Preeclampsia

Expression and Epigenetic Alterations of Placental Serine Protease Inhibitors

SERPINA3 Is a Potential Marker of Preeclampsia


Abstract—Preeclampsia is the major pregnancy-induced hypertensive disorder. It modifies the expression profile of placental genes, including several serine protease inhibitors (SERPINs). The objective of this study was to perform a systematic expression analysis of these genes in normal and pathological placentas and to pinpoint epigenetic alterations inside their promoter regions. Expression of 18 placental SERPINs was analyzed by quantitative RT-PCR on placentas from pregnancies complicated by preeclampsia, intrauterine growth restriction, or both and was compared with normal controls. SERPINA3, A5, A8, B2, B5, and B7 presented significant differences in expression in ≥1 pathological situation. In parallel, the methylation status of the CpG islands located in their promoter regions was studied on a sample of control and preeclamptic placentas. Ten SERPIN promoters were either totally methylated or totally unmethylated, whereas SERPINA3, A5, and A8 presented complex methylation profiles. For SERPINA3, the analysis was extended to 81 samples and performed by pyrosequencing. For the SERPINA3 CpG island, the average methylation level was significantly diminished in preeclampsia and growth restriction. The hypomethylated CpGs were situated at putative binding sites for developmental and stress response (hypoxia and inflammation) factors. Our results provide one of the first observations of a specific epigenetic alteration in human placental diseases and provide new potential markers for an early diagnosis. (Hypertension. 2007;49:76-83.)

Key Words: preeclampsia ■ intrauterine growth restriction ■ placenta ■ serine protease inhibitors ■ epigenetics

Hypertension during pregnancy includes preeclampsia (PE), gestational hypertension, and pre-existing chronic essential hypertension. From a clinical point of view, PE associates with hypertension, proteinuria, and, in the majority of cases, mild-to-severe edema. It is the most prevalent obstetric complication (≥10% of the pregnancies)1 and may lead to maternal and fetal morbidity and mortality.2 The physiopathological process includes endothelial dysfunctions,3 oxidative stress,4,5 exaggerated inflammatory responses,6 thrombosis, and renin–angiotensin–aldosterone system activation.7 This multisystemic disease can lead to severe clinical conditions, such as eclampsia, when generalized seizures appear, and hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome when a hemolytic process is observed. In >25% of the cases, the reduced vascular flow at the fetomaternal interface induces an intrauterine growth restriction (IUGR). Early caesarean delivery of the fetus is the classical treatment for PE, leading to iatrogenic low-weight newborns. Thus, PE contributes to the risk of developing a metabolic syndrome later in life.8 Although preeclamptic women typically develop clinical signs and symptoms from midgestation, it is now widely admitted that the physiopathological process starts as early as the end of the first trimester of pregnancy.9 In PE, cytotrophoblasts fail to invade deeply spiralized uterine arteries.9–11 This shallow invasion leads to localized ischemic lesions and hypoxia.12,13 Consequently, placental endothelial residues14,15 originating from the fetoplacental unit are released into the maternal blood flow, probably inducing the hypertensive state.16

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The etiology of PE remains poorly understood. Medically assisted procreation increases the risk of PE, suggesting that early gamete alterations (of epigenetic origin) may be sufficient to induce placental defects. Genetic factors are also involved in the pathogenesis of PE, because several genomic regions have been linked to the disease. Recently, STOX1 missense mutations have been shown to predispose to PE.

Today, no univocal predicting indicator has been described to complete clinical data for each individual (summarized in the Table). We classified the patients in 3 groups: isolated PE (PE), PE and IUGR, and isolated IUGR (IUGR). The inclusion criteria used for IUGR was weight at birth less than the 10th percentile. We used a nested PCR approach; therefore, we designed 2 couples of primers for each SERPIN to obtain a final product of ~500 bp. For primer design, the criteria were as follows: (1) annealing with bisulphite-treated DNA without covering any CpG, (2) a similar temperature (~56°C), and (3) a primer length close to 20 bp. PCR primer couples for SERPINAs 5'-ATACTATAAACACTCTCCTC-3' for the primary PCR reaction, and 5'-GGAGAATTTTGATTTAGGAAAA-3', for the secondary PCR reaction.

