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Noninvasive Prenatal Nucleic Acid Diagnostics of Down Syndrome

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1. Introduction

Molecular composition of pregnant women blood plasma/serum is an indispensable tool for biochemists in the field of routine prenatal diagnostics aimed at the detection of the most common aneuploidies.

Predictive power of some biochemical markers respectively their sum is an important guide for the assessment of the status of the fetus and pregnancy.

But the diagnostic potential of maternal plasma is much broader.

Fragmented and apoptically degraded fetal cells and nucleic acid molecules, which overcame the placental (feto-maternal) barrier and got under the influence of the immune system of the mother, offer other options for molecular biologists.

Some of them will focus on the clarification of rules between the quantity of fetal molecules and pathology of the placenta and the fetus others are engaged in direct diagnostic potential.

In the maternal blood circulation can be detected fetal genetic material of a different integrity ranging from the nuclear DNA of living cells through fetal apoptically partially degraded molecules more or less packed in nucleoides to completely fragmented DNA molecules mostly of size between 100 - 400 bp. Cell free fetal (cff) mRNA molecules from trophoblast are also presented in detectable concentration in maternal plasma.

While the presence of fetal cells in maternal circulation is quite rare (1 fetal cell in 1 million maternal cells), the quantity of cffDNA molecules in plasma is considerably higher (typically 3 % - 6 %).

2. Source and transport of cff nucleic acid molecules into maternal plasma

The ways in which cffDNA forms and how gets into the maternal circulation, are not yet fully clarified. There are several hypotheses on the origin of cff DNA.

The main source of free DNA appears to be a placenta (Wataganara & Bianchi, 2004). Most likely the cff DNA forms by disintegration of placental cells and the direct transition to the maternal circulation.

Another possibility is that the placenta functions as an mediator of transition for DNA circulating in the fetal blood circuit into maternal tissues.

In this case, there should be a two-way traffic, however, the amount of analyzed fetal DNA in maternal plasma is much greater than the amount of free maternal DNA in umbilical plasma in childbirth (Sekizawa et al., 2003). It is indicative of an unequal transfer of free DNA from fetus to the mother. Another source of fetal molecules may be fetal hematopoietic cells which get into the maternal circulation (Bianchi, 2004). The fetal DNA is apoptically released into maternal plasma due to the effect of maternal immune system (Pertl et al., 2000). Apoptotic fetal cells were found in detectable concentrations also directly in the plasma (Van Wijk et al., 2000). Apoptosis proven in maternal plasma (Sekizawa et al., 2003) or in cord blood (Hristoskova et al., 2001) suggests that this mechanism could be an important source of fetal material. Another hypothesis assumes a simple diffusion of shorter DNA molecules from the amniotic fluid through placenta or membranes into maternal circulation. This DNA then comes from various fetal tissues.

Cff DNA was also demonstrated in the maternal plasma even before feto-maternal circulation establishment. This means that the DNA could be of the trofoblastic origin (Bianchi et al., 2004). In general, it could be assumed that cff DNA in maternal circulation is of different origin but its large portion likely comes from the trophoblastic placental tissue.

The transfer of fetal molecules through the feto-maternal barrier is continuous during pregnancy and after the delivery they are quickly eliminated from plasma (16 min halftime of degradation (Lo et al., 1999).

Free fetal RNA allows non-invasive prenatal profiling of gene expression and offers a number of research and diagnostic applications on the basis of easily detectable mRNA transcripts from placentally expressed genes in maternal plasma (Ng et al., 2003).

Considering serious degradation as a result of the activities of the ribonucleases, the stability of mRNA molecules in fetal free plasma is surprising. Plasma RNA molecules are much more stable than isolated and purified RNA molecules. Mechanisms that protect the circulating free fetal RNA are not fully clarified at present time. However their conjunction with the sub cellular particles could prevent their degradation (Hasselmann et al., 2001).

They can form complexes with proteins, lipids, lipoproteins, phospholipids bound to the DNA in nucleosomes or within protected apoptotic corpuscles or other vesicular structures (Halicka et al., 2000; Hasselmann et al., 2001; Tsui et al., 2002; Sisko et al., 2001).

The stability of placental mRNA molecules in maternal plasma is a promising assumption that fetal markers at the level of mRNA will be clinically useful.

It could be useful for prenatal detection of some pregnancy pathologies, including pre-eclampsia and certain chromosomal aneuploidies (Ng et al., 2004).

