Hypermethylated *RASSF1A* in maternal plasma as a non-sex-dependent marker for monitoring of pre-eclampsia

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Objective **Our aim is to investigate whether the quantification of cell-free fetal DNA (cffDNA) using the RASSF1A system will be effective for the detection of severity of pre-eclampsia.**

Materials and methods **We performed real-time PCR after methylation-sensitive restriction enzyme digestion to detect placental-derived RASSF1A sequences (fetal DNA) in the plasma of 120 pregnant women.**

Results **Hypermethylated RASSF1A sequences were detectable in the plasma of all 120 pregnant women. There was an increase in the level of hypermethylated RASSF1A (cffDNA) in mild and severe pre-eclampsia.**

Conclusion **Hypermethylated RASSF1A is a non-sex-dependent marker for predicting pre-eclampsia and can be a useful marker for severity.** ***Med Res J*** **12:53–57**

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Keywords: cell-free fetal DNA, hypermethylated RASSF1A, pre-eclampsia, severity

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Introduction

Pre-eclampsia is a multisystem disorder specific to pregnant women. It remains one of the most important causes of maternal and fetal mortality and morbidity in developed countries [1]. Although the pathogenesis of this condition is not fully understood, it is now widely accepted that vascular endothelial cell dysfunction is part of the maternal phenotype; restricted intrauterinal growth and preterm delivery with increased mortality and postnatal morbidity is part of the the fetal syndrome of preeclampsia [2]. The pathogenesis of preeclampsia is still unclear, although poor placentation and subsequent oxidative damage in the placenta are known to play a role [3]. Epidemiological studies have shown that risk factors for preeclampsia include nulliparity, obesity, and extremes of maternal age [4]. Preterm birth is more often and mortality is higher among male fetuses [5]. Two large retrospective studies concluded that male sex of the fetus is an independent risk factor for adverse pregnancy outcome [6,7]. This might be related to the sex-specific differences in microvascular functions [8]. In addition, induced vasodilatation was found to be altered by preeclampsia and male fetus [9].

Circulating nucleic acids of fetal origin have been found in maternal plasma [10]. The concentration of free fetal DNA is higher in pre-eclamptic pregnancies [11]. There is a correlation between fetal DNA levels and severity of symptoms [12]. The dynamics of fetal DNA concentrations during pregnancy is rather complex, suggested to be because of placental necrosis or apoptosis and also as a result of decreased DNA elimination [13]. Cell-free fetal RNA has also been identified in maternal circulation [14]. No differences were found between the analyzed mRNA from plasma of women with pre-eclamptic and healthy pregnancies [15].

Recently, the use of differential methylation patterns between the promoter of the maspin gene in maternal blood cells and the placenta as the first universal fetal DNA marker in maternal plasma was reported [16]. This observation was based on the rationale that fetal DNA molecules in maternal plasma are mostly derived from the placenta [17] whereas background maternal DNA originate from maternal blood cells [18]. After the discovery of maspin as a cell-free fetal DNA (cffDNA) marker, an opposite differential methylation pattern, namely, hypomethylation in blood cells and hypermethylation in the placenta, of the promoter of the *RASSF1A* gene was reported [19]. These patterns allow the placental-derived hypermethylated *RASSF1A* in maternal plasma to serve as a universal fetal marker irrespective of fetal sex and genetic variations.

We aim to investigate whether the quantification of cffDNA using the *RASSF1A* system will be effective for detection of the severity of pre-eclampsia.

Materials and methods

The current study included 120 pregnant women [60 age-matched women with normal pregnancy representing the control group and 60 cases representing the pre-eclampsia group (30 mild and 30 severe)] among cases attending the antenatal care clinic – Kasr Al Aini hospitals (Obstetrics & Gynecology Hospital) and the Medical Service Unit – National Research Center (Reproductive Health Department) after approval from the local ethical committee. Informed consent was

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obtained from 120 pregnant women and they were informed about the nature of the test and any other test that may be necessary. All patients with any other comorbidities were not included in the study from the start. Preeclampsia cases were identified at the time of the disease at gestational age more than 28 weeks.

