Detection of Restriction Enzyme–Digested Target DNA by PCR Amplification Using a Stem-Loop Primer: Application to the Detection of Hypomethylated Fetal DNA in Maternal Plasma

Yu K. Tong, Rossa W.K. Chiu, Tak Y. Leung, Chunming Ding, Tze K. Lau, Tse N. Leung, and Y.M. Dennis Lo

Background: The discovery of cell-free fetal DNA in maternal plasma has opened up new possibilities for noninvasive prenatal diagnosis and monitoring. Among the fetal markers that have been described, methylation markers are sex and polymorphism independent. Methylation-sensitive restriction endonucleases are commonly used to digest hypomethylated DNA molecules, and the hypermethylated molecules remain intact for detection. The positive detection of the cleaved hypomethylated molecules would be useful for certain targets but has not been reported.

Methods: The use of a stem-loop primer in microRNA detection has previously been described. In this study, DNA assays were designed and performed on maternal plasma, which contained the hypomethylated placental serpin peptidase inhibitor, clade B (ovalbumin), member 5 (SERPINB5; maspin) gene in an excess background of hypermethylated maternal SERPINB5. Detection of the enzyme-digested placenta-derived hypomethylated SERPINB5 molecules was achieved by performing stem-loop extension followed by real-time PCR on maternal plasma. The placental origin of the stem-loop–extended SERPINB5 molecules was confirmed by genotyping.

Results: From the real-time PCR results on maternal plasma, stem-loop–extended SERPINB5 promoter sequences were detectable in all 11 enzyme-digested pre-delivery maternal plasma samples. Postpartum clearance was demonstrated. In 9 cases in which the fetal and maternal SERPINB5 genotypes were distinguishable, the placental-specific genotypes were detected in all predelivery maternal plasma samples.

Conclusion: Detection of restriction enzyme–digested hypomethylated placental DNA molecules in maternal plasma by the use of a stem-loop primer represents a novel approach in fetal epigenetic marker detection. The analytical approach may also be generally applicable to the detection of restriction enzyme-digested nucleic acid fragments.

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The discovery of cell-free fetal nucleic acids in the plasma of pregnant women has opened up new possibilities for noninvasive prenatal diagnosis and monitoring (1, 2). Over the past decade, the list of described fetal-specific markers has expanded from sex-dependent Y-chromosomal sequences (3–6) and paternally inherited polymorphic markers (7–10) to universal fetal markers such as placental-specific mRNA transcripts (11–13) and those dependent on placental-specific epigenetic signatures (14–16). In addition to clinical applications, such as prenatal sex determination and prenatal Rh blood group and D antigen (RHD)5 genotyping, researchers have recently demonstrated that trisomy 21 and trisomy 18 pregnancies could be inferred by the analysis of fetal nucleic acids in maternal plasma (2). Although the release mechanism and molecular characterization of plasma nucleic acids remain to be elucidated, a need exists for refining the
methods for the detection of differentially methylated fetal DNA in maternal plasma.

The tumor suppressor gene serpin peptidase inhibitor, clade B (ovalbumin), member 5 (SERPINB5; maspin), which is expressed in the placenta (17) and exhibits a tissue-specific methylation pattern (18), is the 1st universal fetal epigenetic marker reported (14). To detect hypomethylated placental SERPINB5 in an excess background of hypermethylated SERPINB5 of maternal origin in maternal plasma, the use of bisulfite treatment was reported (19, 20). However, it was documented that up to 96% of the DNA would be degraded by bisulfite treatment (21), leaving a minute amount of DNA for analysis. In a recent study, Chan et al. (16) described the discovery and possible utility of another universal fetal marker in maternal plasma. In the placenta and maternal blood cells, this marker, the Ras association (RalGDS/AF-6) domain family 1 (RASSF1A) tumor suppressor gene exhibits a methylation pattern opposite that of the SERPINB5 gene (22). In this case, methylation analysis involving the use of methylation-sensitive restriction endonucleases could be used, thus cutting hypomethylated RASSF1A from maternal blood cells and leaving the hypermethylated placental RASSF1A intact for amplification.

