Large Scale Analysis of Circulatory Fetal DNA Concentrations in Pregnancies Which Subsequently Develop Preeclampsia Using Two Y Chromosome Specific Real-time PCR Assays

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Abstract

Objective: We have recently shown that circulatory fetal DNA levels are elevated in preeclampsia, even before the onset of symptoms. Most studies, however, quantified circulatory male fetal DNA with real-time PCR assays for the single-copy SRY locus on the Y chromosome. We have now examined both, the SRY assay for the single-copy locus and the DYS14 assay for the multi-copy locus in parallel on a cohort of 505 pregnant women, 16 of whom later developed preeclampsia.
Materials and Methods: Pregnant women at approximately 21 weeks of gestation were recruited for this study. Circulatory fetal DNA in maternal plasma samples was quantified by real-time PCR assays specific for the SRY and DYS14 loci.
Results: Circulatory fetal DNA levels were significantly elevated in those patients who subsequently developed preeclampsia when compared to those with uncomplicated deliveries and those complicated by chronic hypertension and IUGR. The SRY and DYS14 real-time PCR assays in this study achieved more than 97% accuracy for identification of fetal gender. The two approaches present a good diagnostic test to predict preeclampsia prior to the onset of symptoms.
Discussion: Our data confirm that circulatory fetal DNA levels are elevated early in pregnancy in those patients who subsequently develop preeclampsia. The data suggest that circulatory male fetal DNA levels can be used as risk markers for early diagnosis of preeclampsia by both the DYS14 and SRY specific real-time PCR assays for the multi-copy and single-copy loci.

Keywords: circulatory fetal DNA, maternal plasma, preeclampsia, prediction validation

Özet

Y Kromozomuna Özgü Gerçek Zamanlı PCR Kullanılarak Sonradan Preeklampsi Gelişen Gebeliklerde Sirkülan Fetus DNA'sı Yoğunluklarının Geniş Ölçekli Analizi

Amaç: Kısa süre önce, sirkülan fetus DNA düzeylerinin preeklampside, daha belirtilerin başlamasından önce arttığını göstermiştik. Ancak, birçok çalışma sirkülan erkek fetus DNA'sı, Y kromozomunda lokalize tek kopya SRY lokusunu gerçek zamanlı PCR kullanarak ölçmüştür. Bu çalışmada, 16'sında sonradan preeklampsi gelişen 505 gebe kadında, eşzamanlı olarak hem tek kopya lokus için SRY testi, hem de çok kopyalı lokus için DYS14 testi değerlendirilmiştir.
Materyal ve Metot: Bu çalışmada gebeliğin ortalama 21. haftasında olan kadınlar kullanıldı. Anneden alınan plazma örneklerindeki fetal DNA'lar, SRY ve DYS14 lokuslarına özgü gerçek zamanlı PCR ile ölçüldü.
Sonuçlar: Komplikasyonsuz gebelikler, kronik hipertansiyon ve IUGR (intrauterine growth retardation: rahim içinde büyüme geriliği) komplikasyonu bulunan gebelerle karşılaştırıldığında, sonradan preeklampsi gelişen hastalarda sirkülan fetus DNA seviyeleri daha yüksekti. Fetus cinsiyetin belirlenmesinde SRY ve DYS14 gerçek zamanlı PCR testlerin kullanımı %97'den yüksek oranda doğru sonuç vermiştir. Bu iki değerlendirme şekli de, preeklampsinin daha semptomları ortaya çıkmadan saptanması için iyi birer tanısal testtir.

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Tartışma: Elde ettiğimiz veriler sonradan preeklampsi gelişen hastaların erken gebelik döneminde fetus sirkülan DNA'sı seviyelerinin daha yüksek olduğunu doğrulamaktadır. Verilerimiz sirkülan erkek fetus DNA seviyelerinin multipl kopya (DYS14) ve tek kopya (SRY) lokusları için özgül gerçek zamanlı PCR'nin, preeklampsinin erken tanısında risk belirleyiciler olarak kullanılmasının mümkün olabileceğini göstermektedir.

Anahtar sözcükler: sirkülan fetus DNA'sı, maternal plazma, preeklampsi, tahmin edebilme gücü

Introduction

The analysis of circulatory fetal DNA in maternal plasma and serum has proven to be the route of choice for the non-invasive assessment of fetal genetic traits, such as fetal RhD status or fetal gender in those at risk for an X-linked disorder (1). Indeed, this approach has proven to be so reliable for the determination of these fetal loci, which it is already being offered clinically by several centres. A further advantage of these analyses is that by the use of real-time PCR, it has been possible to quantify the amount of circulatory fetal DNA in a reliable and reproducible manner (2). Using this approach, elevations in circulatory fetal DNA concentrations have also been noted in a number of pregnancy related disorders including preeclampsia (3), preterm labour (4) and pregnancies with aneuploid fetuses (5).

