Promoter hypomethylation of TIMP3 is associated with pre-eclampsia in a Chinese population

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Submitted on October 11, 2012; resubmitted on November 5, 2012; accepted on November 9, 2012

ABSTRACT: A study by Yuen RK, Penaherrera MS, von Dadelszen P, McFadden DE, Robinson WP. DNA methylation profiling of human placentas reveals promoter hypomethylation of multiple genes in early-onset preeclampsia. Eur J Hum Genet 2010;18:1006–1012 based on a Canadian population found the tissue inhibitor of the metalloprotease 3 (TIMP3) gene to be hypomethylated in pre-eclampsia (PE) placentas and to be a potential prenatal marker for early onset PE. To further explore the role of TIMP3 in PE and to investigate whether the TIMP3 promoter shows the same methylation pattern in the Han Chinese population, we analyzed a complete methylation assay of TIMP3 including the promoter region studied in the Canadian report and the neighboring CpG island in placentas (cases n = 41, controls n = 22) maternal peripheral blood (cases n = 3; controls n = 6) and umbilical cord blood (cases n = 7; controls n = 8) using MassArray EpiTyper (Sequenom, San Diego, CA, USA). Our results confirmed the finding of aberrant TIMP3 promoter methylation in PE placentas (mean = 0.405) compared with those in controls (mean = 0.534, P = 9.40 × 10−7). A tissue-specific methylation pattern between placentas (mean = 0.459) and bloods (mean = 0.961, P = 6.91 × 10−13) was also demonstrated in our clinical samples. Furthermore, a nearly 2-fold increase in TIMP3 expression for the hypomethylated promoter was found in PE placentas (P = 0.007), pointing to a negative relationship between TIMP3 methylation and the expression (R = −0.758, P = 0.029). In conclusion, we replicated the findings of Yuen et al. in our Han Chinese-based study, confirming that TIMP3 is likely to be involved in the etiology of PE and that hypomethylated and placenta-specific TIMP3 may be a potential marker for early diagnosis of PE in maternal plasma.

Key words: pre-eclampsia / prenatal marker / prompter methylation / replication / TIMP3

Introduction

Pre-eclampsia (PE; MIM 189800) is a pregnancy-associated disorder characterized by de novo hypertension and proteinuria at or after 20 weeks’ gestation (Redman and Sargent, 2005; Sibai et al., 2005). The disease is a major contributor to perinatal maternal-fetal morbidity and mortality (Redman and Sargent, 2005) affecting 5–8% of nulliparous pregnancies worldwide (Wang et al., 2012), as well as increasing the risk of preterm birth and intrauterine growth retardation. Patients with PE also exhibit multisystemic symptoms, including edema, renal/liver failure and disturbance of hemostasis (Grill et al., 2009).

The precise etiopathogenesis of PE has been a focus of interest to both clinicians and scientists for decades. Recent studies have suggested that epigenetic alterations, including changes in DNA methylation status, and environmental factors play a role in the onset of PE (Kanayama et al., 2002; Chelbi et al., 2007; Knox and Baker, 2007). Consistent with this, women who conceive with assisted reproduction techniques or who have preexisting hypertension, diabetes, obesity or smoking have a higher risk of developing PE (Wang et al., 2002). On the other hand, multivitamin supplements containing folic acid and vitamin B12 are correlated with reduced risk of PE (Bodnar et al., 2006), indicating the potential role of DNA methylation in the pathophysiology of the disorder. Given the complex pathogenic factors and heterogeneity of PE, no effective predictive measures have so far been identified for the disorder. The application of cell-free fetal DNA (cfDNA) with epigenetic differences between maternal blood and fetal (placental) DNA in noninvasive prenatal diagnosis and monitoring of PE are currently being investigated (Chim et al., 2005; Chan et al., 2006). The APC (Muller et al., 2004), RASSF1A (Chim et al., 2005) and SERPINA3 (Chelbi et al., 2007) genes with aberrant promoter

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methylation have been suggested as potential markers in prenatal diagnosis of the disease.

In a recent genome-wide study, Yuen et al. (2010) investigated the methylation differences between pre-eclamptic placentas and normal samples using an Illumina GoldenGate Methylation panel 1 array. Their results highlighted several genes with gene-specific hypomethylation in early onset pre-eclampsia (EOPET) placentas, especially the promoter of the tissue inhibitor of metalloproteinase 3 gene (TIMP3; \( P = 0.00001 \)). They also showed that TIMP3 with a tissue-specific methylation in bloods and placentas provides a potential prenatal diagnostic marker for EOPET.