Fragments (10 to 20 mm) from the placental subchorial zone were dissected as described previously. Maternal membranes were eliminated, and floating villi were washed in Hank's balanced salt solution. A fraction of these samples was kept in TRizol (5 mL/g, Invitrogen), and total RNA was extracted, treated with RNase-free DNase, and quantified by spectrophotometry. Genomic DNA was extracted from chorionic villi after mechanical grinding using an electric Ultraturrax homogenizer in lysis solution (NaCl 50 mmol/L, Tris-HCl 10 mmol/L [pH 7.4], EDTA 10 mmol/L, and 0.2% SDS) and treated with Proteinase K (1 g/L final concentration) for 3 to 12 hours at 55°C. The DNA was precipitated, washed with 70% ethanol, and quantified.

Primers and Quantitative RT-PCR Conditions

Reverse transcription was carried out according to a standardized protocol. Briefly, 2 µg of total DNase-treated RNA was reverse transcribed in a volume of 25 µL at 39°C using the Superscript reverse transcriptase (Invitrogen) during 1 hour. Quantitative RT-PCR was carried out in duplicates on 8 control, 9 PE, 7 PE+IUGR, and 8 IUGR placentas. The amplification kit Platinum SYBR Green Mastermix (Invitrogen) was complemented with MgCl2 (4 mmol/L final concentration), BSA (0.05 g/L), and primers (10 ^3 mmol/L) to a final volume of 17 µL. The reaction was performed in a Light Cycler Thermocycler (Roche). Primers (Table I supplementary data, please see http://hyper.ahajournals.org) were designed for the coding sequences (GENBANK) of the different SERPINs using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primeR3). The different couples were chosen to recover all of the previously described isoforms and aligned with basic local alignment search tool software to avoid nonspecific annealing. We used the PCR program: 50°C for 120 s and 95°C for 120 s followed by 35 cycles of 3 temperature steps (94°C for 5 s, 58°C for 10 s, and 72°C for 30 s). Finally, samples were submitted to a progressive temperature elevation (from 65 to 99°C at 0.1°C/s), resulting in a fusion curve, enabling us to check the PCR products homogeneity. In addition, amplification products were systematically controlled by agarose gel electrophoresis. The threshold cycle number (Ct) values were collected with the LightCycler software (Roche) in the exponential phase of the PCR reaction. These Ct values were normalized by the Ct values obtained for the succinate dehydrogenase subunit A (SDHA) used as a reporter gene and shown previously to be stable and highly expressed in the placenta.

Methylation Analysis of CpG Islands Inside the Promoter Regions by Sequencing

DNA was denatured during 5 minutes at 100°C and incubated 20 minutes at 37°C in a 0.2-mol/L NaOH solution. Deamination was carried out in the dark with sodium bisulphite (4.6 mol/L final concentration) and hydroxyquinone (120 mmol/L final concentration) for 4 hours at 55°C. The DNA was then purified with the Wizard DNA clean-up kit (Promega) and rinsed 3 times with isopropanol. The bisulphite conversion was completed by incubation with 0.3 mol/L of NaOH during 20 minutes at 37°C. The DNA was then precipitated (0.5 volumes of 6 mol/L of ammonium acetate, 1 µL of glycogen (Invitrogen), and 2.5 volumes of absolute ethanol), washed with 70% ethanol, dried, and resuspended in distilled water.

For each SERPIN gene, CpG islands were screened on the 10 kb upstream of the transcription start site and defined by a CpG dinucleotides density >50 in 1000 bp (Figure I, available online at http://hyper.ahajournals.org). When no CpG island was detectable under these conditions, we searched for local CpG density and putative binding site(s) for the hypoxia-inducible factor α subunit (HIF-1α). We used a nested PCR approach; therefore, we designed 2 couples of primers for each SERPIN to obtain a final product of ~500 bp. For primer design, the criteria were as follows: (1) annealing with bisulphite-treated DNA without covering any CpG, (2) a similar temperature (~56°C), and (3) a primer length close to 20 bp. PCR primer couples for SERPINAs 5'-AAAGGGTAGAGGATAAGGG-3', 5'-ATACTATAAACACTCTCCTC-3' for the primary PCR reaction, and 5'-GGAGAATTTTGATTTAGGAAAA-3', for the secondary PCR reaction.