3. Placental trophoblast and trisomy 21

Trophoblast with chromosome 21 trisomy shows disturbed both cell fusion and formation of syncytiotrofoblast (ST) (Frendo et al., 2000b; Massin et al., 2001). An activation of caspases is necessary for formation of ST in early stage of differentiation (Huppertz and Kingdom, 2004). If ST is inadequate, the individual trophoblastic cells with trisomy 21 could continue on in the cascade of apoptic events and release more fragmented cff DNA than disomic trophoblastic cells. Another possible cause for the release of larger amounts of fetal molecules in pregnancy with trisomy 21 is a continuation of trophoblastic cells in the improper proliferation, without initiating events leading to the formation of ST. In this case maternal immune system would regulate the division by increased degradation of fetal cells.

4. Fragmentation profile of cffDNA

Publications, relating to the characterization of the DNA fragments in the plasma, are aimed to the level of fragmentation of fetal molecules related to the maternal ones in the course of pregnancy. For pregnant women were described in plasma longer fragments in comparison with non-pregnant women (Chan et al., 2004).

Real-time PCR is the most commonly used procedure for the analysis of fetal DNA fragments in present time (Alberry et al., 2009; Ariga et al., 2001; Lo et al., 1998). Honda et al. (2002) described the sensitivity of the detection of fetal DNA for this method and determined it at 5,38 copies/ml of the peripheral blood.

Molecules of fetal origin are more degraded and shorter than the maternal and their size is approximately in the range from 100 bp - to 700 bp. (Zhong et al., 2000; Koide et al., 2005). Fragments above 1 kb belong mostly to the mother (Li et al., 2004).

We have evaluated fragment analysis using three methods (capillary electrophoresis of STR loci, capillary electrophoresis and Real-time PCR of gonozomal sequences) from two different perspectives. The first relied on direct analysis of the size of fractions and the second assessed PCR efficiency with respect to the size of amplified DNA molecules (Vodicka et al., 2010).

Direct detection of fetal molecules has confirmed a large heterogeneity in the individual fractions both by capillary electrophoresis and Real-time PCR.

A direct dependency of fetal molecules on the week of pregnancy has been suggested only in the size fraction of 500 - 760 bp. Regression modeling at STR and gonozomal analyses was the most accurate in the pD system, where the size of molecules ranged from 395 bp to 440 bp. Statistical significance of regression declined towards the smaller molecules.

The fragment size was 200 bp to 223 bp in the D21S1446 system and 157-188 bp in D21S1435. Regression analysis was inconclusive in the AMELY (molecule size 109 bp).

If we compare analysis of the size of fractions to the results of the effectiveness of the STR and gonozomal analyses (Vodicka et al., 2008a), where was demonstrated statistically significant increase of fetal molecules in all 3 STR systems, we found out that it was confirmed an interesting trend, which indicates an increase in the larger fetal molecules during pregnancy while the number of smaller molecules of fetal origin does not change.

In the case of quantification using SRY probe, whose length is 64 bp it was observed even reverse trend and the number of amplified molecules in 150-300 bp fraction was in indirect relation to the week of gestation.

The tendency has decreased for larger fractions and in total plasma the trend has stopped even slightly reversed.

On the basis of these observations, we can therefore assume the most sensitive detection of fetal material using short probes. But these probes are not much suitable for quantitative analysis. Fractions from 400 bp above are the most appropriate for the assessment of the amount of fetal material, although in these fractions the relative amount of fetal DNA is less.

The importance of this study resides in decision-making, whether, from a diagnostic point of view, it is more important a detection of fetal molecules or their quantification (Vodicka et al., 2010).

5. The extraction of cffDNA/RNA from maternal peripheral blood

5.1 CffDNA isolation procedures

An essential precondition for the success of all subsequent procedures is a sufficient yield, reproducibility and the purity of the isolated plasma cffDNA. Isolation procedures are most

often based on double centrifugation and subsequent binding (adsorption) of DNA molecules on a silica surface of membrane or magnetic particles.

5.1.1 Isolation by binding to a silica-gel membrane
The adsorption on silica surface is one of the most common techniques which is able to capture free fragmented DNA.

Silicate polymer in the presence of chaotropic salts (e.g. sodium iodide or guanidine thiocyanate) specifically binds to the DNA molecule on the surface of silicon dioxide (usually specially prepared glass) and in the presence of water or other elution reagent, which has a very low content of salt, this molecule releases from its surface.