The TIMP3 protein has been identified as a member of the TIMP family that antagonizes matrix metalloproteinases (MMPs; Langton et al., 1998). As a homeostasis guardian, TIMP3 is associated with a wide range of physiological processes, such as cell growth, apoptosis, invasion, angiogenesis and metastasis (Baker et al., 1999; Qi et al., 2003; Cruz-Munoz et al., 2006). TIMP3 is expressed in a variety of tissues, such as the heart, kidneys, lung, liver and brain, with the highest expression levels found in the placenta (Boon et al., 2002). Several placenta-related diseases have been associated with abnormal TIMP3 methylation (Feng et al., 2004; Xue et al., 2004; Yuen et al., 2010), which may indicate the involvement of TIMP3 in trophoblast invasion. As failure of trophoblast invasion has been associated with PE, it is likely that TIMP3 is involved in the pathophysiology of PE.

It should be noted that Yuen’s findings (Yuen et al., 2010) were based on relatively few samples comprising different ethnic groups. And replications with larger sample sizes within single ethnic populations based on relatively few samples comprising different ethnic groups are therefore needed to confirm the role of TIMP3 in PE. Here, we carried out a methylation assay of TIMP3 in a Han Chinese population using a total of 87 subjects. The relationship between promoter methylation and gene expression of TIMP3 in placentas with and without PE in additional samples were also examined to explore the mechanism of the development of PE.

### Materials and Methods

#### Patients and samples

Twenty-two normal placentas and 41 preeclamptic placentas were used for the case–control studies. Standard diagnostic data were collected for PE patients, namely systolic pressure > 140 mmHg, diastolic pressure > 90 mmHg and proteinuria > 0.3 g over a 24-h period. The controls consisted of healthy women undergoing Caesarean section. The clinical characteristics of all participants are shown in Table I. All clinical placentas from normal and pathological pregnancies were collected immediately after Caesarean section. Two ~1 cm³ fragments were dissected from the placenta, after removal of maternal blood by vigorous washing in phosphate buffered saline and the tissues were maintained in centrifuge tubes and RNA later (Ambion Inc., Austin, TX, USA), then stored frozen at –80 °C. In addition, umbilical cord blood samples (seven samples from pregnancies with PE and eight samples from controls) and maternal peripheral blood samples (three samples from pregnancies with PE and six control individuals) were collected. The study protocols were reviewed and approved by the Fudan University Ethical Committee, and informed consent was obtained from all participants.

#### DNA preparation and bisulphite conversion

Genomic DNA was isolated from placentas and bloods using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) or the QIAamp Blood DNA Mini Kit (QIAGEN). DNA was bisulphite-converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer’s protocol.

#### Quantitative methylation analysis

Quantitative methylation of TIMP3 was performed using MassArray EpiTyper (Sequenom, San Diego, CA, USA) based upon MassCLEAVE base-specific cleavage and MALDI-TOF mass spectrometry. To fully investigate the methylation patterns of TIMP3, the neighboring CpG island (CGI) of the corresponding region investigated in Yuen’s study were also examined. The amplicons cover the regions with most CpG sites and the target regions are shown in Fig. 1. Primers were designed using the online software Epidesigner (http://www.epidesigner.com) shown in Table II. The quantitative methylation data for each CpG site or aggregates of multiple CpG sites obtained from MassARRAY were analyzed on the EpiTYPER software (Sequenom).

#### RNA isolation, reverse transcription and quantitative real-time PCR

Total RNAs were extracted from placentas using mirVana™ PARIS™ Kit (Ambion) in accordance with the manufacturer’s instructions. Reverse transcription (RT) was conducted on 1 μg of RNA using M-MLV

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PE (n = 48)(^a)</th>
<th>Control (n = 31)</th>
<th>(P)-value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>30.61 ± 5.73</td>
<td>28.63 ± 4.02</td>
<td>0.164</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>35.39 ± 3.33</td>
<td>39.22 ± 1.05</td>
<td>3.31 × 10⁻⁶</td>
</tr>
<tr>
<td>Pregnancy BMI (kg/m²)</td>
<td>29.82 ± 3.43</td>
<td>30.15 ± 3.85</td>
<td>0.921</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>151.64 ± 18.45</td>
<td>111.91 ± 12.92</td>
<td>3.05 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>100.70 ± 21.55</td>
<td>71.45 ± 10.16</td>
<td>3.38 × 10⁻⁹</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
<td>4.78 ± 7.12</td>
<td>0</td>
<td>1.14 × 10⁻¹³</td>
</tr>
<tr>
<td>Infant birthweight (g)</td>
<td>2409.41 ± 967.40</td>
<td>3494.44 ± 374.81</td>
<td>6.59 × 10⁻⁶</td>
</tr>
<tr>
<td>Placenta weight (g)</td>
<td>387.03 ± 135.79</td>
<td>626.82 ± 107.52</td>
<td>5.09 × 10⁻⁶</td>
</tr>
</tbody>
</table>

All results are presented as mean ± SD.