If the same approach were to be applied to the detection of the placenta-derived hypomethylated SERPINB5 molecules, an endonuclease that exhibits an opposite action to the methylation-sensitive restriction endonucleases described above would be needed. The McrBC enzyme, which is an endonuclease, was recently reported to cleave methylcytosine-containing DNA sequences (23), but little is known about the exact molecular action of this enzyme, and the length of the cleavage spacing can range from 32 bp to 2 kb (24). For experiments that require high precision, as in the case of detecting fetal DNA in maternal plasma, such an enzyme would not be reliable. Alternatively, if methods of amplifying fragmented DNA were available, methylation-sensitive restriction endonucleases could be used to detect the digested hypomethylated placental DNA molecules in maternal plasma. We hypothesize that with an additional step of stem-loop primer extension before PCR amplification, the methylation-sensitive restriction enzyme-digested, placenta-derived hypomethylated molecules, e.g., SERPINB5, in maternal plasma can be detected.

Materials and Methods

STEM-LOOP PRIMER-MEDIATED AMPLIFICATION OF ENZYME-DIGESTED DNA

Rationale for the stem-loop design. Conventionally, the use of methylation-sensitive restriction endonucleases involves the digestion of unmethylated CpG sites in a DNA sequence, so as to avoid selective PCR amplification of the uncut DNA sequence containing methylated CpG sites. For cases in which the unmethylated DNA is the target, the use of bisulfite conversion seemed to be an unavoidable step. Here, we built on the idea from Chen et al. (25), who used a stem-loop primer to reverse transcribe a microRNA sequence before PCR amplification. We investigated whether the stem-loop extension step would work as well in the priming of short DNA fragments. The mechanism of the stem-loop primer extension on fragmented DNA is illustrated in Fig. 1, A–C.

After restriction enzyme digestion, the DNA would be cut at specific restriction sites. The stem-loop primer was designed in such a way that the last few nucleotides on the 3' tail were complementary to the 3' end of the enzyme restriction site on the target DNA (Fig. 1B). In our assay, we used the same stem-loop sequence as that of the microRNA detection assay by Chen et al. (25). The tail of the stem-loop to anneal to the target DNA fragment had 6 complementary bases, whereas the later-reported stem-loop sequence had 8 complementary bases for annealing (26). The specificity of the stem-loop primers to detect enzyme-digested DNA fragments was evaluated by genotyping of the SERPINB5 –156 single-base variation of placental-maternal DNA mixtures with different fetomaternal genotypes (15). Results showed that the 1st design had a higher specificity for our DNA assays (unpublished data).

Stem-loop assay primer design. Details of the assay design are illustrated in Fig. 1. Our target sequence was the SERPINB5 promoter, which is hypomethylated in the placenta and hypermethylated in maternal blood cells (14). Enzyme digestion was performed by HpaII, a methylation-sensitive restriction endonuclease that recognized and cut the DNA sequence CCGG and whose action would be blocked by CpG methylation. After enzyme digestion, a specific cut site would be created on the hypomethylated placental SERPINB5 DNA molecule, which would allow the stem-loop primer to anneal after DNA denaturation. For the hypermethylated DNA molecules that remained intact after enzyme digestion, the efficiency for stem-loop primer extension would be low. This process is analogous to the TaqMan® microRNA assays, which specifically target the 3' end of the mature microRNA molecules. When the assays were tested on the much longer microRNA precursor molecules, there was a delay in the threshold cycle of 7 or more (25). In our assays, the stem-loop primer annealed to the minus strand of the enzyme-digested SERPINB5 promoter and proceeded with the extension reaction. This extension product served as a template for the subsequent PCR. The detection of the enzyme-digested, stem-loop–extended hypomethylated fetal DNA molecules in 3rd-trimester predelivery maternal plasma was demonstrated by real-time quantitative PCR (Fig. 1D), and the fetal origin of the detected molecules was further confirmed by genotyping the SERPINB5 –156 single-base variation (15) (Fig. 1E).

STUDY PARTICIPANTS

Women with singleton uncomplicated pregnancies who attended the Department of Obstetrics and Gynaecology,
Fig. 1. Schematic diagram of the stem-loop assay design targeting the placenta-derived hypomethylated SERPINB5 promoter in maternal plasma. (A), the hypomethylated SERPINB5 molecule is digested by the HpaII enzyme. HpaII digestion cannot proceed if the target is hypermethylated. The stem-loop primer anneals to the 3' end of the enzyme digestion site (B) and extends the target sequence (C). The extension will unfold the stem-loop, with the extension product serving as a template for PCR. Binding of the stem-loop primer to the undigested long DNA molecule is inefficient, and no stem-loop extension product is expected to be generated from the hypermethylated DNA molecule. (D), quantities of the detectable enzyme-digested, stem-loop–extended SERPINB5 molecules are measured by the real-time PCR assay. (E), the placental specificity of the detectable enzyme-digested, stem-loop–extended SERPINB5 molecules in maternal plasma is verified by the genotyping assay on the SERPINB5 –156 single-base variation.
Prince of Wales Hospital, Hong Kong, were recruited between September 2006 and December 2006. Informed consent was given by all participants, and ethics approval was obtained from the institutional review board. Placental tissue samples were collected from 3rd-trimester pregnancies after elective cesarean delivery. Twelve milliliters of maternal peripheral blood samples were collected into EDTA tubes from all women before cesarean section. An additional 12 mL of blood was collected into EDTA tubes from each of these women 24 h after delivery to demonstrate the postpartum clearance of the fetal DNA sequences.