High specificity of the link between DNA molecule and silicate material is useful in eliminating other extracellular substances.

5.1.2 Isolation by binding to the magnetic particles
Methods based on the (para) magnetic particles (MPs) are other approach to the cffDNA isolation. MPs are particles of size from 5 nm to 100 μm, consisting of a metal core, it is usually γ-Fe2O3 (maghemit) or Fe3O4 (magnetite) but could be also for example Au.

The core is coated with a layer that has specifically prepared surface for binding the molecules that we want to isolate.

The size of the MPs can be adapted according to the molecules which we are isolating: 5-50 nm proteins; 20-450 nm nucleic acids; 10-100 μm viruses.

MPs respond to the external magnetic field and are capable to bind different bioreaktive molecules, because of their affinity to the modified surface, directly from the biological material. The isolation is then as follows.

MPs are added to the sample where bind with the targeted molecule. Modified MPs are then attracted by magnet to the wall of the tube and the remaining solution with non-bind substances is removed.

The MPs with joined molecules are subsequently washed and released in added solution.

Bound molecules are separated from the MPs by some physical-chemical step (denaturing or change in pH).

This way we obtain targeted molecules which could be afterward analyzed (Húska et al., 2008).

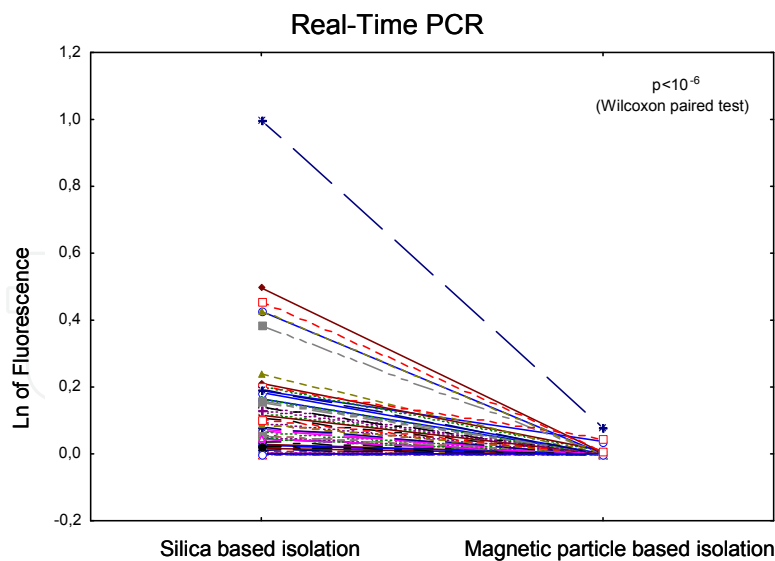### 5.1.3 Comparison of silica-gel to magentic particles methods
Both above mentioned isolation procedures were tested in our workplace.

For a comparison, cff DNA (male fetus) was measured and quantified both by Real-time PCR in SRY locus and by quantitative fluorescent (QF) PCR and capillary electrophoresis separation in AMELY locus in 38 samples from the 1st and 38 samples from the 2nd trimester. While the concentrations of total DNA measured spectrophotometrically were similar there were substantial differences in yield of cffDNA between tested DNA extraction methods (graph 1,2).

Isolation based on the separation of magnetic particles had significantly lower recovery compared with the amount of cffDNA extracted by binding on the silicate membrane.

According to our current findings "silica membrane" based method seemed to be clearly more suitable for isolation of cffDNA from the plasma of pregnant women.

DNA extraction using magnetic particles should present a simple way how relatively easily and without contamination obtained the cffDNA.