\(a\)Diagnoses criteria used for PE patients were as follows: systolic pressure > 140 mmHg, diastolic pressure > 90 mmHg, and proteinuria > 0.3 g in a 24 h collection.

\(b\)Obtained using the Mann–Whitney U test on SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).
Reverse Transcriptase (Promega, Madison, WI, USA). Quantitative real-time PCR (qRT–PCR) was performed to determine the mRNA expression of TIMP3 using FastStart Universal SYBR Green master (ROX) reagent (Roche Diagnostics, Basel, Switzerland) in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. Optimal real-time PCR assays for TIMP3 and GAPDH (PCR primers listed in Table II) were designed on PrimerBank (http://pga.mgh.harvard.edu/primerbank/). All qRT–PCR reactions were performed in replicate in a final volume of 10 μl containing primers, FastStart Universal SYBR Green master (ROX) reagent and cDNA samples. Relative expression of TIMP3 in samples was expressed as the averaged normalized Ct value of each sample compared with the GAPDH Ct value of the corresponding sample based on the 2^−ΔΔCt method.

**Statistical analysis**
Quantitative data are presented as mean ± SD. Statistical significance was determined by the non-parametric Mann–Whitney U test for two group comparisons. A P-value of <0.05 was considered statistically significant.

**Results**

**Aberrant DNA methylation of the TIMP3 proximal promoter in patients with PE**

The previous Yuen study on a Canadian sample had identified hypomethylation of TIMP3 in pregnancies with PE (Yuen et al., 2010). To examine whether TIMP3 showed a similar methylation status in the Han Chinese population, we performed methylation analyses of the region (−928 to −502 bp upstream from the transcription start site) investigated in that study. For the additional investigation of the methylation patterns in the neighboring region, two amplicons were performed to evaluate the methylation status of the CGI (579–1465 bp downstream from the transcription start site). The CGI and promoter regions analyzed contained 69 and 5 CpG-sites, respectively, but only 55 and 2 CpG were available for analysis. In our study, the methylation status of CpG sites in the CGI region of TIMP3 showed no differences as between normal and pathological placental samples (average methylation level was 0.074 and 0.076, respectively; P = 0.841; Supplementary data, Fig. SI). The two analyzed CpG sites (−699 and −880 bp upstream of the transcription start site, respectively) in the promoter region were significantly hypomethylated in placental samples with PE (mean methylation = 0.342, 0.494, respectively) than those in normal placentas (mean methylation = 0.405, 0.534, respectively; P = 9.40 × 10^{-7}), which was close to the methylation level identified in Yuen’s study (Yuen et al., 2010) although the participants in our study were of different genetic and environmental background.

**Figure 1** Schematic diagram of the TIMP3 gene. The locations of the target regions including promoter region upstream from transcription start site and the CGI are noted. Positions and orientation of the MassARRAY primers are indicated by black arrows.

**Table II** Sequences of PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MassARRAY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP3-1</td>
<td>tag-FW</td>
<td>aggaagagagTGGTTATTTTGAATTGGGATTTTTT</td>
<td>434</td>
</tr>
<tr>
<td></td>
<td>T7-RV</td>
<td>cagtaatacgactcactatagggagaaggctAAAAACAAATAAACATACCCCCCAT</td>
<td></td>
</tr>
<tr>
<td>TIMP3-2</td>
<td>tag-FW</td>
<td>aggaagagagTTGTTATTTGTTGAGGAGGGG</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>T7-RV</td>
<td>cagtaatacgactcactatagggagaaggctCCCAAACTCAAACACCTACCCAAA</td>
<td></td>
</tr>
<tr>
<td>TIMP3-3</td>
<td>tag-FW</td>
<td>aggaagagagTTTTTTTGGGTAGTTGGAGGTTTG</td>
<td>315</td>
</tr>
<tr>
<td></td>
<td>T7-RV</td>
<td>cagtaatacgactcactatagggagaaggctACTCCCTCAACCACCTATAATTCCC</td>
<td></td>
</tr>
<tr>
<td>qPCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP3</td>
<td>FW</td>
<td>TGCAACTCCTGGAAGGTTG</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>AGGTGATACCGATAATTCACAACCG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>FW</td>
<td>AAGGTGAAGGTCGGAGTCAAC</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>GGGGTCATTGGATGCAACATA</td>
<td></td>
</tr>
</tbody>
</table>

FW, forward; RV, reverse.
We also applied correlation analysis to evaluate whether any clinical characteristic such as period of gestation, maternal age and BMI were correlated with TIMP3 promoter methylation in preeclamptic samples and controls separately. The results showed no statistically significant correlation between clinical characteristics and TIMP3 promoter methylation (all \( P > 0.05 \)). It may indirectly imply that the aberrant TIMP3 promoter methylation is associated with the disease state (Supplementary data, Table SI).

