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TIMPs Expression as A Maternal Cell Free Plasma Biomarker of Severe Preeclampsia: A Case-Control Study

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Abstract

Objective: Preeclampsia (PE) is a pregnancy related disorder with prevalence of 6-7%. Insufficient trophoblastic invasion leads to incomplete remodeling of spiral arteries and consequent decrease in feto-placental perfusion. Altered placental expression of tissue inhibitors of matrix metalloproteinase (TIMPs) is considered to be involved in this process while the balance between matrix metalloproteinases (MMPs) and TIMPs contributes to remodeling of the placenta and uterine arteries by degradation and refurbishing of extracellular matrix (ECM). Therefore, TIMPs, fetal expression pattern was evaluated with the aim of its potential to be used as a determinant for the (early) detection of PE.

Materials and Methods: In this case-control study, cell free fetal RNA (cffRNA) released by placenta into the maternal blood was used to determine expression patterns of *TIMP1, 2, 3* and *4* in the severe preeclamptic women in comparison with the normal pregnant women. Whole blood from 20 preeclamptic and 20 normal pregnant women in their 28-32 weeks of gestational age was collected. The second control group consisted of 20 normal pregnant women in either 14 or 28 weeks of gestation (each 10). cffRNA was extracted from plasma and real-time polymerase chain reaction (PCR) was done to determine the expression levels of *TIMP1, 2, 3* and *4* genes.

Results: Statistical analysis of the results showed significant higher expression of *TIMP1-4* in the preeclamptic women in comparison with the control group (P=0.029, 0.037, 0.037 and 0.049, respectively). Also, an increased level of *TIMPs* expression was observed by comparing 14 to 28 weeks of gestational age in the normal pregnant women in the second control group.

Conclusion: An increased cffRNA expression level of *TIMPs* may be correlated with the intensity of placental vascular defect and may be used as a determinant of complicated pregnancies with severe preeclampsia.

Keywords: Cell Free Fetal RNA, Gene Expression, Preeclampsia, Tissue Inhibitors of Matrix Metalloproteinase

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Introduction

Preeclampsia (PE), a multi-system disorder of pregnancy, is diagnosed by new onset of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive woman. A systolic blood pressure>160 mmHg or diastolic blood pressure >110 mmHg defines severe preeclampsia. PE is one of the leading causes of maternal/fetal morbidity and mortality, which affects 6-7% of all pregnancies (1). The main etiology of PE is still unclear, but it seems that several factors such as placenta are largely involved. While there is not a general agreement on the amount of proteinuria to define the severity (2) although, several other symptoms such as edema, renal or liver failure and the hemolysis, elevated liver enzymes and low platelet counts (HELLP syndrome) may also develop based on organ involvement (1). PE can lead to early-onset of severe hypertension accompanied with a fetal growth restriction that requires pregnancy termination before 34 weeks of gestation. Late-onset mild hypertension that needs to terminate the conception or after 34 weeks of gestation with a normally grown fetus may also represent as a milder form of the disease (3). It is assumed that the poor cytotrophoblastic invasion in the PE leads to abnormal remodeling of the uterine arteries and inadequate oxygen delivery to developing utero-placental unit (4). This will consequently lead to placental endothelial dysfunction and oxidative stress (5). Delivery of the placenta and fetus is the only treatment to prevent maternal organ injury (6), while early detection of PE helps to plan appropriate monitoring and clinical

management (7).

The recent discovery of fetal cells and cell free fetal nucleic acids in the maternal blood has provided a new possibility for a non-invasive prenatal diagnosis (8). Analysis of fetal RNAs in the maternal plasma has also produced valuable information about the condition of gene expression in fetal tissues in the complicated pregnancies. The cell free fetal RNA (CffRNA) was not expected to be present in the plasma due to the presence and action of ribonuclease (RNAses) (8, 9). However, recent studies revealed an altered level of placental-specific mRNA coding for corticotrophin releasing hormone (CRH) in the PE (10). In addition, cell-free mRNA concentrations of CRH, PLAC1, and P-selectin are increased in the plasma of pregnant women with preeclampsia (11).Subsequently, it was postulated that the placental-expressed mRNAs were encapsulated within a syncytiotrophoblast-derived microvesicle (STBM), which provides RNase resistance to "cell free" fetal RNA in the maternal plasma in comparison with the maternal RNA (11). It is known that tissue inhibitors of matrix metalloproteinase (TIMPs) adjust the matrix metalloproteinases (MMPs) activity in different stages of pregnancy (12, 13). So during pregnancy, the level of MMPs/TIMPs activity plays an important role in the uterine spiral arteries remodeling through governing cytotrophoblastic invasion (14, 15). This suggests that MMP/TIMP imbalances could have an important role in the implantation failure and placental development (16).