**PROCESSING OF BLOOD AND TISSUE SAMPLES**

Maternal peripheral blood samples were centrifuged at 1600g for 10 min at 4 °C, and the plasma portion was recentrifuged at 16 000g for 10 min at 4 °C (27). The blood cell portion was recentrifuged at 2500g, and any residual plasma was removed. DNA from the peripheral blood cells and that from maternal plasma was extracted with the blood and body fluid protocol of the QIAamp DNA Blood Mini Kit (Qiagen). DNA samples from the placentas were extracted with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s tissue protocol.

**METHYLATION-SENSITIVE RESTRICTION ENDONUCLEASE DIGESTION**

To create a specific end for the stem-loop primer to anneal to and extend on the target DNA, HpaII (New England Biolabs), a methylation-sensitive restriction endonuclease, was used to digest the hypomethylated DNA. Extracted DNA was digested with the HpaII enzyme at 37 °C for 16 h in a 20-μL reaction mixture. For placental tissue and maternal blood cells, 10 U of HpaII enzyme was used to digest 100 ng and 50 ng of DNA for the real-time and genotyping assays, respectively. For plasma samples, 10 U of the HpaII enzyme was used to digest the DNA from 1.6 mL plasma. A mock-digested aliquot, labeled as undigested, was included for each sample. For mock digestion, an equal amount of DNA was subjected to the same digestion condition without the addition of enzyme.

**STEM-LOOP EXTENSION AND REAL-TIME ASSAY ON MATERNAL PLASMA**

For stem-loop extension, 5 μL of the digestion products were mixed with 10 nmol/L stem-loop extension primer (Integrated DNA Technologies; Table 1A), 1× reverse transcriptase buffer (Applied Biosystems), and 0.25 mmol/L each of dNTPs (Promega), and then the volume was made up to 14 μL with water. This mixture was denatured at 95 °C for 5 min, snap cooled on ice, and 50 U of MultiScribe reverse transcriptase (Applied Biosystems), which worked on both RNA and DNA as templates, was added. This 15-μL reaction was incubated at 4 °C for 5 min, 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. All samples, including no-template controls, were run in duplicate, and the mean quantity was calculated. Placental DNA and maternal blood cell DNA were included as positive and negative controls, respectively.

Real-time TaqMan assay for the SERPINB5 promoter sequence was performed on the HpaII digested, stem-loop–extended DNA; 1.33 μL of the extension product was used for the real-time assay. Each 20-μL real-time PCR included 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 40 nmol/L TaqMan probe (Applied Biosystems), 300 nmol/L forward primer (Inte-

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**Table 1. Oligonucleotide sequences for the real-time stem-loop assay and the genotyping stem-loop assay on the SERPINB5 gene promoter.**

**A. Real-time SERPINB5 assay.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem-loop primer</td>
<td>GTCGTATCCAGTGCGAGGTCCCGAGGATTCCGACTGGATACGACGTCTCTG(^a)</td>
</tr>
<tr>
<td>TaqMan probe</td>
<td>6-FAM-GCAGCTGGGATACGACGTCTC((MGB))</td>
</tr>
<tr>
<td>Forward primer</td>
<td>TTAATGCAGATCGCTACAGACATCGCG</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>AGTGCAGGCTCCAGGATAT</td>
</tr>
<tr>
<td>Calibrator</td>
<td>AGTTACAGTACGCTACAGACATCGCTACGGCAACGCCCATCGAGGAC</td>
</tr>
</tbody>
</table>