Graph 1. Comparison of silica gel and magnetic particles isolation effectiveness by Real-time PCR quantification.
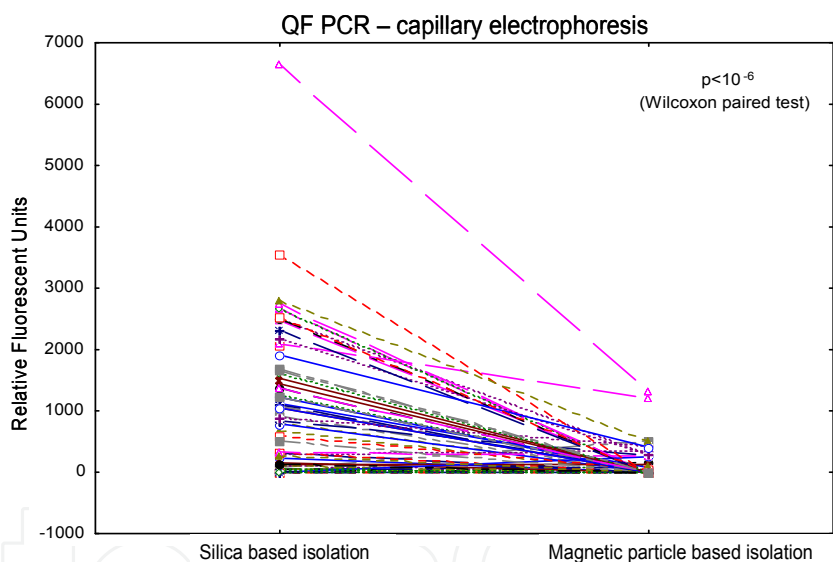

Graph 2. Comparison of silica gel and magnetic particles isolation effectiveness by capillary electrophoresis quantification

However, for the isolation of short fragments of cffDNA this method did not work, although the manufacturer declares, that it should be able to detect the DNA molecules of the size from 50 bp to 1,5 kb. Low efficiency of cffDNA capture can be caused by strong bond of free fragmented fetal DNA on magnetic particles, or by contraries, by weaker bond of these fragments on magnetic particles and their wash out during isolation.

To explain this, we re-isolated unbound DNA from supernatant. The subsequent quantification by capillary electrophoresis has demonstrated again only the maternal sequences, which suggests a stronger link of fragmented fetal DNA on magnetic particles.

If cffDNA was succsessfully detected by both methods, the amount of DNA has correlated to each other (Graph 3).
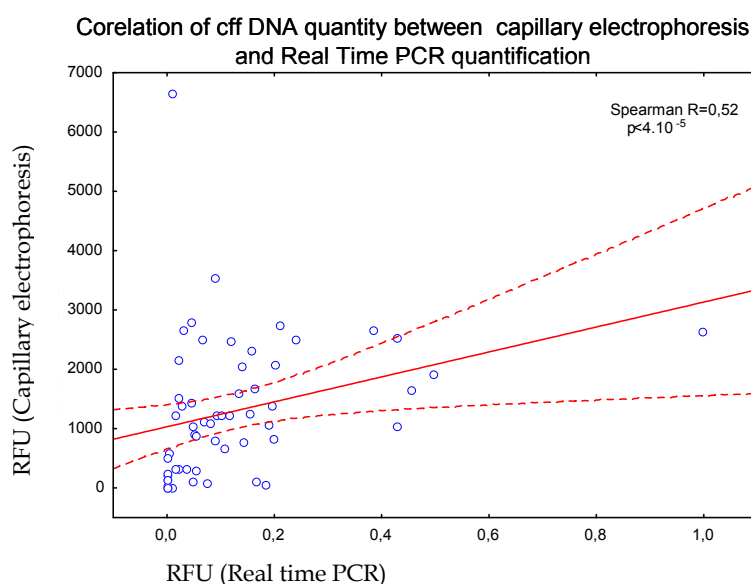
Graph 3. Correlation of the results of free fetal DNA quantification by capillary electrophoresis and real-time PCR (RT-PCR). The Y axis shows the Relative fluorescence units (RFU) obtained from capillary electrophoresis, X axis shows the fluorescence scanned in Real time system.

5.2 Cff mRNA isolation procedures

To maintain integrity of obtained free fetal RNA, it is important to use isolation techniques which have minimum isolation steps, are quick and contain RNA stabilizers.

Currently there are no commercially produced kits standardized for isolation of free fetal RNA directly from maternal plasma, but there are available kits specialized for isolation of viral RNA, which could be, with little modification, applied to the isolation of free fetal RNA.

Double centrifugation in cooled centrifuge or ultra centrifugation is necessary for separation of cellular component of blood prior to the isolation of free fetal RNA.

The combination of viral RNA Kit and vacuum pumps seems to be an optimal system for obtaining free fetal RNA in the highest possible quantity and the quality, with minimal risk of contamination during isolation, unlike centrifugation steps. Microfilters can be used to increase the concentration of free fetal RNA.

6. The use of cff DNA for diagnostic purposes

The resolution of the fetal genotype and precise quantification of cffDNA are crucial for diagnostic purposes.

