy. RHD was used as a test-bed to prove the effectiveness of detecting a paternally inherited allele carried bty circulating free fetal DNA in maternal plasma (Lo et al., 1997). Maternal plasma-based RHD genotyping has been succesfully implemented in our laboratories for use as a non-invasive prenatal diagnosis where alloimmunization has occured (Gunel et al., 2010). Real-time PCR has been the choice diagnostic test used. Fetal RhD detection can be applied routinely to Rh (-) women and the use of human anti –D can be significantly reduced (Hahn et al., 2008). A reduction in anti-D administration also has economic implications. Rh locus is composed of two homologous genes RHD and RHCE (97% homology) closely linked on chromosome 1p34-p36. Each of these genes consists of 10 exones, and they contain 69 kb of DNA. The regions of exone 7 and exone 10 within the RhD gene are the areas of focus. In all positive results, the RhD blood group has also been found positive (Huang, 1998, Wagner and Flegel, 2000). In a white population, D-negative individuals are homozygous for a deletion of RHD. The D-negative phenotype, absence of the whole RhD protein from red cell membrane. Different mechanisms may explain rare phenotypes, including gene conversion events

between homologous RHD and RHCE producing hybrid genes, nonsense mutations, and deletions or insertions interrupting the reading frame of the messenger RNA (Rouillac-Le Sciellour et al., 2004. Gunel et al., 2011).

3.2 Fetal sexing

Researchers had focused on the detection of fetal-derived paternally inherited DNA because Y chromosome of male fetus, which were absent in the genome of the pregnant women (Lo YMD, 2010). Most sex-linked disease are recessive X-linked disesae such as haemophilia and and Duchenne muscular dystrophy. To determine the sex of a fetus by real-time PCR using DYS14 and SRY genes on and Y chromosome. The genotyping of these fetal loci helps preventing invasive procedures in the case of X-linked disorders (Rijinders et al., 2004). CffDNA technology has been used to identify babies at risk of congenital adrenal hyperplasia by identifying fetal sex at the requisite stage in pregnancy in 6-7 weeks (Rijinders et al., 2001).

3.3 Determination of fetal aneuploidy by real-time PCR

The free fetal DNA particles in maternal blood are promising as an important step for diagnosis of numerical and structural chromosome anomalies belonging to fetus without invasive procedures. The direct analysis of cff DNA for the NIPD of trisomies is mainly complicated. The most common chromosomal abnormalities in live births is trisomi 21 and other chromosomes 13, 18 trisomies has rapidly analysis with real-time PCR methods. The full fetal karyotype is usually determined using cultured cells and need two weeks for result. Real-time PCR has rapidly determination of template copy numbers. Tong et. al. had first demonstration of the direct detection of trisomi 18 from maternal serum (Tong et al., 2006). They were achived with use of the epigenetic allelic ratio method. Enrich et.al., had used sequence-specific taq of known chromosomal location as a quantitative representation of individual chromosomes in maternal plasma. Trisomi 21 detection by massively parallel shotgun sequencing (MPSS) in high risk women is complicated. Their overall classification showed 100% sensitivity and 99.7% specificity of detection of fetal trisomi 21 (2011). Lo et al., described a novel method for the NIPD of Down syndrome. Candidate mRNA markers shoul be encoded from genes located on chromosome 21. The PLAC4 gene on chromosome 21 and originating from fetal cells in the placenta and cleared following delivery of the fetus (Lo et al., 2007). If the fetus is euploid, that is containing two copies of the PLAC4 gene, because SNP (single nucleotide polymorphism) alleles would be 2:1. (Lo, 2009). The first large-scale noninvasive prenatal detection of trisomi 21 using multiplex sequencing to analyze fetal DNA from maternal plasma by Chiue et. al., (2011). They show that 100% sensitivity and 97.9% specificity of detection of fetal trisomi 21. They report a % 2.1 false-positive rate. This method replace invasive procedures and would be eliminated unintended procedures fetal losses as well as better targeting of pregnancy-related interventions.

3.4 Pre-eclampsia and cell free fetal nucleic acids

Preeclampsia is a potentially dangerous disorder specific to the second half of pregnancy, affecting about 2.5–3% of women (Redman & Sargent, 2005). The etiology of this disease involves the placenta. Real-time PCR, it is now possible to measure DNA/RNA content

accuracy. The increased presence of cell-free fetal DNA indicates that preeclampsia is associated with damage to the placenta (Zhong et al., 2001) The cell-free fetal DNA levels in maternal blood in preeclamptic patients are significantly higher than those of normal pregnancies, and this elevation precedes the clinical occurrence of preeclampsia by approximately 1-2 weeks. The results of the studies in this area are important for pregnancy follow-up. The change of free fetal DNA levels in maternal blood in healthy pregnancies according to gestation week, was shown in various publications (Zhong et al.,2001, Ferina et al.,2004, Carty et al., 2008). By using real-time PCR technology was shown to be elevated ~ 5-fold in samples obtained from pregnant women with preeclampsia when compared with an eual-sized cohort of normotensive, pregnant women (Lo et al., 1999) All study subjects were pregnant women carrying male fetuses, and investigators amplified sequences from the SRY gene distinguish fetal from maternal DNA (Levine et al., 2004).

4. Summary

The discovery of cell-free fetal DNA (cffDNA) in maternal blood has exciting possibilities for non-invasive prenatal diagnosis (NIPD). Free fetal DNAs stemming from the destruction of fetal cells from seven weeks of gestation transport to the maternal blood via the bi-sidal flow through the placenta.The free fetal DNA particles in maternal blood are promising as an important step for diagnosis of numerical and structural chromosome anomalies belonging to fetus without invasive procedures. The detection and quantification of gene rearrangement, amplification, translocation or deletion is a significant problem, both in research and in a clinical diagnostic setting. Real-time PCR has become a well-established procedure for quantifying levels of gene expression. There are a number non-invasive prenatal diagnosis methods such as sex diagnosis or Rh blood type incompatibility and single-gene disorders have been reported. Different problems associated with placental growth and development result in changed levels of cffDNA. The cell-free fetal DNA levels in maternal blood in preeclamptic patients are significantly higher than those of normal pregnancies. It is possible the diagnostic utility of adding cffDNA concentration to the current panel of biomarkers. The test can be used very early of pregnancy, with no risk to the mother and child.

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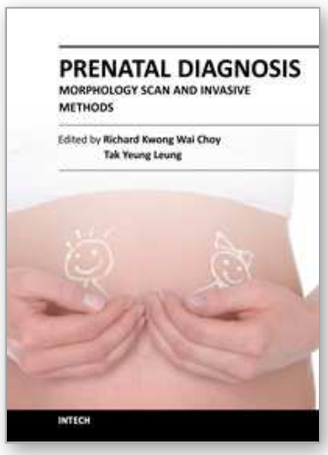

Prenatal Diagnosis - Morphology Scan and Invasive Methods

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