**B. Genotyping SERPINB5 – 156 single-base variation assay.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’ to 3’)</th>
<th>Molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem-loop primer</td>
<td>GTCGTATCGAGGATACGACGTCTCTG(^a)</td>
<td>NR</td>
</tr>
<tr>
<td>Forward primer</td>
<td>AGCTTGGATGCGGAAGAAGGCTGATACGACGTCTC(^a)</td>
<td>NR</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>AGCTTGGATGCGGAAGAAGGCTGATACGACGTCTC(^a)</td>
<td>NR</td>
</tr>
<tr>
<td>Extension primer</td>
<td>AGACATGGTGATCCGCAA</td>
<td>5228.4</td>
</tr>
<tr>
<td>Extension product 1</td>
<td>AGACATGGTGATCCGCAAAT</td>
<td>5516.6</td>
</tr>
<tr>
<td>Extension product 2</td>
<td>AGACATGGTGATCCGCAA</td>
<td>5830.8</td>
</tr>
</tbody>
</table>

\(^a\) Underlined nucleotides indicate the 6 bases on the 3’ end of the stem-loop primer that are complementary to the target sequences for primer annealing.

\(^b\) 6-FAM, 6-carboxyfluorescein; MGB, minor-groove binding; NR, not relevant.

\(^c\) Bold nucleotides indicate the 10-mer tags added to the 5’ end of the primers in such a way that the masses of the primers would fall out of the analytic range of the mass spectrometry.
grated DNA Technologies), and 140 nmol/L reverse primer (Integrated DNA Technologies). The sequences of the probe and primers are listed in Table 1A. The thermal profile was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 64 °C for 1 min. Absolute quantification was determined by running a calibration curve with concentrations ranging from 1.25 × 10^6 copies to 6.25 copies per stem-loop extension reaction. Sequence of the synthetic oligonucleotide (Proligo) is shown in Table 1A.

**GENOTYPING OF THE SERPINB5 –156 SINGLE-BASE VARIATION**

Specificity of the stem-loop assay in amplifying the enzyme-digested, hypomethylated placental DNA molecules was further illustrated by assessing the genotype of the amplified enzyme-digested SERPINB5 molecules in maternal plasma samples collected from informative fe-tomaternal pairs. Paired placenta and maternal blood cell samples were genotyped, by use of a previously described protocol (15), for the SERPINB5 –156 single-base variation, which was an A-to-C variation. Any pairs showing different placental and maternal genotypes were deemed informative. The maternal plasma samples from those informative cases were subjected to analysis by a stem-loop genotyping protocol as described below.

**STEM-LOOP EXTENSION AND GENOTYPING ASSAY ON MATERNAL PLASMA**

The stem-loop genotyping protocol, as apposed to the stem-loop real-time protocol, was aimed at specifically genotyping the enzyme-digested plasma SERPINB5 amplified through stem-loop extension. To maximize the input amount of DNA for genotyping, all reaction products from the enzyme digestion reaction were used for the stem-loop extension as a large volume reaction. In the 56-µL reaction mixture, 10 nmol/L stem-loop extension primer (Integrated DNA Technologies; Table 1B), 1× reverse transcriptase buffer (Applied Biosystems), and 0.25 mmol/L each of dNTPs (Promega) were mixed with the digestion product. The mixture was then denatured at 95 °C for 5 min and snap cooled on ice. We added 200 U of MultiScribe reverse transcriptase (Applied Biosystems) to the denatured DNA mixture and incubated at 4 °C for 5 min, 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

In a 200-µL PCR, 1× HotStar Taq PCR buffer with 1.5 mmol/L MgCl₂ (Qiagen), an additional 1 mmol/L MgCl₂, 200 µmol/L dNTP mix (Promega), 750 nmol/L of the forward primer (Integrated DNA Technologies; Table 1B), 350 nmol/L of the reverse primer (Integrated DNA Technologies; Table 1B), and 4 U of HotStar Taq polymerase were added with 53.2 µL of the stem-loop–extended DNA template. The thermal profile was 95 °C for 15 min, followed by 50 cycles of 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min, plus a final incubation at 72 °C for 3 min. Then 25 µL of the PCR products were taken out and subjected to shrimp alkaline phosphatase (SAP) treatment to remove unincorporated dNTPs as previously described (15). The single-base variation genotyping was then performed on the SAP-treated PCR products by a primer extension reaction, the MassARRAY™ Homogenous MassEXTEND™. In brief, 771 nmol/L of extension primer (Integrated DNA Technologies; Table 1B), 1.15 U Thermosequenase (Sequenom), and 64 µmol/L each of ddATP, ddCTP, ddTTP, and dGTP (Sequenom) in an extension cocktail were added to 10 µL of the SAP-treated PCR products. The thermal profile was 94 °C for 2 min, followed by 95 cycles of 94 °C for 5 s, 52 °C for 5 s, and 72 °C for 5 s. The allele-specific extension reactions were designed to generate products of distinct masses for each single-base variation that could be readily resolvable by MALDI-TOF mass spectrometry analysis. Data acquisition from the SpectroCHIP was done using the MassARRAY Analyzer Compact Mass Spectrometer (Sequenom). Mass data were imported into the MassARRAY Typer (Sequenom) software for analysis.