Severe pre-eclampsia was defined as blood pressure of at least 160 mmHg systolic or 110 mmHg diastolic with proteinuria more than 5 g in 24 h with or without other features such as oliguria (< 500 ml of urine in 24 h), cerebral or visual disturbances, pulmonary edema or cyanosis, epigastric or right upper quadrant pain, impaired liver function, thrombocytopenia, hemolysis elevated liver enzymes low platelets syndrome, or intrauterine growth restriction.

Sample processing and DNA extraction

Ten to 15 ml of peripheral blood was drawn in an EDTA-containing tube and cell-free plasma samples were obtained by centrifugation of whole blood at 1600*g* for 10 min. Plasma was transferred to microcentrifuge tubes and centrifuged at 16 000*g* for 10 min to remove residual cells. Cell-free plasma was stored at −80°C until further processing; thawing was carried out only once before DNA extraction. DNA extraction from 500 µl cell-free plasma samples was carried out using the QIAamp Mini Kit (Qiagen Gmbh, Hilden, Germany) and eluted with 50 µl of H_2O, and 35 µl of plasma DNA were digested with 100 U of *Bst*UI, a methylation-sensitive restriction enzyme, at 60°C for 16 h [20].

Real-time detection of *RASSF1A*

PCR amplifications were performed using 7500 fast real-time PCR (Applied Biosystems, Foster City, California, USA). The sequences of the primers and probes are listed in Table 1. Each reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nmol/l of each primers, and 85 nmol/l probes. We used 7.15 µl of enzyme-digested plasma DNA mixture as a template for PCR. The thermal profile was 50°C for 2 min, 95°C for 10 min, 50 cycles of 95°C for 15 s, and 60°C for 1 min. All reactions were run in duplicate, and the mean quantity was taken. A methylated DNA (Qiagen Gmbh) was used as the standard [20].

Data analyses were carried out using real-time 7500 fast SDS software v. 2.05 (Applied Biosystems).

Statistical analyses

Statistical comparisons were performed using SigmaStat v.3.0.1a (SPSS, Systat Software, Inc., Chicago, Illinois). In general, a *P*-value less than 0.05 was considered statistically significant.

Results

The current study was carried out on 120 pregnant women [60 women with normal pregnancy representing the control group and 60 cases representing the pre-eclampsia group (30 mild and 30 severe)].The age of the women ranged between 20 and 38 years and all of them at more than 28 weeks' gestational age. Demographic data are summarized in Table 2.

There were no statistically significant differences between the two groups studied in the maternal age, parity, or gestational ages ($P = 0.073$, 0.087, and 0.069, respectively).

Because *Bst*UI is a methylation-sensitive restriction enzyme, hypomethylated DNA sequences, such as the RASSF1A molecules derived from maternal blood cells, are digested and not detected after enzyme digestion. Our results showed that there was an increase in the level of cffDNA in mild and severe cases of pre-eclampsia (mean 166.1 ± 130.38 and 283.62 ± 222.64 copies/ml, respectively). In mild cases, the mean difference of cffDNA was 112.296 (with $P < 0.05$). In severe cases, the mean difference of cffDNA was 227.402 (with $P < 0.05$) (Table 3).

The comparison between all three categories of cases in terms of parity status showed that the cffDNA in primigravidas severe cases were significantly increased in comparison with the mild and control cases ($P < 0.05$), whereas multipara cases did not show any significant difference ($P > 0.05$) (Fig. 1).

Discussion

Pre-eclampsia is one of the leading causes of maternal and fetal/neonatal mortality and morbidity worldwide [1]. The disease occurs in 2–5% of pregnancies in the western countries, but it complicates up to 10% of pregnancies in the developing world, where emergency care is often inadequate or even lacking. Therefore, there is a need for widely applicable and affordable tests that can identify women at risk early in pregnancy and subsequently monitor them throughout pregnancy, and thus provide the best prenatal care for these patients and their children. Furthermore, if these tests could provide useful indications as to which women are likely to develop early-onset pre-eclampsia or a severe form of the disorder, this would allow an accurate risk categorization of women and would enable medical care providers to plan a more tailored course of action, for example a referral to a specialist center. This single action alone decreases neonatal mortality by ∼20% [21]. Currently, there is no single reliable parameter for the prediction of pre-eclampsia, and considerable attention has shifted toward the development of noninvasive testing methods,

