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Fetal mRNA in maternal plasma

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Summary

Prenatal diagnosis has become an integral part of obstetric care in the Western world. For the detection of chromosomal abnormalities of the fetus invasive procedures, like amniocentesis and chorionic villus sampling, are necessary to obtain fetal cells. Unfortunately these procedures carry a risk of iatrogenic miscarriage. Fetal trisomy 21, related to advanced maternal age, is the most common reason for women to choose for invasive prenatal tests. The availability of a non-invasive diagnostic test with the same potentials as the present diagnostic test would be ideal. Already for decades development of such a diagnostic test has been a very challenging research goal.

Circulating fetal cells in maternal circulation have been extensively studied but it was not possible to develop a test suitable for population wide implementation. In the blood of pregnant women fetal DNA and placental mRNA can also be detected. This genetic material provides a reservoir of possible biomarkers for the development of non-invasive prenatal tests with diagnostic potentials. In this thesis the studies are directed toward development of a robust non-invasive prenatal test for trisomy 21 using maternal plasma collected in the late first- or early second trimester of pregnancy, preferably easy to implement and at reasonable costs.

Chapter 1 contains a brief introduction on invasive and non-invasive prenatal testing, with the accent on trisomy 21 and describes the outline of the thesis.

In **chapter 2** the cellular origin, biological features and clinical potentials of cell-free fetal nucleic acids, both DNA and mRNA present in maternal plasma and serum during pregnancy are described. For clinical application of cell free fetal DNA in maternal circulation two approaches can be distinguished, a gender and a polymorphism dependent approach. Both already found their way to the clinic. By non-invasive fetal gender assessment an invasive procedure can be avoided and so the number of pregnancies at risk for miscarriage, for example in case of congenital adrenal hyperplasia, are decreased. An example of a polymorphism dependent approach is determination of fetal Rhesus D genotyping in Rhesus negative pregnant women.

In **chapter 3** the recent advancement of technical possibilities for non-invasive aneuploidy tests based on cffDNA and placental mRNA in maternal plasma is reviewed. In small studies and model systems strategies and techniques for potential non-invasive aneuploidy tests are demonstrated. The RNA-SNP allelic ratio strategy seems to be the most feasible test at this

moment with the desirable quality that the result is based on fetal specific genetic material, but has the disadvantage of polymorphism dependency. The combination of several markers might help to enlarge population coverage. Deep sequencing is promising, but at this moment too labour intensive for clinical implementation and at high costs. Great advantage is the fact that the test is gender and polymorphism independent and several aneuploidies can be tested at the same time.

Chapters 4 to 10 contain studies focussing on several aspects of the development of a clinical test for detection of trisomy 21 using mRNA in maternal plasma. At the start we defined criteria for markers for trisomy 21 detection: the gene analysed should be

1. encoded by chromosome 21.
2. located within the Down syndrome critical region (DSCR).
3. expressed in first trimester normal placenta tissue.
4. over expressed by the placenta in trisomy 21 pregnancies.
5. detectable in maternal plasma during early pregnancy.
6. absent in plasma of non pregnant women.

In **chapter 4** the detection of chromosome21-encoded mRNA of placental origin in early placenta tissue and in first trimester maternal plasma was studied. The aim was to identify possible markers for prenatal testing on trisomy 21. Plasma samples were obtained from pregnant women between weeks 9-13 of pregnancy. RNA was isolated from 800 and 1600 μL of plasma by silica-based affinity isolation and, after on-column DNase treatment, subjected to two-step, one tube reverse transcription-PCR with gene specific primers. Three genes which met the above mentioned criteria were tested. One of these chromosome 21-encoded genes, LOC90625, showed strong expression in first trimester placenta and was selected for plasma analysis. RNA from LOC90625 was present in maternal first trimester plasma and could be detected in 60% of maternal plasma samples when 800 μL of plasma was used and in 100% of samples when 1600 μL of plasma was used.

The detection of chromosome 21-encoded mRNA of placental origin in maternal plasma during the first trimester may allow development of plasma-RNA based strategies for prenatal prediction of trisomy 21. LOC90625 is a good candidate gene for this purpose.

In **chapter 5** a large panel of RNA targets, distributed over all chromosomes except for the Y chromosome, known or expected to be present in extra embryonic tissues, was tested for their presence in early placental tissue, presence in pregnant plasma and absence in non-pregnant female plasma. This set included genes coding for transcription factors, genes subject to genomic imprinting, genes coding for non-coding RNA, and other genes with restricted or

