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Cell free fetal DNA in the plasma of pregnant women with preeclampsia

M.M. Seval[1], H.G. Karabulut[2], A. Tükün[2], A. Koç[1]

[1] Ankara University School of Medicine, Department of Obstetrics and Gynecology, Ankara
[2] Ankara University School of Medicine, Department of Medical Genetics, Ankara (Turkey)

Summary

Objective: Insufficient cytotrophoblast invasion to the myometrium is associated with preeclampsia, especially with the early-onset preeclampsia (before 34 gestational weeks). Several investigations have marked changes in the concentration of cell free fetal DNA in the maternal circulation of women with preeclampsia. However, these studies were not performed for early or late preeclampsia subgroups individually. The present authors planned to determine the levels of the cell free both fetal and maternal DNA in the maternal circulation in early preeclampsia subgroup and compare it with normotensive control cohort. *Materials and Methods:* A total of 16 women; eight of these with preeclampsia and eight normotensive control cohorts with singleton male pregnancy between 28 and 32 gestational weeks were included in the study. Real-time PCR analysis was performed for determining the circulating cell free DNA levels. *Results:* Cell free fetal DNA concentrations were higher in early preeclamptic women than control subjects. The authors found no statistically significant difference in each levels of maternal and total DNA between hypertensive and normotensive groups. *Conclusions:* The present findings suggest that the levels of cell free fetal DNA in maternal circulation were higher in pregnancies which are complicated with early preeclampsia than normotensive controls.

Key words: Fetal DNA; Maternal DNA; Early-onset preeclampsia; Real-time PCR.

Introduction

Preeclampsia is characterized with hypertension and proteinuria after 20 weeks' gestation. It is a disease which is specific to pregnancy and affects approximately 3% – 5% of pregnancies [1]. It is the leading cause of maternal deaths in developing countries [2]. Etiopathogenesis of preeclampsia is not yet well understood, but is thought to arise in the abnormal interaction of maternal and fetal tissue at the uteroplacental interface. Reduced maternal blood flow and oxygenization causing placental ischemia due to insufficient cytotrophoblast invasion to the myometrium, and abnormal remodeling of the uterine spiral arteries, are the pathologic changes in the uteroplacental unit [3]. Pathologic changes in uteroplacental unit and abnormal placental morphologic changes however are associated with the early-onset preeclampsia (before 34 gestational weeks) more than the late-onset preeclampsia [4].

Fetal-derived DNA has been found in the maternal circulation firstly in 1997 [5]. Real-time quantitative PCR assay for measuring the concentration of cell free fetal DNA in maternal plasma and serum has been described [6]. Several investigations have marked an increase in the concentration of cell free fetal DNA in the maternal circulation of women with preeclampsia [7,8]. However, generally these studies were not performed for early or late preeclampsia subgroups individually. The present authors planned to determine the levels of the cell free both fetal and maternal DNA in the maternal circulation in early preeclampsia subgroup and compare it with normotensive control cohort.

Materials and Methods

Pregnant women attending the Ankara University Medical Faculty, Department of Obstetrics-Gynecology, were recruited for the study. The study was approved by the Ankara University Medical Faculty Ethics Committee and all subjects provided written informed consent prior to data collection. Women with singleton male pregnancy between 28 and 32 gestational weeks were included in the study. The gestational ages of all studied subjects were confirmed by early ultrasound examination. Pregnant women with fetal aneuploidy, abnormality of cord insertion or maternal complications such as systemic lupus erythematosus (SLE), diabetes mellitus, hyperthyroidism, urinary infection, vaginal bleeding, and pelvic pain were excluded from the study. All subjects were followed until delivery, and all relevant clinical information was recorded.

Antecubital venous blood was collected before the onset of labour from a total of 16 women; eight of these with preeclampsia and eight normotensive control cohorts. Preeclampsia was determined by a blood pressure of ≥140/90 mm Hg in two determinations four hours apart or by a diastolic blood pressure of ≥110 mm Hg and an associated proteinuria of ≥300 mg/24 hours after 20 weeks' gestation. Patients with severe preeclampsia also met at least one of the following criteria: blood pressure >160/110 mm Hg on at least two occasions, proteinuria of >5 g in a 24-hour

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Table 1. — *Primers and probes for SRY and β-globin real-time PCR.*