Table 1 **Primers and probe sequence for *RASSF1A***

Target	Name	Sequence	Primer/probe
RASSF1A	RSF-b151F	5-AGC CTG AGC TCA TTG AGC TG-3	Primer
RASSF1A	RSF-dsgnR	5-ACC AGC TGC CGT GTG G-3	Primer
RASSF1A	RSF-dsgnT	5-FAM-CCA ACG CGC TGC GCAT (MGB)-3	Probe

Table 2 **Demographic data of the studied groups**

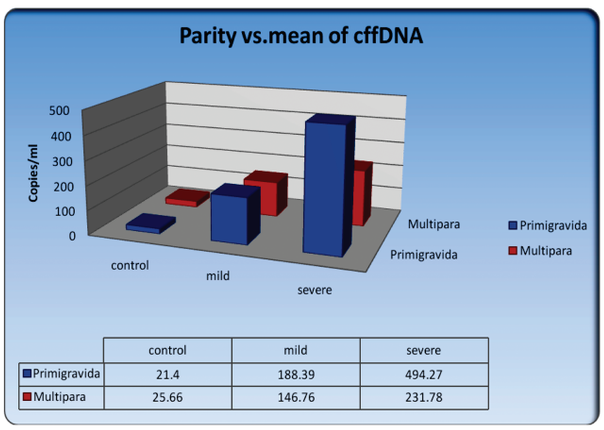
	Mean ± SD		
	Preeclampsia ($n=60$)	Controls ($n=60$)	*P*-value
Maternal age	31.34 ± 5.23	29.12 ± 6.93	0.073 (NS)
Parity	1.4 ± 0.8	1.5 ± 1.0	0.087 (NS)
Gestational age (weeks)	34.4 ± 6.2	33.5 ± 5.1	0.069 (NS)

Table 3 **Results of cffDNA among all different cases**

	Mean (copies/ml)	SD	SE
Control	25.144	19.756	3.85
Mild	166.196	130.38	12.678
Severe	283.6228	222.644	36.544

cffDNA, cell-free fetal DNA.

Fig. 1



Parity status and mean cffDNA in all categories of cases. cffDNA, cell-free fetal DNA.

including ultrasound examination and the quantification of various blood-borne and urinary biomarkers.

The value of cffDNA in maternal plasma as an indicator for preeclampsia was first reported by Lo *et al.* [22], where cffDNA was increased approximately five-fold in women with preeclampsia in the third trimester compared with gestational age-matched controls. Also, the same effect was observed in the second trimester [23]. Levine *et al.* [13] studied 120 pre-eclamptic women and 120 controls: a two-fold to five-fold increase in cffDNA levels was observed starting from week 17 until 3 weeks before the onset of preeclampsia. As the amount of fetal DNA is routinely determined by quantifying Y-chromosome specific sequences, for example *SRY* (sex determining region Y) and *DYS* [24], alternative approaches have been tested to overcome this limitation. Furthermore, approaches to analyze cffDNA independent of fetal sex, using epigenetic differences between maternal and fetal DNA, have been developed, for example the use of the maspin gene, which is hypomethylated in fetal tissue [25] or the hypermethylated fetal promoter sequence of *RASSF1A* [26]. Tsui *et al.* [27] quantified cffDNA using the RASFF1A approach in 10 women with preeclampsia and 20 controls. Our results showed that there was an increase in the level of cffDNA in mild and severe cases of pre-eclampsia (mean 166.1 ± 130.38 and 283.62 ± 222.64 copies/ml, respectively). In mild cases, the mean difference in cffDNA was 112.296 (with $P < 0.05$). In severe cases, the mean difference in cffDNA was 227.402 (with $P < 0.05$).

In the study of Sifakis *et al.* [28], the median cffDNA level was higher in those patients who developed early-onset pre-eclampsia compared with the controls, whereas in the case of late-onset pre-eclampsia, the levels were similar between cases and controls. Interestingly, cffDNA correlated significantly ($P = 0.038$) with the pulsatility index of the uterine arteries, measured by transabdominal color Doppler in patients with subsequent pre-eclampsia, but not in control patients. Thus, the levels of cffDNA appeared to be already increased between 11 and 13 weeks of gestation in patients with subsequent early-onset pre-eclampsia [28,29].