abundant expression in trophoblast cells. RNA was extracted from 1.6 mL of maternal plasma by silica-based affinity isolation with use of the QIAamp MinElute Virus Vacuum system (Qiagen) with minor modifications. The two-step, one tube reverse transcription-PCR assay was performed as described previously, except that for a selected set of genes the number of PCR cycles was increased to 50. Three patterns could be distinguished. Pattern C consisted of detectable amounts in pregnant plasma and not detectable amounts in non-pregnant controls (positive/negative). This was the pattern of interest and was observed in eight genes. Two of these genes (GCM1 and ZDHHC1) code for transcription factors. It was demonstrated that this approach permits rapid screening of a large set of potential new markers, it allows the detection of markers not accessible by conventional antibody-based assays. This greatly increases the number of markers that become available for non-invasive prenatal diagnosis.
The search for possible markers is extended in **chapter 6.** A novel method was tested to identify syncytiotrophoblast-derived RNA products in vitro. RNA was obtained selectively by controlled denudation from syncytiotrophoblast cells of an early second trimester trisomy 21 placenta, that was subsequently analyzed by cDNA cloning and microarray profiling. Given the preponderance of 5' mRNA fragments lacking a poly A tail, the placental RNA products were amplified following polymerase A mediated tailing using a method originally designed for small-sized microRNAs. The RNA recovered following denudation is representative of the RNA expressed by and released from the placental syncytiotrophoblast as indicated by the presence of both high and low abundancy targets, i.e. hPL and LOC90625 were recovered reliable before and after amplification. The RNA isolated can be used for cDNA synthesis including cDNA synthesis of small sized RNAs. The 95 bp microRNA precursor of Hsa-Mir-141 was correctly and consistently identified following cDNA synthesis and cloning. So this approach when combined with cDNA library- or microarray expression screening is a novel *in vitro* method to screen for syncytiotrophoblast-derived RNA products representative of trisomy 21 placental RNA as present *in vivo* in maternal plasma.
The mRNA LOC90625, nowadays called C21orf105, was the most promising candidate found as marker for a non-invasive test for trisomy 21. In the study described in **chapter 7** C21orf105 was tested in maternal plasma of women carrying a fetus with or without trisomy 21. Using quantitative RT-PCR we determined transcript levels of target (C21orf105) and reference (hPL) genes in first-trimester plasma samples. Plasma was obtained from EDTA blood, sampled between 9-15 weeks of gestational age, after two sequential centrifugation steps and stored at -70 ˚C. After RNA extraction, quantitative RT-PCR was performed using Taqman probes. From the 51 samples, 43 samples were conclusive. Comparison of transcript

levels of C21orf105 in both groups showed no significant differences. When expressed as ratios of hPL/C21orf105, the differences between trisomy 21 and normal pregnancies remained non-significant. It was concluded that the amount of C21orf105 mRNA in maternal plasma, although situated in the Down syndrome critical region on chromosome 21 and up-regulated in trisomy 21 placentas, is not higher in women carrying a fetus with trisomy 21.

In the mean time another strategy was described, the RNA single-nucleotide polymorphism (SNP) allelic ratio strategy. In this strategy quantitative comparison of the allelic expression ratio of a chromosome 21-encoded gene (meeting the above mentioned criteria for a trisomy 21 marker) enable the detection of the differences between 2 and 3 copies of chromosome 21. Due to the fact that polymorphism is the crux for discrimination, the RNA-SNP allelic ratio strategy can only be employed to a subset of the population with a heterozygosity for the SNP used. Theoretically an increase in population coverage can be obtained by combining the results of several markers. So the availability of useful SNP's was studied. In **chapter 8** we tested 44 SNP's expressed by 7 chromosome 21-encoded, placenta expressed genes for their potential use in non-invasive prenatal diagnosis. Blood samples were collected in EDTA and PAX gene tubes. Within the transcripts of interest, 44 SNPs were identified. Primers flanking these SNPs were designed with similar thermodynamic characteristics to permit RT-PCR analysis in single runs. All primers were intron spanning, except for primers of PLAC4. Using a sensitive, 2-step, 1-tube RT-PCR assay the marker set was tested in placenta tissue, plasma from pregnant women and non-pregnant women. From RNA isolated from whole blood collected in PAX gene tubes, no SNP marker fulfilled the criterion of absence in non-pregnant blood. Identical analysis of hPL RNA excluded false positivity, because in RNA recovered from whole blood in PAX gene tubes, this marker was clearly present and absent, respectively in samples obtained from pregnant and non-pregnant females. Prenatal use of PAX gene tubes appears to be limited to genes with high relative expression differences between placental tissue and maternal blood cells. With the use of RNA isolated from EDTA plasma, 5 of 44 SNP markers were detectable in maternal plasma and absent in non-pregnant plasma. This result permits an evidence-based selection of target genes and markers to increase population coverage.
In the RNA-SNP allelic ratio strategy study from Lo *et al*, the assay used is based on extension of the polymorphic site to generate small but very specific allele-dependent differences in size. This approach requires highly specialized equipment, which might limit its widespread implementation in routine diagnostic setting. In **chapter 9** we adapted and applied

the Transgenomic WAVE System and the quencher extension (QEXT) for measuring heterozygosity and allelic ratio of placental transcripts. The expressed SNP (rs2187247) in exon 2 of the placentally-expressed, chromosome 21-encoded C21orf105 gene was tested in a trisomy 21 model system. For this, RNA selectively released from the syncytiotrophoblast of normal and trisomy 21 placentas, confirmed by karyotyping, was obtained during first trimester. An exact correlation was seen between the results observed by sequencing and the WAVE system used for the identification of heterozygous samples. With respect to the analysis time needed, the WAVE system was superior. Secondly, as optimized and validated with calibration standards consisting of cDNA amplicons (262 bp) of C21orf105, the real-time QEXT assay was highly accurate in determination of allele ratios following optimization of fragment purification, input DNA- and quencher label concentrations, and reporter signal calculation. Thirdly, the optimized and validated QEXT assay correctly discriminated normal and trisomy 21 placentas as tested in clinically relevant combinations: diploid homozygous (CC), diploid heterozygous (AC), triploid homozygous (AAA), triploid heterozygous (AAC or ACC). In conclusion: The QEXT method, which is directly adaptable to current real time PCR equipment, along with rapid identification of informative samples by the WAVE system, will facilitate routine implementation of the RNA-SNP assay for non-invasive aneuploidy diagnostics.

Finally in **chapter 10** the quencher extension technique is tested in clinical samples to discriminate trisomy 21 plasma samples from controls. Despite the numbers tested were low, the proof of principle was demonstrated that by the use of less elaborate assays the allelic ratio strategy can be used on clinical samples.