Actually the expression pattern of *TIMPs* gene family has been previously evaluated in the PE using samples from placenta or whole RNA (but not the fetal component) from maternal plasma. In this study and for the first time, we used maternal blood and cell free fetal RNA to compare those with and without PE (fetal component) with the aim of its potential application for early and non-invasive prenatal screening/diagnosis.

Material and Methods

Samples were provided by the prenatal unit, Imam Khomeini Hospital Complex, Tehran, Iran. A written informed consent, approved by the Royan institutions' Ethical Committee of the Research Council, Tehran, Iran (IR.ACECR.ROYAN.REC.1394.24) was received from all the participants.

Study groups

In this pilot case-control study, twenty severe preeclamptic women that suffered of average proteinuria of 3+ and systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥110 mmHg) participated and also, twenty normal pregnant women were included as a matched control group in their 28-32 weeks of gestational age. We also evaluated the second control group which consisted of 10 pregnant women in 14 and 10 other pregnant women at 28 weeks of gestational age as an additional investigation.

All participants aged 20 to 45 years old. Chronic hypertension before 20 weeks of pregnancy, history of gestational diabetes and kidney disease comprise our exclusion criteria. Actually, the normal control women were experiencing their first or subsequent pregnancies and all of them did not have any history of PE in their previous pregnancies. The sample size was based on the similar published works (17).

Plasma collection

To find out the probable variability of *TIMPs* cell free fetal expression level, as potential biomarkers for severe preeclampsia, we collected blood samples of the normal pregnancy at 14 weeks of GA and 28 weeks of GA.

Peripheral blood (10 ml) was collected from all 60 participants (20 PE cases, 20 normal pregnant gestational matched controls and 10+10 normal as a second control group at 14 or 28 weeks) in a K3EDTA tube (BD Biosciences, USA). Then, the tubes were centrifuged at 1600 g for 10 minutes at 4ºC. Plasma was carefully separated and transferred into 1.5 mL micro tubes (GUNSTER BIOTECH, TAIWAN). The plasma was re-centrifuged at 16000 g for 10 minutes and 4ºC. Then supernatants were transferred into 2 mL cryovials (SPL LIFE SCIENCES, Korea). The plasma samples were frozen in liquid nitrogen and stored at -70ºC.

Extraction of cell free fetal RNA and cDNA synthesis

The plasma samples were centrifuged at 16000 g for 5 minutes at 4ºC following melting at room temperature. Extraction of cffRNA was done from 3 mL of plasma according to manufacturer's protocol (Cat. No. 55114, QIAamp Circulating Nucleic Acid Kit, Qiagen, Germany). CffRNA was eluted in the AVE buffer (Cat. No. 55114, QIAamp Circulating Nucleic Acid Kit, Qiagen, Germany) and stored at -70ºC until later use. Using reverse transcription process (RT) and following manufacturer's protocol (Cat. No. 11754, SuperScript VILO cDNA Synthesis Kit, Invitrogen, USA.) complementary DNA (cDNA) was produced from each RNA template.

Real time polymerase chain reaction

Primers were designed by the Perl Primer (Table 1) and cell free fetal mRNA of *TIMP* genes were amplified. As housekeeping control gene, *18s* was employed. Real time polymerase chan reaction (PCR) was set up using SYBR green, according to the manufacturer's instructions (Cat No: 4367659, Applied Biosystems by Thermo Fisher Scientific, USA) in a reaction volume of 20 µL (each reaction contained: 5 µL SYBR green, 1 µL forward

and 1 µL reverse primer of 100 Pico mole/µL, 2 µL cDNA with concentration of 50 ng per µL and deionized water). Reactions were prepared in duplicate. In each PCR run, cDNA sample of preeclamptic women and their matched controls were examined under the same condition. cDNA samples from the second control group were evaluated in separate reactions. Real time PCR was carried out as follows: primary denaturation for 4 minutes at 95ºC, 10 seconds of denaturation at 95ºC followed by 1 minute of annealing/extension at 60ºC (repeated for 45 cycles), and ultimately the melt curve analysis for 15 seconds started at 95ºC. Normalization of data was performed using *18s* as housekeeping gene (ΔCt). The comparative threshold cycle method was used to calculate the changes in the relative gene expression ($2^{-\Delta\Delta Ct}$).