Methods

Human Material Samples and Ethics

Placentas from normal and pathological pregnancies (PE and IUGR) were collected after caesarean delivery from 3 maternities (St Antoine, Port-Royal, and Institut de Puériculture, Paris, France) following the indications of the Hôpital Cochin ethical committee. We had access to complete clinical data for each individual (summarized in the Table). The inclusion criteria used for PE patients were the following clinical thresholds: systolic pressure >140 mm Hg, diastolic pressure >90 mm Hg, and proteinuria >0.3 g per 24 hours. The inclusion criteria used for IUGR was weight at birth <10th percentile. We classified the patients in 3 groups: isolated PE (PE), PE associated with IUGR (PE+IUGR), and isolated IUGR (IUGR). The control group was formed by women who underwent caesarean surgery without suffering any disease during pregnancy.
Clinical Characteristics of Patients Analyzed at the Transcriptional Level

<table>
<thead>
<tr>
<th>Placenta No.</th>
<th>Status</th>
<th>Weeks</th>
<th>Mother Age</th>
<th>HTA</th>
<th>Proteinuria (g/24 h)</th>
<th>Uterine Doppler (N= notch)</th>
<th>Oligoaminos</th>
<th>Birth Weight (g)</th>
<th>Percentile</th>
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<td>766</td>
<td>PE</td>
<td>37+4</td>
<td>45</td>
<td>140/90</td>
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<td>N=1/1</td>
<td>nd</td>
<td>2930</td>
<td>&gt;10th</td>
</tr>
<tr>
<td>867</td>
<td>PE</td>
<td>33+1</td>
<td>26</td>
<td>170/90</td>
<td>5.36</td>
<td>nd</td>
<td>0</td>
<td>1930</td>
<td>&gt;10th</td>
</tr>
<tr>
<td>885</td>
<td>PE</td>
<td>30+0</td>
<td>22</td>
<td>175/105</td>
<td>8.68</td>
<td>0/0</td>
<td>0</td>
<td>1080</td>
<td>&gt;10th</td>
</tr>
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<td>898</td>
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<td>33</td>
<td>165/100</td>
<td>4.00</td>
<td>0/0</td>
<td>0</td>
<td>1960</td>
<td>&gt;10th</td>
</tr>
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<td>150/80</td>
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<td>N=0/1</td>
<td>0</td>
<td>1196</td>
<td>&gt;10th</td>
</tr>
<tr>
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<td>29+3</td>
<td>24</td>
<td>140/90</td>
<td>2.70</td>
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<td>0</td>
<td>1390</td>
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</tr>
<tr>
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<td>32+5</td>
<td>36</td>
<td>160/110</td>
<td>14.36</td>
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<td>0</td>
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<tr>
<td>869</td>
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<td>160/80</td>
<td>3.00</td>
<td>N=2/2</td>
<td>0</td>
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<td>&gt;10th</td>
</tr>
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<td>29</td>
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<td>846</td>
<td>PE+IUGR</td>
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<td>32</td>
<td>205/120</td>
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<td>0</td>
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<td>2.10</td>
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<td>1+</td>
<td>1670</td>
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<td>150/90</td>
<td>46.00</td>
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<td>33</td>
<td>140/90</td>
<td>1.04</td>
<td>N=0/2</td>
<td>0</td>
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<td>5th&lt;10th</td>
</tr>
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<td>IUGR</td>
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<td>26</td>
<td>160/90</td>
<td>0.00</td>
<td>N=2/2</td>
<td>nd</td>
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<td>5th&lt;10th</td>
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<td>35</td>
<td>Normal</td>
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<tr>
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<td>IUGR</td>
<td>37+0</td>
<td>33</td>
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<tr>
<td>814</td>
<td>IUGR</td>
<td>29+2</td>
<td>25</td>
<td>Normal</td>
<td>0.00</td>
<td>N=2/2</td>
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<td>Normal</td>
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<td>0/0</td>
<td>0</td>
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<td>Normal</td>
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<td>N=2/0</td>
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<td>0</td>
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<tr>
<td>774</td>
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<td>0/0</td>
<td>nd</td>
<td>2900</td>
<td>&gt;10th</td>
</tr>
</tbody>
</table>

The Notch (N) is represented by 2 values; the first corresponds with a measure at the second trimester and the second value at the third trimester. 1 indicates unilateral notch; 2, bilateral notch; nd, not determined.