6.1 Noninvasive fetal sex determination by cffDNA

So far, the most common and relatively simple for the analysis of fetal DNA was the use of Y specific sequences from male fetuses.

These sequences can be quantified by methods of Real-time PCR and QF PCR.

The methodology for fetal sex detection in maternal plasma is already relatively well developed and reliable (Lo et al., 1998; Honda et al., 2002; Wei et al., 2001; Zhong et al., 2000, Vodicka et al., 2008b).

Our quick and simplify fetal sex detection is based on refined QF PCR. We tested 475 DNA samples isolated from maternal plasma in different weeks of pregnancy ranging from 4th w.g. to 37th w.g. Y chromosomal sequences in AMELY were tested and quantify by comparison to AMELX using capillary electrophoresis.

The method is able to distinguish even less than 1 % of Y chromosomal sequences of artificial mixtures. Fetal sex was detected with 4.05 % of false positivity and 7.15 % of false negativity (Vodicka et al., 2008).

The methodology has very high detection sensitivity, which is comparable with a sensitivity of real-time PCR methodology (Sekizawa et al., 2001; Honda et al., 2002).

Possible contamination of the samples during collection and in the course of the isolation procedure is the main cause of false-positive detection of the male sex.

Minimization of any contamination is fundamental in sensitive DNA analyses in which it is necessary to capture the genotype in quantities of about 5%.

Mistakes in determination of male fetus (false-negative results) come mostly from early stages of pregnancy, when the concentration of cffDNA is still very low, so rather than non-compliance could be assessed these results as missing fetal DNA.

Capillary electrophoresis has proven to be uniquely able to capture even 1 % genotype artificial admixture.

The result of examinations of our group of pregnant women can be considered very satisfactory. The methodology is prepared for practical use, however, further improvement of isolation robustness and following laboratory procedures may increase specificity, sensitivity, and the yield of fetal DNA.

6.2 Noninvasive RHD genotyping by cffDNA

RHD genotype of the fetus can be identified from maternal plasma already at the beginning of pregnancy using molecular analysis of cffDNA.

Clinical significance of determination of fetal RhD status is dual:

1. Noninvasive fetal RHD determination from peripheral blood of Rh- alloimmunized pregnant women at the beginning of pregnancy allows to determine the fetuses which are really threatened by development of hemolytic disease of fetus and newborn.

2. If anti-D antibodies are not present at the beginning of pregnancy, RhD negative woman should be provided with anti-D immunoglobulin during pregnancy in sufficient dosage for each potentially sensitizing events including completion of antepartal prophylaxis at 28th week of gestation. Potentially sensitizing event means any situation in which may occur intersection of RhD positive fetal erythrocytes to the mother's blood, and subsequent development of the RhD alloimmunization (chorionic villus sampling, amniocentesis, cordocentesis, another intervention of the prenatal diagnosis and fetal therapy, obstetric hemorrhage in the 2nd and 3rd trimester, intrauterine death of fetus). Prevention of RhD alloimmunization should be provided to RhD negative pregnant woman by delivery of anti-D immunoglobulin in sufficient dosage in all above-mentioned cases. The administration of anti-d immunoglobulin is necessary if the fetus is RhD-positive, on the contrary, if the fetus is RhD negative, the pregnant woman is not threatened by the development of the RhD alloimmunization and administration of anti-D immunoglobulin is not necessary.

RHD genotyping of cffDNA is most often done using Taq man Real time PCR with probes from exone 7, 10, 4 or 5. System of negative and positive PCR control in addition to internal

control of PCR amplification is necessary for the reliability of detection considering great sensitivity of the method. At the same time, you must have created calibration quantitative profile for exact specification of detection limit of RhD positivity.

6.3 Noninvasive cffDNA analysis of monogenic inherited diseases

CffDNA can be also used for examination of monogenic inherited diseases.

Many genetic abnormalities, for example autosomal dominant myotonic dystrophy (Amicucci et al., 2000), Huntington's disease (Gonzalez-Gonzalez et al., 2003) or gene mutations such as achondroplasia (Saito et al., 2000) has already been examined from cffDNA. Furthermore, it is possible to detect some autosomal recessive diseases, such as cystic fibrosis (Gonzalez-Gonzalez et al., 2002) or β-talasemia (Chiu et al., 2002).

7. Cff nucleic acid quantitation for noninvasive Down syndrome diagnostics

Targeted molecular analysis of fetal aneuploidy with maternal plasma utilization is more complicated and applicable differentiation of maternal and fetal genotypes within the desired chromosome brings with it many challenges.