Table 1: Primer sequences for *TIMPs* real time polymerase chain reaction reactions

Gene	Primer sequences (5´-3´)	Product size (bp)
18s	F: GTAACCCGTTGAACCCCATT	151
	R: CCATCCAATCGGTAGTAGCG	
TIMP1	F: GAA GTC AAC CAG ACC ACC T	181
	R: TTC CAG CAA TGA GAA ACT CCT	
TIMP2	F: CGACATTTATGGCAACCCT	217
	R: GCACGATGAAGTCACAGAG	
TIMP3	F: CAAGCAGATGAAGATGTACCGA	228
	R: GTGATACCGATAGTTCAGCCC	
TIMP4	F: CTGCCAAATCACCACCTG	193
	R: CGATGTCAACAAACTCCTTCC	

Statistical analysis

The results were analyzed using SPSS software (Version 16.0, IBM, USA). Normalization of the result was validated by the one sample K-S test (normal distribution: P>0.05). T test was used to compare the relative expression of *TIMP* genes in preeclampsia, first and second normal groups. The P≤0.05 were considered as statistically significant.

Results

The main demographic and biophysical features of the study groups are summarized in Table 2. Totally, 60 samples were analyzed, including 20 preeclamptic and 40 normal pregnant women. We evaluated cell free expression patterns of *TIMP* genes of maternal plasma. The results indicated a significant increase in the *TIMPs* expression of the preeclamptic group in comparison with the matched normal group (P≤0.05, Fig.1).

To find out the probable variability of *TIMPs* cell free fetal expression level, as potential sever PE biomarkers, we collected blood samples of the normal pregnancy at 14 weeks, of GA and 28 weeks of GA. In second control group the results confirmed the presence of all *TIMP*s expression in both 14 and 28 weeks of normal pregnancies while the level of expression was relatively increased from 14 to 28 weeks of gestational age. Enhanced expression in 28 weeks compared to 14 weeks (along with the increase in the placental mass) confirms the placenta as the source of fetal cell free RNAs (Fig.2).

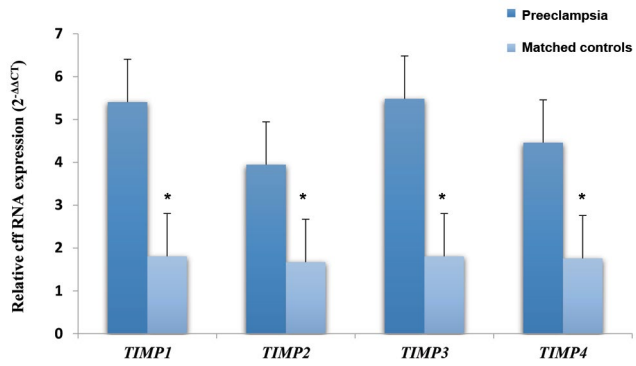

Fig.1: Plot of real-time polymerase chain reaction analysis of *TIMP1, TIMP2, TIMP3,* and *TIMP4* gene expression in maternal plasma of preeclamptic (PE) pregnancies versus their matched control group using cell free fetal RNA (cffRNA). All *TIMPs* showed significant increase in PE pregnancies compared to matched control. *; P=0.029, 0.037, 0.037, and 0.049, respectively.

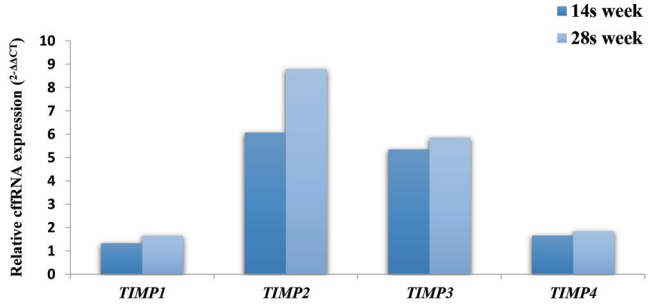

Fig.2: Plot of real-time polymerase chain reaction analysis of *TIMP1, TIMP2, TIMP3,* and *TIMP4* gene expression in maternal plasma of 14 weeks healthy pregnancies in comparison with 28 weeks normal pregnancies using cell free fetal RNA. Enhanced expression in 28 weeks compared to 14 weeks (along with the increase in the placental mass) indicates placenta as the source of detected cell free RNAs.