The reports concerning preeclampsia are of considerable interest, as these have indicated that elevations of fetal DNA in maternal circulation not only occur in cases with manifest preeclampsia, but also early, before the onset of symptoms, in those pregnancies which subsequently develop pre-eclampsia (6-7). It has therefore been suggested that the quantitation of circulatory fetal DNA may serve as a new screening marker for this disorder before the onset of symptoms (8-9).

However, in the first and second trimesters, cell-free DNA concentrations are very low (10). Most studies quantified circulatory male fetal DNA with real-time PCR assays for the single copy SRY locus on the Y chromosome (2-5). The concentrations of single-copy genes are close to the detection limits of PCR methods (10). Recently, our group developed an assay specific to the multi-copy sequence DYS14 (10). We observed that this assay is more accurate than that for the single-copy SRY locus when examining DNA concentrations close to the detection limit (10-11). We have now examined these two assays in parallel in a large scale cohort of 505 pregnant women, 16 of whom later developed pre-eclampsia.

Materials and Methods

Patient cohorts: This study was performed in a retrospective manner. Approval for the study was granted by the Institutional Review Board (Basel, Switzerland). Five hundred and five plasma samples were collected from pregnant women in the second trimester at the Department of Obstetrics and Gynecology, University of Greifswald, Germany. Pregnancy outcome was subsequently determined from the case histories.

Following groups are the study and control groups:
1) Preeclampsia was defined by a blood pressure of ≥140/90 mmHg in 2 determinations 4 hours apart in pregnant women, who were previously normotensive, and an associated proteinuria of ≥300 mg/24 hr after 20 weeks' gestation (12). 2) Chronic hypertension was defined as a blood pressure exceeding 140/90 mmHg before pregnancy or before 20 weeks' gestation without superimposed preeclampsia during pregnancy (12). 3) Intrauterine growth retardation (IUGR) was defined as a birth weight <2500 gram in full-term infants. The IUGR cases which were not associated with smoking, undernutrition, preeclampsia or gestational hyper-

Table 1. The cell-free fetal and maternal DNA in the four study groups by Kruskal-Wallis-Test

	1	2	3	4	*p* value
Cases	n=16	n=14	n=6	n=390	
Male fetuses (Mean)	8	10	4	186	
GA (Mean)	21	21	21	21	0.650
GAPDH GE/ml					0.190
(Mean)	1807	1899	3209	1811	
(Range)	731-4376	243-23 071	910-20 995	388-48 903	
SRY GE/ml					0.014
(Mean)	102	32	45	40	
(Range)	15-169	15-842	33-86	8-194	
DYS14 GE/ml					0.012
(Mean)	253	146	147	132	
(Range)	119-265	59-2322	95-251	19-590	

1: Preeclampsia; 2: Chronic hypertension; 3: IUGR; 4: Normal control; GA: Gestational age at blood taking

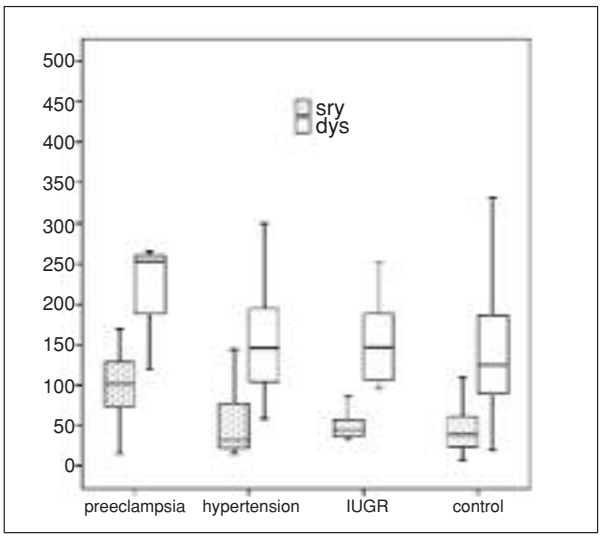

Figure 1. Elevations of cell-free fetal DYS14 and SYR DNA in maternal plasma occur before onset of PET.
Box plots showing concentrations of cell-free DYS14 and SRY DNA in plasma samples obtained from pregnant women with preeclampsia, chronic hypertension, IUGR and normotensive controls. The medians are indicated by a line inside each box, the 75th and 25th percentiles by the box limits; the upper and lower error bars represent the 10th and 90th percentiles, respectively.