Considering differences between the quantity of total cffDNA and cfDNA, in the fetuses with Down syndrome (DS) pathology can be observed a greater quantity of cffDNA compared to the physiological pregnancies (Vodicka et al, 2008a).

The total amount of cffDNA cannot be considered as unambiguous marker due to large variation in the amount of cffDNA in physiological pregnancies and, rather, it is possible to include it to the system of ultrasound and biochemical markers.

Direct examination of the loci responsible for the DS brings a substantial improvement of the diagnosis. Currently, there are several approaches or their combinations respectively, aimed to unambiguous resolution of pathological fetus with the DS.

7.1 Noninvasive cffDNA diagnostics of Down syndrome using genotype differences between paternal and maternal genotypes

DS analyses and quantifications of cffDNA are based on the resolution of paternal from maternal genotypes. The fetus must be always heterozygous for the monitored loci. Previous genotyping of the parents is therefore important for the selection of appropriate markers.

Targeted resolution assumes a sufficient number of reliable analyzable markers of paternal (fetal) origin on chromosome 21 and at the same time a sufficient number of reliable markers outside the chromosome 21.

The genotype of the fetus can be distinguished by means of DNA sequence variation using male specific sequences for male fetus, short tandem repeats (STR) or single nucleotide polymorphisms (SNP).

For the estimation of the relative amounts of cffDNA originating in chromosome 21 it is necessary to choose a strategy that combines the creation of sensitive and accurate calibration standards for each marker and reciprocal measuring of cffDNA amount from the locus on chromosome 21 and cffDNA quantity from autosomal locus or locus on chromosome Y respectively.

If the measurement is accurate enough, then the amount of cffDNA of chromosome 21 related to the quantity of the cffDNA locus outside the chromosome 21 should differ in the

case of trisomy 21 from disomy 21. It is clearly distinguishable in the case of trisomy of paternal origin, where the extra chromosome comes from the father.

The methodology of quantitative calculations for STR loci on chromosome 21, where was mainly taken into account the relative length of alleles and preferential amplification of the shorter alleles has been developed at our department.

In pregnancies with trisomy 21, it was found statistically more cffDNA for systems in which the length of PCR products ranged mostly between 200 bp - 400 bp (Vodicka et al., 2008).

In total six genes that are specifically expressed in trofoblastic tissues (placenta) has been selected for quantification and genotyping on the DNA level in loci responsible for Down syndrome and for reference SNP genotyping (Böhmova et al. 2010).

These genes are DSCR4, KRTAP26-1, PLAC4, PLAC1, PAPPA and PSG11 of them DSCR4, KRTAP26-1 and PLAC4 are localized on chromosome 21 in the area responsible for Down syndrome. In total 12 SNPs were selected for genotyping in these genes.

Balanced allelic frequency for particular SNP alleles reported in the database of the NCBI website was the criterion for the SNP selection. For individual SNP variants have been defined TaqMan probes. Individual genotypes were identified initially by sequencing of the SNP regions for 30 randomly selected maternal DNA samples. Subsequently TaqMan Real Time PCR optimization and calibration was carried out.

Quantifiler kit (Applied Biosystems) has been used for verification of the quality of the dilutive series of calibration standards. The quantification itself was carried out on 240 DNA samples isolated from plasma of pregnant women in the 1st and 2nd trimester.

Heterozygozity in particular SNP ranged from 14 % to 55 % and the probability of appropriate genotypes capture for each SNP was stated in the range from 12 % to 28 %. Sensitivity of minor genotype capture depends on fluorescence background of non-specifically hybridized probe.

Taqman SNP probes were able to capture as low as 1 percent of artificial genotypic mixture and by the most sensitive systems we have detected 0,22 % artificial genotypic mixture.