Table 2: Demographic and biophysical variables of case and control group

Charactristics	Normal pregnant women (matched control group)	Severe preeclamptic women (case group)
Sample size (n)	20	20
Age (Y)	28.2 ± 4.7	32.7 ± 5.2
Systolic blood pressure (mmHg)	112.0 ± 7.1	165.0 ± 14
Diastolic blood pressure (mmHg)	80.5 ± 6.0	111.5 ± 8.7
Proteinuria (1-4 plus), median (IQR)	-	+3 (+2 to +4)
Gestational age (weak)	30.4 ± 1.5	30.6 ± 1.5

Data are presented as mean ± SD. SD; Standard deviation and IQR; Interquartile range.

Discussion

PE, an important cause of prenatal morbidity, leads to at least 50 thousands of annual maternal deaths all around the world. It is clinically diagnosed by the de novo hypertension and proteinuria after 20 weeks of gestation (18). The trophoblastic cells invasion reduction is considered as the central process of the PE pathophysiology which in turn leads to an incomplete remodeling of maternal spiral arteries and ultimately, the poor placental invasion (5). Currently, the only treatment to prevent maternal organ injury is pregnancy termination as soon as possible and delivery of the placenta and fetus (6).

It is known that in the normal pregnancies, artery remodeling changes, including angiogenesis and trophoblastic invasion are maintained by the specific enzymes, MMP/TIMP (19). In pregnancies complicated by preeclampsia, an imbalance in MMP/TIMP activity may cause poor placental perfusion (13, 15). There are different TIMPs protein in human (TIMP1-4) which all act as endogenous inhibitors of active MMPs protein (20). TIMP proteins are crucial enzymes for the invasion processes of different types of cells, including cytotrophoblasts invading the uterus in human placentation (21).

There is not available, a clinically efficient screening test to accurately predict the occurrence of PE before its clinical onset (7). Discovery of placental/fetal mRNA in the maternal plasma may be a promising noninvasive biomarker (22). Cell free fetal mRNA unlike cffDNA is not dependent on the fetal gender and genotype (23). Also, a placenta derived mRNA, that is detectable in the maternal plasma at 4 weeks of gestation, is rapidly cleared after delivery (6).

Several studies have previously examined the expression patterns of *TIMP* genes at the protein level and reported significant changes in preeclamptic mothers (14, 24-26). To the best of our knowledge, there are no report to examine maternal plasma and fetal fractions of cell free RNA. The present study has provided a compilation of the cffRNA expression of *TIMP* genes ofthematernal plasma. Wecomparedit in the preeclamptic affected with thenormal pregnancies. Accordingly, wereport herefor the first time,a significant change in the degree of cffRNA expression in plasma of PE pregnancies.

TIMP1, a specific inhibitor of MMP9 and a proteolytic enzyme, mainly degrades the extracellular matrix (ECM). It also has an important role in a trophoblastic invasion (27). In pregnancies complicated with PE, an increased level of TIMP1 protein prevents the endothelial cell migration in the uterine vessels which may lead to an incomplete remodeling of spiral arteries (13, 14, 28). For the first time, we evaluated the expression of *TIMP1* gene in the maternal plasma using cffRNA, which showed a significant increase in the PE women in comparison with the normal matched control group. This is consistent with previous studies that reported an increased levels of TIMP1 protein in the placental cells of PE groups (29).

TIMP2, the specific inhibitor of MMP2 (30), acts as the first mediator of trophoblast invasion into the endometrium. It also participates in the remodeling of arteries and angiogenesis in early pregnancy (31, 32). In normal pregnancies, physiologic remodeling of the endothelial layer and degradation of elastic muscular-vascular tissues may increase the blood supply to the developing fetus (14). However, in preeclampsia, collagen aggregation reduces the vessel wall elasticity and blood supply to the fetus where ultimately hypoxic damage happens. Increased level of TIMP2 and or decreased amounts of MMP2 may lead to the collagen aggregation (14) which is the triggers of preeclampsia. According to our data, the expression level of *TIMP2* cffRNA in the PE group showed a significant increase in comparison with the normal matched control group. This is also in agreement with the results of previous studies of placenta samples (13, 14).