Primer	Probe
SRY	
SRY-109F	5'-TGGCGATTAAGTCAAATTCGC-3'
SRY-245R	5'-CCCCCTAGTACCCTGACAATGTATT-3'
SRY-142T	5'-(FAM)AGCAGTAGAGCAGTCAGGGAGGCAGA (TAMRA)-3'
b-globin	
b-globin-354F	5'-GTGCACCTGACTCCTGAGGAGA-3'
b-globin-455R	5'-CCTTGATACCAACCTGCCCAG-3'
b-globin-402T	5'-(FAM)AAGGTGAACGTGGATGAAGTTGGTGG (TAMRA)-3'

urine collection, eclampsia, hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome (total bilirubin concentration >1.2 or lactate dehydrogenase activity >600 IU/L; aspartate aminotransferase >70 IU/L; <100,000 platelets/dl), unremitting headache, right upper-quadrant pain, or intrauterine growth at <5th percentile. Early-onset preeclampsia was defined when the clinical symptoms occurred before 34th gestational weeks.

Maternal blood samples (ten ml) were collected with vacutainer system into tubes containing ethylene diamine tetraacetic acid (EDTA) from the maternal antecubital vein. Plasma separation was performed within one hour after collection. In brief, blood samples were centrifuged at 3,000 g for ten minutes. Plasma samples were then transferred into plain polypropylene tubes and recentrifuged at 3,000 g for ten minutes. The supernatants were collected into fresh tubes and stored at -80°C until used for DNA extraction.

DNA was extracted from 900 µl plasma using a MagNA pure LC DNA isolation kit–large volume with a MagNA pure LC instrument. DNA was eluted into 200 ml elution buffer and stored at -20°C until further testing. Five ml of eluted DNA was used as template in real-time PCR analysis.

Real-time PCR analysis was performed using a specific instrument. The amount of circulating cell free fetal DNA was determined with the use of specific primers for the SRY gene located on Y chromosome. β-globin which is present in all genomes was used to determine the total amount of circulating cell free DNA present in the maternal plasma. Primer and probe sequences are shown in Table 1.

TaqMan amplification reactions were set up in a reaction volume of 20 ml using a specific kit according to the manufacturer's instructions. TaqMan probes and primers were custom-synthesized by TIB MOLBIOL. Primers and probes were used in 0.1 mM and 0.05 mM final concentrations, respectively. Each sample was analyzed in duplicate. A calibration curve was run in parallel and in duplicate with each analysis. The conversion factor of 6.6 pg of DNA per cell was used for expression of results as copy numbers.

Identical thermal profiles were used for both the SRY and the β-globin TaqMan systems. Thermal cycling was initiated by a first denaturation step of ten minutes at 95^{0}C and then 45 cycles of 95^{0}C for ten seconds, 60^{0}C for 30 seconds, and 72^{0}C for three seconds. Amplification data collected by a specific instrument were then analyzed by use of the LightCycler Software version 3.5.

The mean quantity of each duplicate was used for further calculation. The concentration, expressed in copies per milliliter, was calculated by use of the following equation:

$$C = Q \times (V_{DNA}/V_{PCR}) \times (1/V_{ext})$$

C= Target concentration in plasma (copies per milliliter)

Q= Target quantity (copies) determined by the LightCycler instrument in real-time PCR

V_{DNA}= Total volume of DNA obtained after extraction (200 ml in our DNA extraction protocol)

V_{PCR}= Volume of DNA solution used for PCR (five ml in the present real-time PCR set-up)

V_{ext}= Volume of plasma extracted (900 ml in the present DNA extraction protocol).

The data were analyzed using SPSS software. Mann-Whitney rank-sum test was performed to compare nonparametric data between hypertensive and normotensive pregnant women. The alpha level was considered as 0.05.

Results

Eight preeclamptic pregnant and eight control pregnant were considered for the study. Each pregnant had singleton male fetuses and male fetuses were confirmed after birth for each case. Maternal and gestational ages were similar between groups. There were no HELLP syndromes and eclampsia in the study group. Maternal ages, gestational ages, and blood pressures are shown in Table 2.

A series of amplification curves of SRY and b-globin were produced by real-time quantitative PCR on a serial dilution of template prepared from a normal adult male DNA (Figure 1). A linear relationship was observed when

Table 2. — *Maternal age, gestational age, blood pressures, and cell free DNA levels in maternal plasma of the groups.*