TIMP3 could be used as a fetal DNA marker for noninvasive prenatal diagnosis

The discovery of cffDNA in maternal plasma provides a novel approach for noninvasive prenatal diagnosis, which requires universal fetal DNA markers. Previous studies (Flori et al., 2004; Masuzaki et al., 2004) have focused on the fetal DNA of placental origin. Chim et al. (2005) proposed that epigenetic differences between placenta and maternal blood could be used as markers to discriminate cffDNA from maternal origin. We investigated the methylation status of TIMP3 in placentas and maternal bloods. In the promoter region (−928 to −502 bp) of TIMP3, a significantly low level of methylation (mean methylation = 0.459) was found in placentas (normal and preeclamptic), while the level of methylation of maternal bloods (maternal peripheral blood and umbilical cord blood, normal and preeclamptic) were almost completely methylated (mean methylation = 0.961; \( P = 6.91 \times 10^{-13} \); Fig. 3). Consequently, the unmethylated TIMP3 sequences could be used to measure the enrichment of cffDNA in maternal plasma, which suggest that the methylation status of TIMP3 could have potential as a universal prognostic marker in noninvasive prenatal diagnosis.

TIMP expression is elevated in placental samples with PE

qRT–PCR of the TIMP3 gene was performed on a range of normal \((n = 8)\) and pathological \((n = 9)\) placentas to assess whether the gene expression pattern of TIMP3 was associated with methylation patterns. The results, consistent with our expectation, indicated that the TIMP3 gene showed a nearly 2-fold increase of expression in respect to the preeclamptic placentas with a low level CpG methylation compared with those in normal samples \((P = 0.007; \text{Fig. 4})\). Notably, a high degree of gene expression variations was observed in placentas from pregnancies with PE.

We also investigated whether gene expression was related to DNA methylation in placental tissues. A negative relationship between TIMP3 methylation and mRNA expression was indicated regarding the overall TIMP3 promoter methylation status and gene expression \((R = -0.758, P = 0.029; \text{Supplementary data, Fig. S2})\).

Discussion

PE is recognized as a multisystem disease leading to perinatal maternal-fetal morbidity and mortality. To date, a variety of PE-susceptible genes with aberrant DNA methylation and/or gene expression have been identified, such as the apoptosis-related genes, cytokine-receptor genes and obesity-related genes (Reimer et al., 2002; Tsoi...
TIMP3 hypomethylation in pre-eclampsia

**Figure 4** Expression analysis of TIMP3 in the placentas from pregnancies with PE (n = 9) and healthy controls (n = 8). The relative mRNA level of TIMP3 was measured by the qRT–PCR. The average mRNA level of TIMP3 in healthy controls was defined as 1. **p < 0.01.

et al., 2003; Pang and Xing, 2004). TIMP3 with aberrant promoter methylation in placentas with PE is thought to be involved in the etiology of the disease (Yuen et al., 2010). In the present study, we were able to successfully replicate the results found by Yuen et al. (2010) although the participants in the two studies had different genetic and environmental backgrounds. Previous studies have identified ethnic variations in DNA methylation levels related to lifestyle and dietary differences (Enokida et al., 2005; Das et al., 2006). As a consequence, the uniformity of the TIMP3 promoter methylation in the two studies strengthens the association between TIMP3 promoter methylation and PE.

In the current study, TIMP3 showed a nearly 2-fold elevated expression in PE placentas compared with normal placentas, which is in accordance with the results in a study by Pang and Xing (2003). Moreover, TIMP3 mRNA expression has been found to be inversely correlated with the TIMP3 promoter methylation index as also reported by Yuen et al. (2010), confirming the likely role of methylation in the regulation of TIMP3 expression. Similarly, Feng et al. (2004) identified the involvement of TIMP3 promoter hypermethylation in the down-regulation of TIMP3 in choriocarcinoma, which suggests a negative correlation between DNA methylation and mRNA expression of TIMP3. It is generally recognized that promoter methylation blocks transcription and mRNA expression by preventing transcription factor binding. Hence, we speculated that the hypoxic environment in the placenta gives rise to stress that induces relevant expression of putative transcription factors, and thereby mediates elevated expression of TIMP3.