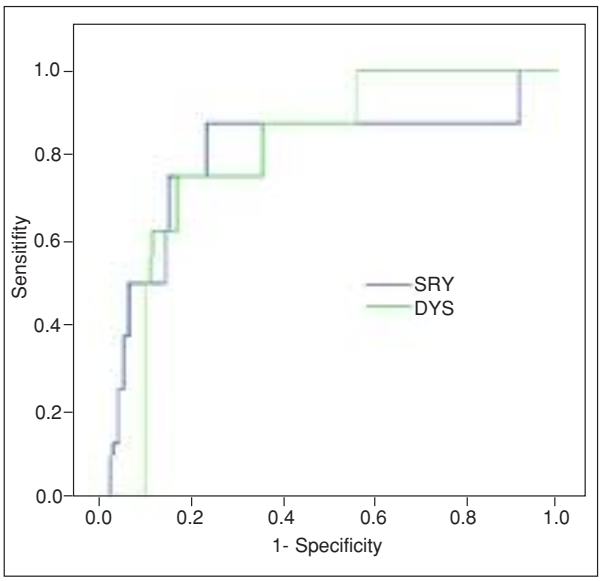

Figure 2. The receiver operating characteristic (ROC) curves. The graph shows two ROC curves, for DYS14 and SRY plotted on the same graph. The area under the ROC curve is 0.8 for both the DYS14 assay and SRY assay.

tension, and congenital malformations were considered as unexplained IUGR (15). 4) Normal controls were defined as gestational age matched normal pregnancies which were not complicated by pregnancy- and non-pregnancy related medical problems.

Processing of blood samples and DNA extraction: Plasma DNA was extracted from 800 µl plasma using the High Pure PCR template kit (Roche Diagnostics, Switzerland) according to the manufacturer's instructions. The DNA was eluted into 80 µl elution buffer, of which 5 µl was used as template for the PCR reaction.

TaqMan real-time PCR analysis: Cell-free fetal DNA and total DNA in the maternal plasma were analysed by TaqMan real-time quantitative PCR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, ABI). The quantities of male fetal DNA were determined using the multiplex TaqMan assay for both the SRY and DYS14 sequences as described previously (10-11). The total levels of cell-free DNA, which indicate the levels of cell-free maternal DNA, were determined using a real-time PCR assay for GAPDH (glyceraldehype-3-phosphodehydrogenase), which is present in all genomes.

The primers and probes used in this study were as described previously (2,10,11).

These TaqMan assays were carried out in 25 µl of total reaction volume using a 2 minute incubation at 50°C, followed by an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 1 minute at 60°C and 15 seconds at 95°C, as described previously (2,10,11).

To determine the genome equivalents (GE) of male DNA and total maternal DNA present in the plasma, a standard dilution curve using known concentrations of male genomic DNA was used. For the conversion to genome equivalents, 6.6 pg was used as described previously (2).

Statistical analysis: The data concerning levels of circulatory DNA were analysed using SPSS for Windows with both the Kruskal-Wallis- and Mann-Whitney-Test, and are described as box plots (Figure 1). The degree of correlation between the levels of fetal SRY and DYS14 circulatory DNA was examined by Pearson's correlation coefficient. Receiver-operating characteristic (ROC) curves were used to determine cut-off points for distinguishing between affected and non-affected individuals (Figure 2).

Results

Accuracy of the two PCR assays for male DNA determination

In this study, we measured cell-free male fetal DNA in the 505 samples from pregnant women with 257 female and 248 male fetuses. The SRY PCR and DYS14 PCR assays in this study achieved 97.42% and 98.02% accuracy for identification of fetal gender, respectively. The concentrations of DYS14 and SRY were significantly correlated (p=0.000, r=0.875). The mean DYS14 DNA levels were found to be 3 fold higher than SRY DNA levels.

Cell-free fetal DNA in maternal circulation increased prior to the onset of preeclampsia

From the four groups of pregnant women (preeclampsia, chronic hypertension, IUGR, normal control), where we analysed the cell-free fetal DNA levels in plasma samples (Table 1). The Kruskal-Wallis-Test, which is used to compare many populations, showed the significant differences between the medians of the cell-free fetal DNA concentration in the four groups (p=0.014 for SRY and p=0.012 for DYS14) (Table 1). Mann-Whitney-Test, which is used to compare two populations, confirmed that only in those patients who subsequently developed preeclampsia, the cell-free fetal DNA concentration in these plasma samples was significantly increased compared to the normal control group (SRY: 102 vs. 40 GE/ml, p=0.007; DYS14: 253 vs. 132 GE/ml, p=0.005).

In those women who had chronic hypertension before their pregnancies and unexplained IUGR, the cell-free fetal DNA concentration in plasma samples was not elevated compared to the normal control group.

The amounts of cell-free maternal DNA (GAPDH) determined did not show any differences between the groups.