5’-CCCCCTAATTCCATTCTC-3’ for the secondary PCR reaction. The complete list of postbisulphite primers is given as supplementary data (Table IB). The products were either gel purified using QIAquick columns (QIAGEN) for direct sequencing or ligated into pCR4 Topo vector (Invitrogen), cloned into electrocompetent Top10 bacteria (Invitrogen), and sequenced. In either case, analyses were carried out on 5 control and 5 PE placentas. Sequences were analyzed using the BioEdit software (sequence alignment editor) on the raw and interpreted data as well.

Methylation Analysis of CpG Islands Inside the Promoters Regions by Pyrosequencing

For SERPINA3, amplicons encompassing the selected CpG island were designed with 1 of the 2 primers being biotinylated. Forty-seven placental DNAs from PE, 16 from IUGR, and 18 from control subjects were treated with sodium bisulphite and amplified by PCR. The template for pyrosequencing was purified, rendered single stranded, and analyzed by serial pyrosequencing to evaluate the methylation percentage at each CpG position.37,38

Promoter Composition Analysis

The putative transcription factor binding sites inside the SERPINA3 promoter sequence were obtained using the Genomatix software (http://genomatix.de).39

Statistical Analysis

The averaged normalized Ct values of each pathological group were compared with the averaged Ct value obtained for the control group, using Student’s t test. Details about multivariate principal component analysis are given as supplementary data (available at http://hyper.ahajournals.org), together with their statistical validation methods.

Results

Analysis of SERPIN Expression in Normal and Pathological Placentas

Quantitative RT-PCR was carried out for 18 SERPINs on a panel of nonpathological and pathological near-term placentas. The expression levels varied on 4 orders of magnitude.
between the different SERPINs, on average, the SERPIN A clade (composed of SERPINA1, A3, A5, and A8 in our panel) is significantly underexpressed compared with the SERPIN B clade (or ovalbumins, SERPINB1, B2, B5, B6, B7, B8, and B9) by a factor of 30 on average ($P_{/\text{H}11005}0.036$). We did not find evidence of a relationship between expression level and chromosome location. Indeed, although several SERPINs map in clusters (Figure IB), wide differences in expression can be observed between consecutive genes.

The expression level was analyzed in the 3 pathological states. The ratio of the average expression levels between each pathological situation (PE, PE/IUGR, and IUGR) and the control are shown in Figure 1. Although several SERPINs vary similarly in the 3 pathological conditions (SERPINA3, A5, B2, and B7), 3 distinct SERPIN expression profiles distinguish the 3 pathological states.

For a refined description of the data we performed a multivariate analysis by principal component analysis (Figure II). This analysis indicates that, on the whole, 6 SERPIN do not vary much with the pathological state (SERPINB1, B9, B6, E1, G1, and F1). Conversely, SERPINB5, B7, A5, A3, and B2 are the most widely modified. This analysis also permitted us to classify efficiently the disease states according to the pathological condition. On the graph (Figure II), isolated preeclamptic samples were clearly separated from isolated IUGR samples. Furthermore, PE+IUGR were the clinical situations where expression alterations were the highest. Interestingly, part of these samples (placentas 846, 853, 871, and 872) were closer to the PE-only situation, whereas others (placentas 772, 775, and 887) were closer to the IUGR-only situation.