R^2 values were in the range of 0,995 to 0,999 and the inclination (angular coefficient) assessing the effectiveness of the PCR (tendency around the values of -3.3 shows the optimum, 100% PCR amplification) from -3.2 to -3.6. All standard calibration curves were appropriate for the cffDNA quantification (Picture 1).
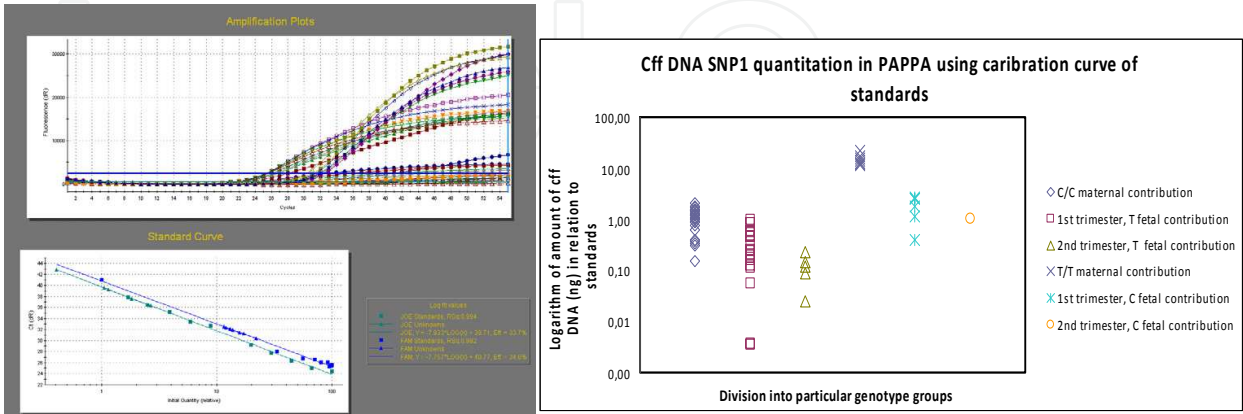
Picture 1. Quantity assessment of cffDNA using calibration curve in SNP1 of PAPPA

Cff DNA was analyzed only for the detectable genotypes. The detection ability ranged from 11.8 % to 100 %. The amount of cffDNA was very variable (from about 0.02 ng to 13 ng

cffDNA in 200 ul of the plasma). All 12 selected SNP are able to detect cffDNA with the desired probability. Parameters of the calibration curve are suitable for the precise quantification of cffDNA (Picture 1) (Böhmova et al., 2010).

7.2 Noninvasive cff mRNA analysis for Down syndrome diagnostics

Placental tissue is ontogenetically much unrelated to blood cells and may express other genes whose expression is missing in maternal blood cells. The first work, which confirmed the RNA of fetal origin, was published in 2000 (Poon et al., 2000).

Using microarray technology in the maternal plasma have been detected RNA markers whose expression is specific to the placenta (Tsui et al., 2004). The same approach was used in the study, which described the cff mRNA transcribed from gene PLAC4 (placenta-specific 4), which is located on chromosome 21.

On the basis of the assumption of uniform batch theory (3 alleles of PLAC4 gene, without allele-specific preferential transcription, express 1/3 more mRNA than 2 alleles) quantitative detection of disomic and trizomic dose was developed. With the use of the SNP in the heterozygous state in the gene coding for the PLAC4 there were detected disomic fetus ratio 1: 1 and trizomic ratio 2: 1 with 90% of the diagnostic sensitivity and 96.5% specificity of trisomy 21 capture by mass spectrometry technique (Lo et al., 2007a).

The disadvantage of this method is relatively labour-intensive preparation of high-quality and reproducible matrix for mass spectrophotometry RNA analysis. Another limiting factor is the real amount of cff mRNA molecules in the isolate from plasma, which could affect the evenness of distribution of mRNA molecules transcribed from individual alleles. Only sufficient number of SNPs can assure desired heterozygozity. Therefore it is necessary to test and introduce new mRNA SNP markers (Go et al., 2007; Yang et al., 2008).

Another approach of measuring allelic SNP ratios in cff mRNA has been tested using digital PCR. It is based on the assumption that, in the PCR reaction is either 1 or no molecule of cDNA. PCR is carried out in parallel in many hundreds to thousands of mixtures. According to the number of positive responses, you can estimate an initial number of cff RNA molecules with a particular SNP and by measuring of the ratios between the SNP from RNA derived from chromosome 21 and another autosome, you can detect chromosome 21 trisomy with high sensitivity (Lo et al., 2007b).

7.3 Noninvasive Down syndrome diagnostics by cffDNA epigenetic detection and quantification

Methodology relies on different methylation of maternal and fetal or placental sequence. Sequences, which are subject to methylation are on principle situated in CpG islands of promoter areas. These islets may regulate the transcription of the gene by variable degree of methylation. In different tissues there are promoters of genes whose expression is tissue specific, and which are methylated differently, and specifically for the type of tissue.