Vlkova *et al.* [30] hypothesize that fetal nucleic acids present in the maternal circulation can enter and be expressed by maternal cells. Especially, monocytes might phagocyte the subcellular particles that protect fetal RNA from degradation. This RNA can be used for translation of encoded proteins potentially breaking the immune tolerance against own maternal antigens (such as the angiotensin receptor 1). This may explain why the cffDNA level is significantly increased in primigravida on comparing multigravida cases in which the body tolerates the exogenous nucleic acids, which might lead to novel alternatives for prevention of pre-eclampsia including the use of RNases *in vivo* [30].

The current hypothesis to explain the increased levels of cffDNA long before the onset of the clinical symptoms of pre-eclampsia proposes that the failure of transformation of uterine spiral arteries during the early stages of placentation may induce aberrant placental perfusion [31,32]. As a consequence, the placenta may become chronically hypoxic, or most likely, alternating periods of hypoxia/reoxygenation within the intervillous space may subsequently trigger tissue oxidative stress and may increase placental apoptosis and necrosis [33]. What likely follows is an increased shedding of necrotic and/or apoptotic subcellular syncytiotrophoblast debris that contain fetal DNA into the maternal circulation [34]. Further evidence that cffDNA may originate from the placenta by apoptosis comes from an in-vitro study examining the effects of oxidative stress induced by 0.5% oxygen for 1 h, followed by reoxygenation, on trophoblastic tissue. Also, the concentration of cell-free β-globin DNA in the tissue supernatant was significantly increased 20 h after hypoxia–reoxygenation [35]. In addition to the evidence for an increased release of cffDNA into the maternal circulation in pre-eclampsia, there is also solid proof of reduced renal clearance of these molecules in this pregnancy condition. In normal pregnancy, cffDNA is detectable as soon as 5 weeks after

coitus, its levels increase with gestational age, and it completely disappears after 2 h following delivery (the mean half-life for circulating fetal DNA is 16.3 min, range 4–30 min) [36]. In contrast, Lau *et al.* [37] reported a much slower clearance rate in pre-eclamptic patients, with a median half-life of cffDNA clearance that was four times longer than that in unaffected women (114 min in the pre-eclamptic group vs. 28 min in the control group). The fast disappearance rate may be because of an efficient renal clearance in normotensive women, which is impaired in pre-eclampsia. However, other organs, such as the liver, may also contribute toward the impaired clearance of circulating DNA in pre-eclamptic patients [38].

Conclusion

The current study shows that hypermethylated *RASSF1A* (fetal DNA) in maternal plasma of pre-eclampsia represents a useful noninvasive approach that is sex and polymorphism independent for assessment of pre-eclampsia.

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Conflicts of interest

There are no conflicts of interest.

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الملخص العربي

البروتين دي إن أيه الحر الخاص بخلايا الجنين كعامل مبين لحدة ارتفاع ضغط الدم أثناء الحمل

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ارتفاع ضغط الدم أثناء الحمل هو اضطراب مرتبط بالحمل، وقد أبلغ عن زيادة الخلايا الجنينية الأنوية وارتفاع في الحمض النووي الحر في دم الأمهات اللاتي يعانين هذا الاضطراب. لقد أجريت هذه الدراسة على عدد 120 سيدة حامل من المترددات على عيادة متابعة الحمل (مستشفى التوليد وأمراض النساء – القصر العيني وقسم بحوث الصحة الإنجابية وتنظيم الاسرة، المركز القومي للبحوث. نتائجنا تظهر أن هناك زيادة في مستوى الحمض النووي الجنيني الحر في الحالات المعتدلة وخطيرة من تسمم الحمل. يتبين لنا في الدراسة الحالية للانحرافات الكمية للجين في بلازما الأم المصابة بتسمم الحمل أنه نهج مفيد غير اجتياحي وغير معتمد على الجنس وتعدد الأشكال لتقييم ما قبل الولادة لتسمم الحمل. إن الفائدة الإكلينيكية لهذا النهج يجب أن تتم على نطاق أوسع في حالات ذات المراحل المبكرة من تسمم الحمل، بالإضافة الى ذلك إن التقدم السريع في مجال بحوث ما بعد الجينوم يفتح أفاقاً كثيرة لتشخيص ما قبل الولادة غير الاجتياحي لذلك، مع استخدام تسلسلات غير معتمدة على الجنس، قد تكون الأحماض النووية الجنينية شيء أساسي في العالم في الرعاية الروتينية للحوامل واقعاً ملموساً في غضون سنوات قليلة.