**STATISTICAL ANALYSIS**

Statistical analyses were performed using the SigmaStat 3.0 software (SPSS).

**Results**

**STEM-LOOP EXTENSION AND REAL-TIME PCR ASSAY ON MATERNAL PLASMA WITH AND WITHOUT HpaII ENZYME DIGESTION**

The methylation-sensitive restriction enzyme HpaII was expected to cut the hypomethylated SERPINB5 DNA sequences from the placenta, creating ends necessary for stem-loop primer extension and subsequent PCR detection. On the other hand, the SERPINB5 promoter was hypermethylated in maternal blood cells. In this case, we would expect no fragmentation and no stem-loop extension from the digestion reaction either with or without restriction enzyme. As a result, no signal was expected to be detectable from the subsequent PCR step.

When the placental DNA was digested with the HpaII enzyme, the observed percentage recoveries of detectable SERPINB5 promoter sequence were 12.7%, 3.3%, 1.9%, and 5.8% for 4 separate runs. The percentage recovery was the quantity of detectable SERPINB5 promoter sequences expressed as a percentage of the original amount of DNA input into the reaction. Without HpaII enzyme digestion, no detectable signal was observed for the placental DNA.

When subjected to HpaII digestion followed by stem-loop primer extension, DNA from the maternal blood cells had not been showing any detectable signals. In one particular run of the stem-loop protocol, however, one of the duplicated wells gave a low level of detectable enzyme-digested, stem-loop–extended SERPINB5 promoter sequences, with a percentage recovery of 0.56%.

Eleven pairs of pre- and postdelivery maternal plasma samples were subjected to the stem-loop real-time PCR assay. Comparison was made between HpaII-digested
and undigested predelivery maternal plasma. Results showed that after HpaII enzyme digestion, the detectable SERPINB5 promoter sequence concentration in the predelivery maternal plasma ranged from 52 to 400 copies/mL plasma (Table 2). The median SERPINB5 concentration was 90 copies/mL plasma (interquartile range, 80–183). Without enzyme addition, the SERPINB5 promoter was not detectable in 9 of the 11 cases (median, 0; interquartile range, 0–0). (Table 2). A statistically significant difference in the stem-loop–extended SERPINB5 promoter concentrations was observed between HpaII-digested and undigested predelivery maternal plasma ($P = 0.001$, Wilcoxon signed-rank test), as shown in Fig. 2A.

After confirming the effect of enzyme digestion on creating an extendable end on the placental SERPINB5 promoter in the maternal plasma, we proceeded to compare the enzyme-digested pre- and postdelivery maternal plasma. As described above, stem-loop–extended SERPINB5 promoter was detectable in all 11 predelivery maternal plasma samples after HpaII enzyme digestion (Table 2). For the paired postdelivery maternal plasma after enzyme digestion, postpartum clearance was observed ($P = 0.001$, Wilcoxon signed-rank test), as shown in Fig. 2B.

**GENOTYPING OF THE SERPINB5 −156 SINGLE-BASE VARIATION**

Different placenta and maternal genotypes were observed in 9 of the 32 pairs of placentas and maternal blood cells genotyped for the SERPINB5 −156 single-base variation. Three pairs had a placental genotype of heterozygous AC and a maternal genotype of homozygous AA. The remaining 6 pairs had an opposite fetomaternal genotype combination. Maternal plasma samples from these 9 individuals were chosen for the genotyping of the placenta-derived, enzyme-digested hypomethylated SERPINB5 in maternal plasma.

**Table 2. Real-time quantitative PCR detection of HpaII-digested, stem-loop–extended SERPINB5 promoter in maternal plasma.**

<table>
<thead>
<tr>
<th>Case</th>
<th>Undigested</th>
<th>HpaII digested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predelivery</td>
<td>Postdelivery</td>
</tr>
<tr>
<td>M2275</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M2469</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>M2492</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M2494</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M2505</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M2509</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M2521</td>
<td>0</td>
<td>58</td>
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<tr>
<td>M2526</td>
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<td>0</td>
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<tr>
<td>M2535</td>
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<td>0</td>
</tr>
<tr>
<td>M2551</td>
<td>36</td>
<td>125</td>
</tr>
<tr>
<td>M2570</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Bold numbers indicate the measured quantities of the samples that are expected to have detectable enzyme-digested, stem-loop–extended SERPINB5 molecules.