	Preeclampsia (n=8)	Normal (n=8)	*p* *
Maternal age (years) - *Median [IQR]*	28.5 [24.5 - 31.75]	32.5 [25.5 - 36.5]	NS
Gestational age (weeks) - *Median [IQR]*	31.0 [30.0 - 32]	30.0 [28.2 - 31.0]	NS
Diastolic blood pressure (mmHg) - *Median [IQR]*	90 [90 - 90]	70 [70 - 70]	0.001
Systolic blood pressure (mmHg) - *Median [IQR]*	150 [145 - 157.5]	110 [110 - 117.5]	<0.001
Mean blood pressure (mmHg) - *Median [IQR]*	110 [107 - 111.2]	83.3 [80.8 - 88.3]	0.001
Fetal DNA (genomeequivalents/ml) - *Median [IQR]*	604 [413.0 - 821.0]	310.0 [100.0 - 423.0]	0.018
Maternal DNA (genomeequivalents/ml) - *Median [IQR]*	5,848.5 [847.7 - 12,992.5]	2,362.0 [1,378.0 - 40,223.0]	0.834
Total DNA (genomeequivalents/ml) - *Median [IQR]*	6,700.0 [1,280.2 - 13,605.7]	2,710.0 [1,479.2 - 40,227.0]	0.916

* Mann-Whitney rank-sum test performed.

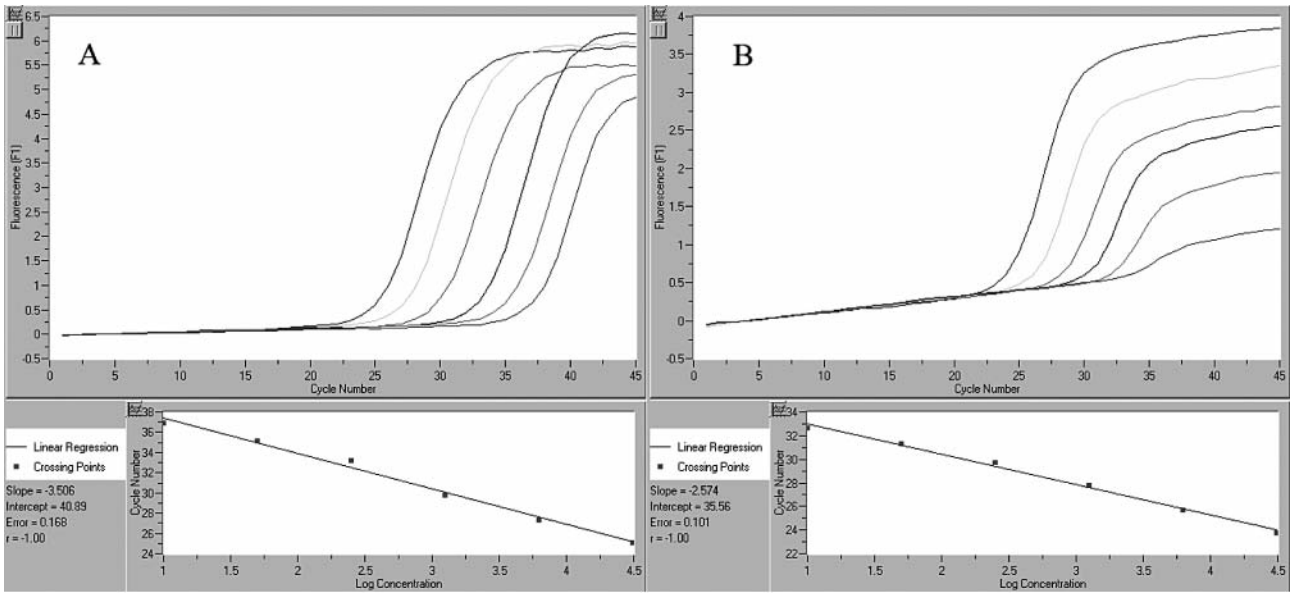

Figure 1. — A series of amplification curves of SRY (A) and b-globin (B) were produced by real-time quantitative PCR on a serial dilution (10 – 31.250) of template prepared from a normal adult male DNA.

the threshold cycle was plotted against the input target quantity, with the latter plotted on a common logarithmic scale. The linearity of the plot indicates that the threshold cycle value could be used to quantify the starting copy number of unknown samples over a wide dynamic range. There was a good correlation between the cycle number and the gene concentration in every assay ($r \geq 0.99$). After confirming the absence of dimmer formation in PCR, the authors calculated the number of SRY copies in each sample using Light Cycler System software. The system was able to detect the DNA equivalent from a single target cell. Positive amplification signals were seen in all tested samples subjected to a TaqMan assay for the beta-globin gene, thus confirming the quality of the DNA samples. This is accepted the control for the ability of serum-extracted DNA to be amplified.