Validation of the tests to predict preeclampsia prior to the onset of clinical symptoms

Using the ROC curve, the results of the two approaches for predicting preeclampsia before onset of clinical symptoms was evaluated. Figure 2 shows two graphics with the sensitivity plotted on the y coordinate vs. 1-specificity or the false positive rate plotted on the x coordinate. The optimal discrimination threshold as cut-off point has been selected according to the binary classifier system. The cut-off values of 65.3 SRY GE/ml and 207.5 DYS14 GE/ml were chosen by ROC curve analysis, which provided a sensitivity of 87.5% and a specificity of 76.4% for the SRY assay, and a sensitivity of 75% and a specificity of 82.4% for the DYS14 assay to discriminate between the cases at risk for preeclampsia and the normal controls (Figure 2).

Accuracies of the two approaches for the prediction were measured by the area under the ROC curves. The area under the ROC curve for both SRY and DYS14 assays was 0.8 (Figure 2), indicating a good diagnostic test according to the traditional academic point system.

Discussion

Our large scale analysis of circulatory cell-free fetal DNA concentrations in pregnancies using two Y chromosome specific real-time PCR assays showed that cell-free fetal DNA in the maternal circulation is increased in those pregnancies which subsequently develop preeclampsia, but not in those of chronic hypertension during pregnancy or IUGR.

The pathogenesis of preeclampsia and IUGR remains unclear. Both preeclampsia and IUGR have been often assumed to be related to placental insufficiency (13-14). In our study, however, we found high levels of cell-free fetal DNA in the pregnant women at risk of preeclampsia, but not in those at risk of unexplained IUGR, suggesting a different placental mode of release of cell-free DNA into the circulating system between the two conditions.

In preeclampsia, poor placental perfusion leads to placental ischaemia, from which the aged nuclei and genetic material are apoptotic-necrotically liberated and shed as cell-free debris into the maternal circulation. The cell-free apo-necrotic debris could possibly alter endothelial and vascular function and cause widespread intravascular inflammatory response, which is a typical symptom of preeclampsia (15-16).

In our previous studies, we had found that both the cell-free fetal DNA and placental specific mRNA in maternal circulation were increased in preeclampsia (17). The increments corresponded to the degree of disease severity (3,17). Cell-free fetal DNA and placental specific mRNA has been observed to be associated with placental syncytiotrophoblast microparticles (STBM) (18), which are released into the maternal blood stream during pregnancy. This suggests that exaggerated shedding of placental apoptotic and/or necrotic materials in preeclampsia may cause the high levels of cell-free fetal and placental specific nucleic acids. Subsequently, two groups, Leung et al. and Zhong et al. (6-7) found that cell-free fetal DNA increased prior to the onset of preeclampsia, implying that the cell-free fetal DNA in the maternal circulation could serve as new markers to detect preeclampsia before the onset of symptoms.

In our large scale analysis, we evaluated the diagnostic possibilities of using cell-free fetal DNA in maternal circulation as a risk marker to facilitate the early detection of preeclampsia. We used two approaches to detect the fetal Y-chromosome from maternal plasma which achieved more than 97% accuracy for the identification of fetal gender. To predict preeclampsia before the onset of symptoms, an area under the ROC curve for both SRY and DYS14 assays was 0.8, suggesting that the two tests represent a good diagnostic potential. We selected optimal cut-off points indicated by the ROC analysis for both cell-free SRY and DYS14 fetal DNA levels, suggesting that there was good discriminating power to differentiate between the cases at risk for preeclampsia and the normal controls. Using the levels of plasma SRY and DYS14 as markers to predict the condition before the onset of symptoms, a sensitivity of 75-87.5% and a specificity of 76.4-82.4% to discriminate between the cases at risk for preeclampsia and the normal controls could be achieved, when the optimal cut-off points were selected. The sensitivity and specificity is higher than in the other methods, such as the uterine artery Doppler for preeclampsia screening (19).

In our previous work, we have reported that the DYS14 real-time PCR assay showed a higher sensitivity when compared to the SRY real-time PCR assay (11). For plasma samples from women in the first trimester of pregnancy, better results were obtained by amplifying DYS14 compared with those amplifying SRY (10). In this study, we examined samples

from women in the second trimester of pregnancy. The measurements of male DNA by the DYS14 assay for the multi-copy locus and by the SRY assay for the single-copy locus are comparable. The two assays showed a high accuracy and a good concordance for identification of fetal gender and for the determination of the cell-free fetal DNA levels in maternal plasma.

In conclusion, we performed the first large scale analysis of cell-free fetal male DNA in maternal plasma determined by both DYS14 and SRY real-time PCR. We determined that cell-free fetal DNA in maternal circulation is increased in pregnancies at risk for preeclampsia, but not in condition of unexplained IUGR and chronic hypertension during pregnancy. We evaluated the accuracy and possibilities of using cell-free fetal DNA in maternal plasma as risk markers for predicting preeclampsia prior to the onset of clinical symptoms. The approaches applied in this study are reproducible and simple to perform, and is suitable for large scale clinical screening. Our work presents significant progress in the transition of this research application towards routine clinical use.

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