Different fetal (placental) and maternal methylation of regions on chromosome 21 were tracked for the purposes of Down Syndrome non-invasive prenatal diagnostics. Different methylation showed 22 out of 114 assessed genomic regions on this chromosome (Chim et al., 2008).

This relatively large number of methylation specific fetal DNA sequences offers a promising source of prenatal biomarkers independent on maternal and fetal genotype.

Large loss of genetic material (90 %) in the course of DNA modification, based on cytosine deamination using bisulphite, is a disadvantage of this epigenetic way of examination.

The modification procedure, somewhat laborious, in addition increases the risk of contamination. Additional distortion of results could be due to heterogeneous methylation of CpG islands.

Some laboratories are trying to test methods that would eliminate the a forementioned shortcomings (Chan et al., 2006; Tong et al., 2007).

Digital methyl sensitive PCR, methyl sensitive HRM and, probably, massive parallel DNA sequencing are the most promising prospective technologies for precise quantification of epialleles.

7.4 Combination of genetic/epigenetic/cff mRNA methods

To increase the diagnostic sensitivity it is possible to combine methodological approaches, based on cffDNA, cff mRNA and hyper/hypomethylated placental DNA.

Tong et al (2010) published the epigenetic quantification of hypermethylated placental DNA from chromosome 21 (locus HLCS) using digital PCR and compared it to the male specific genetic marker of the Y chromosome.

The relative quantities of the epigenetic marker were shown to be increased for fetus with trisomy 21 compared to disomic fetus. This approach is very sensitive even for the DS diagnosis in the first trimester.

It will be appropriate to use different paternal genetic polymorphic markers at the level of the SNP or STR instead of male specific genetic marker.

To increase the reliability of resolution of trisomy 21 from disomy 21 is also possible to perform relative quantification of cffDNA/cff mRNA using exonal SNP of chromosome 21 and exonal SNP from the reference autosome.

8. Tasks for future

For the successful development of high accurate detection of DS and other frequent trisomies it is necessary to attend to the following tasks:
- Choice of isolation procedures for plasmatic DNA fragments length of which corresponds to the fetal molecules.
- Protection of plasmatic molecules from the disintegration of maternal lymphocytes.
- Prevention of free plasmatic fetal RNA degradation.
- Loss minimization during modification of hyper/hypomethylated DNA.
- Provision of sufficient number of reliable genetic and epigenetic fetal markers.
- More sensitive optimization of calibration and quantification for HRM, Real time PCR and QF PCR.
- Introduction of automation of digital PCR and digital HRM for quantification of CffDNA/cff mRNA/modified cffDNA.

9. Conclusion

Almost 15 years have passed since the first work, which pointed to the presence of the cff DNA in maternal plasma of pregnant women (Lo et al., 1997).

Since then many authors gradually contributed more and more theoretical and methodological knowledge.

Nowadays, also thanks to introduction of new and more robust technologies we can boldly predict that direct clinical outcome of this multi-year effort of noninvasive prenatal diagnostics of Down Syndrome will be feasible in very near future.

10. Acknowledgment

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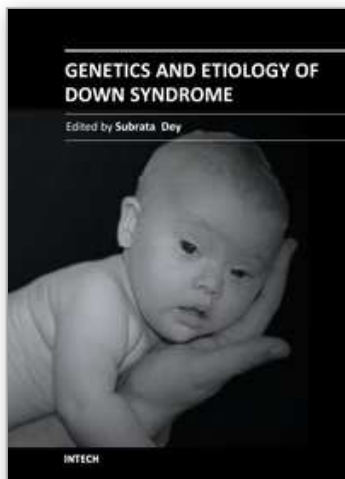
Genetics and Etiology of Down Syndrome

Edited by Prof. Subrata Dey

This book provides a concise yet comprehensive source of current information on Down syndrome. Research workers, scientists, medical graduates and paediatricians will find it an excellent source for reference and review. This book has been divided into four sections, beginning with the Genetics and Etiology and ending with Prenatal Diagnosis and Screening. Inside, you will find state-of-the-art information on: 1. Genetics and Etiology 2. Down syndrome Model 3. Neurologic, Urologic, Dental & Allergic disorders 4. Prenatal Diagnosis and Screening Whilst aimed primarily at research workers on Down syndrome, we hope that the appeal of this book will extend beyond the narrow confines of academic interest and be of interest to a wider audience, especially parents and relatives of Down syndrome patients.

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