**Epigenetic Regulation/Dysregulation of SERPIN in Normal and Pathological Placentas**

We attempted to correlate levels of expression with promoter methylation, because in the classical view, a high methylation level is associated with gene silencing. One or 2 CpG islands were chosen for each SERPIN (Figure I and Table IB in the supplementary data). The direct sequencing of the amplicons showed that the CpG islands of SERPINB1, B9, and B8 were totally unmethylated, whereas those of SERPINA1, B7, D1, F1, and H1 were totally methylated. For SERPING1 and II, 2 CpG islands were studied, showing discrepant results (the CpG island located closest to the transcription start site is unmethylated in both cases, whereas the one located farther was totally methylated). On average, the unmethylated promoter was associated with an increased (8 times) expression level. For SERPINA5, A8, A3, and E1, the direct sequencing approach did not clearly identify a methylation profile. Thus, we used a cloning/sequencing protocol after bisulphite treat-
ment for analyzing the SERPINA5, A8, and A3 CpG islands. Five control and 5 PE placentas were analyzed, and 10 to 14 clones were sequenced for each of them. For SERPINA5 and A8, the cloning/sequencing procedure revealed a marked heterogeneity between consecutive CpGs and among individuals. Nevertheless, no correlation with the disease status was observed (data not shown). By contrast, the CpG island of SERPINA3 seemed significantly undermethylated in PE (paired $t$ test; $P < 0.03$). To validate this observation, we decided to use the more precise pyrosequencing technique on a larger group of individuals (47 PE, 16 IUGR, and 18 control placental DNAs). As shown in Figure 2, we confirmed the observation of a hypomethylation of this region in pathological placentas using a paired $t$ test comparing all of the CpG between the controls and the pathologic samples (PE versus control, $P = 0.00019$ and IUGR versus control, $P = 0.029$).

The analysis was also carried out by a standard $t$ test to evaluate whether given CpG dinucleotides were specifically affected by the pathology-associated hypomethylation. We demonstrated a significant hypomethylation in PE, especially at positions 98, 129, 149, 167, and 497 ($P = 0.00006$, $0.0004$, $0.017$, $0.008$, and $0.01$, respectively). Interestingly, the positions located at the start of the analyzed sequence encompass 2 putative, consecutive HIF-1α binding sites (positions 128 and 137). The first significant CpG positions encompass putative binding sites for homeodomain-containing transcription factors (BRNF, HOXC13, HOXF, and OCTB). The CpG dinucleotide in position 98 is included in a myc-associated Zn finger protein binding site. Finally, the CG dinucleotide in position 497 lies in the only retinoic acid receptor (RXR) binding site of the promoter.

**Discussion**

Early diagnosis of placental diseases, such as PE, is a major challenge for being able to dispense the existing treatments (aspirin and low molecular weight heparin). Recent advances suggest that methylation alterations of trophoblastic circulating DNA in the maternal blood is a promising marker for such early detection. However, the choice of the right promoter DNA to be analyzed is crucial. This choice can be guided by strong expression modifications of placental genes. In this study, we focused our interest on placental SERPINs, several of which, indeed, display such alterations. These are probably related to the physiopathogenesis of placental diseases, such as an exaggerated inflammatory response and thrombosis. In PE and PE/IUGR, proinflammatory cytokines and substances provoking inflammatory damages, such as specific proteases, are increased. We found an increase of SERPINA3 expression consistent with its inhibitory role on chymotrypsin to counterbalance inflammation. On the other hand, recent studies about this SERPIN indicate that it is a specific inhibitor of elastase. Elastase belongs to the matrix metalloprotease family, which plays a crucial role during the implantation process. This may suggest that an increased SERPINA3 synthesis may inhibit the matrix disintegration necessary for an efficient implantation. Such an effect would be very consistent with the admitted pathogenesis of PE and could imply a very early impact of SERPINA3 in the onset of placental diseases. If so, the epigenetic modifications observed for this specific gene correspond with a disease-predisposing state rather than a secondary consequence. The SERPINA5 (protein C inhibitor) is secreted to inactivate several proteases of the fibrinolytic system. Therefore, the reduction observed for SERPINA5 may serve to control thrombosis. Similarly, SERPINB2 that participates in fibrinolysis regulation is reduced in placental diseases. Our results are consistent with previous studies, because it has been shown that SERPINB2 decreases in both PE and IUGR in the maternal serum and that this decrease also correlates with a diminished level of SERPINB2 mRNA in IUGR placentas. For SERPINE1 and SERPINA8, different studies have shown a PE-induced increase in maternal serum.
our observations, the increase was not significant at the placental level probably because of interindividual heterogeneity, a very common feature of human placentas.\textsuperscript{53} Moreover, the placental level of \textit{SERPINE1} mRNA may not be sufficient to reveal alterations observed in the maternal serum, because \textit{SERPINE1} is secreted from other sources than placental villi.\textsuperscript{54} For instance, \textit{SERPINE1} is highly expressed in decidual cells, contrary to \textit{SERPINB2}.$^{54}$ \textit{SERPINA8} (AGT) encodes angiotensinogen and is involved in hypertensive diseases. The T235M substitution\textsuperscript{55} is a risk factor for PE, because it occurs at higher frequency in white and Japanese preeclamptic women\textsuperscript{56–58} and is linked to higher basal levels of AGT. Thus, the high intervariant patterns that we observed might be correlated to genetic heterogeneity.