Increasing evidence supports the view that the local balance between MMPs and TIMPs is crucial for their biological significance and that any dysregulation in the functional balance may affect a number of pathological situations such as the pathology of reproduction (Knox et al., 1997; Spinale, 2002; Karthikeyan et al., 2012). Overexpression of TIMP3 has been linked with inhibited trophoblast invasion in a number of studies. We therefore hypothesized that a preexisting or early established demethylation of TIMP3 promoter may induce increased expression, reducing the function of MMPs. This, in turn, would lead to suppression of the matrix disintegration necessary for efficient implantation and inhibit the trophoblast invasion leading to incomplete modification of the spiral arteries (Meekins et al., 1994). In particular, impaired remodeling of the spiral artery has been considered as a major contributor to PE (Brosens et al., 1972).

A high degree of gene expression variation has been observed in placentas from pregnancies with PE (Fig. 4). This may be partially because of high interpatient variations with respect to the physical quality of the condition, such as a high-fat or low-calorie diet, or maternal underweight or obesity. It also points to the likelihood that specific maternal factors might be associated with the onset, severity, and progression of PE (Huppertz, 2008).

The search for non-invasive PE-specific markers that could predict the development or assist in the detection of the disease is presently one of the most rapidly growing areas in gynecology and obstetrics research (Liu et al., 2009). Nowadays, most of the routinely used biomarkers for PE are proteins, which exhibit low specificity and sensitivity in the most frequently used blood tests. DNA methylation-based markers occur early during disease progression and are readily traceable in the maternal blood flow (Gabory et al., 2011), constituting a promising tool for noninvasive prenatal diagnosis. Similar to the SERPINB5 gene (Chim et al., 2005), the TIMP3 promoter is known to be partially methylated in placental tissues, and almost completely methylated in maternal blood DNA. The unmethylated DNA sequences are a measure of the enrichment of cffDNA in maternal plasma. Accumulating evidence has confirmed a 2- to 5-fold increase of cffDNA in maternal plasma in women with PE (Lo et al., 1999; Zhong et al., 2002; Levine et al., 2004). Moreover, the TIMP3 promoter presents a lower methylation level in placentas with PE than that in control pregnancies, which suggests that the unmethylated TIMP3 sequence is measurably higher in maternal plasmas of pregnancies with PE. Consequently, quantitative aberrations of unmethylated TIMP3 sequence in maternal plasma may serve as a means for predicting PE. It is fairly obvious that gene markers, especially those described in small case studies with selected populations, would not be suitable for widespread use in early PE detection, whereas the consistency of TIMP3 methylation patterns between our Han Chinese population and Yuen’s mixed population (Yuen et al., 2010) would support the widespread use of TIMP3 in PE early screening. To provide an adequate positive predictive value, a large number of valid markers are urgently needed. There is therefore a critical need to conduct large-scale studies to exploit markers in well-defined population cohorts to achieve an effective screening test for PE.

In summary, we successfully replicated the findings of Yuen et al. (2010) in a Han Chinese group of clinical samples, thus providing convincing support for the involvement of the gene in PE etiopathology and its use as a marker for PE detection or surveillance. The correlation between the hypomethylation of the TIMP3 promoter and up-regulating gene mRNA levels indicate the credible role of aberrant gene methylation in the pathophysiology of PE. Consequently,
genome-wide methylation analysis should be conducted to explore the etiology of PE, which will not only help us to identify increasing epigenetic markers of PE, but also offer us new diagnostic perspectives for pregnancies at risk and thus provide the best prenatal care for the affected women and their children.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Acknowledgements

We thank Dr Aman Zhao (Shanghai Information Center for Life Sciences) and Dr Junyu Zhang (Fudan University) for excellent technical assistance. We thank Dr Yan Zhang (Provincial Hospital Affiliated to Shandong University) for the assistance in samples collection.

Authors’ roles

Y.X.: performed the experiments, data analysis and interpretation and final drafting the article. X.Z. and Q.L.: data analysis and interpretation, clinical samples collection. J.X., X.Z., T.W.: data acquisition, analysis and interpretation. Q.X., Y.L. and L.W.: revising the article for important intellectual content. L.H.: revising the article for important intellectual content and final approval of the manuscript.

Funding

This work was supported by the 973 Program (2009CB526006, 2010CB529600), the National Key Technology R&D Program (2012BA101B09), the National Natural Science Foundation of China (30800616, 81100256, 81212001), the Shanghai Municipal Commission of Science and Technology Program (09DJ1400601) and the Shanghai Rising-Star Program (09QA1400500).

Conflict of interest

None declared.

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