TIMP3 is expressed in various tissues, and shows highest expression in the placenta. TIMP3 is considered as an effective factor in the process of fetal implantation and decidualization through regulating trophoblast invasion (25). Increased expression of TIMP3 protein inhibits the ECM degradation which is necessary for an efficient implantation through inhibition of MMPs (28, 32). Also,

TIMP3 protein prohibits the trophoblast invasion and leads to a defective remodeling of spiral arteries in the PE women (25, 33). Our results confirm a significant increase in the *TIMP3* expression in maternal blood of the PE group in comparison to the normal matched control group, which is in accordance with the previous reports (24, 25, 29).

The present study also indicates a significant increase in the *TIMP4* expression of the plasma cffRNA content of the preeclamptic participants in comparison with our normal matched control group. This finding verifies the result of the only previous study that showed elevated plasma protein levels of TIMP-4 in the PE pregnancies in comparison with the chronic hypertensive cases (26). The relatively higher potency of TIMP4 action on the MMP2 pathway compared with other *MMPs* gene, suggests that *TIMP4* similar to *TIMP2* effects more specific on the *MMP2* gene (34). As important role players in the ECM, MMPs develop the endothelial cell migration and trigger angiogenesis. TIMP4 has an anti-angiogenesis activity and prevents formation of endothelial cell tube (35, 36). Therefore, the increased expression of *TIMP4* in PE could be a possible reason of the spiral arteries invasion limitation, which in turn will reduce the blood supply to the fetus and triggers hypertension in the pregnant women (14, 34).

Co-expression of *MMPs* and *TIMPs* in the trophoblasts suggests that the invasive and lytic properties of cells effects on the ECM depend on the MMPs/TIMPs balance (37). Increased expression of *TIMP*s can possibly reduce MMPs function which in turn may suppress matrix disintegration necessary for efficient implantation. This can also inhibit the trophoblast invasion that leads to an incomplete modification of spiral arteries. Particularly, an impaired remodeling of the spiral arteries have been considered as a major contributor to the PE (24, 25).

For a quite long time, non-invasive PE-specific markers have been introduced. For the first time in the present study, the *TIMPs* cffRNA expression of maternal plasma was evaluated and we observed a significant elevated expression in the PE patients in comparison with the normal matched control pregnancies. In addition, we detected the presence of *TIMP*s expression in the 14 weeks GA as well as 28 weeks GA in the healthy second control group pregnant women that showed a relative increase in the latter one. Our finding shows that *TIMPs* expression is detectable from early pregnancy due to the placenta origin of the cell free nucleic acid. An increase in gestational age associated with the subsequent placental size may be accompanied by higher amounts of cell free fetal RNA in the maternal plasma. Taking all together, the expression level of *TIMPs* cell free fetal may determine placental health and may reflect the severity of the preeclamptic disorders. Further studies are needed to give promise of biomarkers for early detection/prognosis of complicated pregnancies such as preeclampsia. Also, a follow-up case control study is needed to be able to clearly determine the cause and effect relationship. Although, due to the limitations in recruitment of the patients/controls, current setup was used. Therefore, further studies will be required to validate our results and to define the detection thresholds through integrated bioinformatics calculations.

Conclusion

Cell free fetal RNA derived from the placenta and released to maternal plasma creates a promising approach for noninvasive prenatal diagnosis independent of fetal gender and genotype. Here, we concluded that the pattern of fetal *TIMPs* expression within maternal plasma can be used as a potential PE biomarker in early pregnancy.

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Authors' Contribution

S.S.; In charge of practical lab works, drafting the manuscript, and was responsible for sample collection. H.N., M.Sh.; Involved in developing the concept and experimental design. F.Gh.V., F.R.; Involved in clinical management and sample collection. R.F.; Involved in the laboratory part of the project and helped in primer designing. A.Gh.; Involved in statistical analysis of data. E.E.; Involved in data generating helped in sample collection. M.Z.; Designed and supervised the project, data interpretation, and drafting and revision of the manuscript. All authors read and approved the final manuscript.

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