PE and IUGR are sustained pathological conditions (the duration of pregnancy) especially dependent on cell proliferation, differentiation, and invasiveness. The invasive and erosive role of the syncytiotrophoblast is extremely important for the implantation of the young blastocyst at \textasciitilde1 week postfertilization. Despite the tight control governing these processes, they seem similar to the development of malignant tissues.\textsuperscript{59,60} In cancer, epigenetic modifications (abnormal DNA methylation and abnormal chromatin structure) are common. DNA methylation can be laid on DNA during cell divisions. In PE, an active cytотrophoblastic division phase is concomitant with the onset of the disease. Therefore, epigenetic alterations in such a rapidly expanding tissue may be expected. Thus, we explored the possibility of PE- and IUGR-induced epigenetic modifications on \textit{SERPIN} promoters. We brought to light significant position‐specific CpG demethylations in the \textit{SERPINA3} promoter in pathological states. This hypomethylated pattern might contribute to render the chromatin accessible to transcription factors. Several studies reported that the absence of CpG methylation at HIF-1\textalpha binding sites is required to allow gene transcriptional induction.\textsuperscript{61,62} Thus, demethylation may facilitate interaction of this transcription factor with the \textit{SERPINA3} promoter in the hypoxic environment generated in PE. In addition, demethylated CpG in this promoter region is prominently recognized by developmental regulators, such as homeodomain-containing proteins and inflammatory modulators (Myc-associated Zn finger protein and RXR). RXR\textalpha/PPAR\gamma heterodimers have been shown to have crucial roles in trophoblast invasion and placental nutrient transport.\textsuperscript{63,64} The presence of such binding sites in the \textit{SERPINA3} promoter suggests, therefore, an early function of this gene in placental development. In our experiments, the \textit{SERPINA3} mRNA was increased at a higher level in IUGR or PE+IUGR than in isolated PE. This observation is consistent with a multistep scenario of transcriptional activation: first, in PE but also in isolated IUGR, a promoter demethylation occurs. Then, in IUGR either isolated or combined with PE, the transcription factor environment enables a higher activation of the \textit{SERPINA3} promoter than in isolated PE.

Perspectives

The observed methylation alterations are quantitative and could, therefore, reflect heterogeneities between the cell types of the placental villi. We intend to address this point by purifying specific cell types (eg, cytотrophoblasts and perivascular smooth muscle cells) from normal and pathological placentas and studying their methylation level. In addition, the links between methylation alterations and protein expression will be tested by immunohistochemistry using an available antibody. Finally, some recent advances suggest that fetoplacental methylation alterations could be detected in the maternal blood.\textsuperscript{65} Therefore, we also intend to evaluate these modifications as markers able to contribute to an early diagnosis. On the other hand, several reports indicate that \textit{SERPINA3} is present in the chromaffin neurosecretory vessels.\textsuperscript{66,67} Therefore, this protein may also be secreted in the maternal blood, and its level could be altered in PE. This issue could be tested in the future on collections of sera of pregnant women.

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Disclosures

None.

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