Table 2 shows median cell free fetal, maternal, and total DNA concentrations in preeclamptic and control pregnancies. Cell free fetal DNA concentrations were higher in preeclamptic than control subjects (604 [413–821] *vs* 310 [100-423], $p = 0.018$). There was no statistically significant difference in each levels of maternal and total DNA between hypertensive and normotensive groups.

Discussion

The present authors are able to confirm that the level of cell free fetal DNA was significantly higher in pregnancies complicated by early preeclampsia than normotensive control group. This study also confirms that real-time quantitative PCR is a reliable and reproducible method for the detection and quantification of cell free fetal and maternal DNA [9]. The authors did not detect any false-positive or negative results. Obtaining quantitative information at the threshold cycle before the plateau phase is one of the main advantage of the real-time PCR against the conventional PCR [10]. This feature enables to determine target quantity and accurate and efficient method to PCR.

The definite mechanism of the releasing free extracellular DNA into the circulation is not clear, yet. However it has been hypothesized that fragments from apoptosis and some other form of cell deaths are the most suspected causes of the releasing free extracellular DNA into circulation [11]. Even release of DNA may be a part of physiological process; it would also be increased pathologically with increase cell death or necrosis. Obviously the amount of circulating DNA was generally found to be higher in patients with malignant disorders or injuries, who have higher rates of cell death or damage, than in healthy control subjects [12, 13].

Destruction of circulating fetal cells and liberation from placenta are the suspected sources of the cell free fetal DNA in maternal circulation. Current consensus, major component of cell free fetal DNA in maternal circulation is placental origin [14, 15]. Evidences like very rapid clearance of cell free fetal DNA against fetal cells after birth [16], postpartum presence of cell free fetal DNA in cases with placenta increate [17] suggest theory of placental origin. The classical way of liberation of trophoblast material into maternal circulation is apoptotic release of syncytial knots [18].

Elevation in cell free fetal DNA in preeclampsia may help to increase understanding of enigmatic disorder. Although pathogenesis of preeclampsia is not clear yet, low placental perfusion, hypoxia, and uteroplacental ischemia by the impaired trophoblast invasion are focused mostly on the pathogenesis. It is also indicated that especially early-onset preeclampsia (before 34 gestational weeks) was associated with impaired placental invasion and abnormal placental morphology by studies which investigate placentas of preeclamptic cases morphologically [4]. Association between preeclampsia and increased apoptosis of cytotrophoblasts among placental bed has been reported in recent studies [19]. It is also well understood that placental hypoxia favours necrotic rather than apoptotic shedding of syncytial fragments into the maternal circulation [18]. In addition, cell free DNA has been accepted an indicator of hypoperfusion, tissue hypoxia and cell death [20, 21]. Thus it is suggested that the increased amounts of cell free fetal DNA is liberated from necrotic or apoptotic areas in the placenta [10]. The present authors though increased amounts of cell free fetal DNA in the maternal circulation in preeclampsia is an evidence of increased cell damage in placental bed caused by hypoxia and low perfusion which is very important for the pathogenesis of early preeclampsia.

The present authors could not detect statistically significant difference in cell free maternal DNA between preeclamptic and normotensive groups. In contrast to this study, several investigators indicated increase in the concentration of circulating cell free fetal and maternal DNA together in the plasma of women with preeclampsia, compared with normotensive women [7, 8, 22]. The present authors thought that they could not prove statistically significant difference probably because of the low number of patients in this study. Actually an important limitation of this study is the insufficient number of pregnant included.

The present findings suggest that the levels of cell free fetal DNA in maternal circulation were higher in pregnancies which are complicated with preeclampsia than normotensive controls. The present authors had poor number of patient inclusion and because of this they did not had a chance to compare their results with late preeclampsia cases. This was the major limitation to this study. However they believe that this study is valuable for future reviews with the content of fetal DNA PCRing in a subgroup of early preeclampsia population. Further studies which will compare the level of cell free fetal DNA between both early and late preeclampsia subgroups that have different etiopathogenesis will be helpful to explain pathogenesis of enigmatic disorder. Since cell free fetal DNA firstly detected in maternal circulation in 1997 [5], in such a short period, laboratories in some European Countries have already started to use cell free fetal DNA routinely as part of their prenatal testing for determining fetal RhD status of the RhD-negative pregnant patients [23]. When this rapid progress is considered, using cell free fetal DNA in the routine clinical practice of obstetrical complications like preeclampsia should not be a surprise.

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Address reprint requests to:
M. M. SEVAL, M.D.
Ankara University School of Medicine
Department of Obstetrics & Gynecology
Tıp Fakultesi Street, 001
Mamak, Ankara (Turkey)
e-mail: seval@ankara.edu.tr