(A), results from the real-time stem-loop assay showed that the stem-loop–extended SERPINB5 molecules in predelivery maternal plasma were detectable after enzyme digestion. The median SERPINB5 concentration is depicted by a dotted line, and the 25th and 75th percentiles are depicted by solid lines. Without enzyme addition, there were no/low levels of detectable signals from the same maternal plasma samples ($P = 0.001$, Wilcoxon signed-rank test). (B), postpartum clearance was observed when comparing the enzyme-digested, stem-loop–extended SERPINB5 promoter in pre-vs postdelivery maternal plasma ($P = 0.001$, Wilcoxon signed-rank test). Related data for Fig. 2 are listed in Table 2.

Fig. 2. Detection of enzyme-digested, stem-loop–extended hypomethylated fetal DNA molecules in maternal plasma samples.

STEM-LOOP EXTENSION AND GENOTYPING ASSAY ON MATERNAL PLASMA

DNA samples from the placenta and maternal blood cells of known genotypes (heterozygous AC) were used as controls. Results showed that after enzyme digestion, correct genotypes were consistently identified in the placental DNA. In the case of maternal blood cell DNA, nonspecific signals were occasionally detected.

For our maternal plasma analysis, corresponding placental genotypes were correctly identified in all 9 $HpaII-$
digested, stem-loop–extended predelivery maternal plasma DNA (Table 3). Representative mass spectra are shown in Fig. 3.

### Discussion

In this study, we evaluated a new technique for amplifying and detecting DNA fragments with cutting sites created by restriction enzymes. Using the SERPINB5 promoter as a model, we successfully amplified and detected the hypomethylated placental SERPINB5 molecules after methylation-sensitive restriction enzyme digestion. This stem-loop technique represents a useful technological advance for methylation studies, because the protocol does not involve the use of bisulfite treatment, and it allows a selective amplification of the fragmented DNA molecules produced by restriction digestion.

The idea of using a stem-loop primer to extend a nucleic acid fragment was originated from the microRNA detection assays developed by Chen et al. (25). One obvious difference between microRNA and our fragmented DNA target is that the former is single stranded and the latter is double stranded. During the stem-loop extension step, our enzyme-digested DNA must be denatured in such a way that the 3’ end of the digested fragment would be exposed for annealing to the 3’ tail of the stem-loop primer. In our protocol, the enzyme-digested DNA was denatured with the extension mixture, including the stem-loop primer, to maximize the chance of stem-loop and target binding. The heat-sensitive RNA/DNA polymer-

### Table 3. Genotyping results for HpaII-digested, stem-loop–extended SERPINB5 promoter in maternal plasma.

<table>
<thead>
<tr>
<th>Case</th>
<th>Maternal blood cells</th>
<th>Placenta</th>
<th>HpaII-digested maternal plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2484</td>
<td>AA</td>
<td>AC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AC</td>
</tr>
<tr>
<td>M2552</td>
<td>AA</td>
<td>AC</td>
<td>AC</td>
</tr>
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<td>M2508</td>
<td>AA</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td>M2297</td>
<td>AC</td>
<td>AA</td>
<td>AA</td>
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<td>M2922</td>
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<td>M2547</td>
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<td>M2549</td>
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</tr>
<tr>
<td>M2550</td>
<td>AC</td>
<td>AA</td>
<td>AA</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bold genotypes indicate the placental-specific genotypes.
ase was added after denaturation, and the whole mixture was then subjected to the stem-loop extension step.

Our quantitative real-time PCR results demonstrated that the hypomethylated placental SERPINB5 molecules were detectable by adding a stem-loop primer extension step after methylation-sensitive restriction enzyme digestion. On the other hand, detection of the hypermethylated maternal SERPINB5 molecules was of a low efficiency. The low level of signal detected from maternal DNA might be due to HpaII digestion on the rare maternal DNA molecules that were unmethylated at the site of enzyme cleavage. Previous results showed that the SERPINB5 promoter was close to, but not 100% methylated in, maternal blood cells (14). If these discrete cleavage events in maternal DNA were caught by the stem-loop primer, stem-loop extension and PCR amplification would continue.

The maternal plasma analysis showed that after HpaII enzyme digestion, stem-loop extension products were successfully detected in predelivery maternal plasma, which contained the hypomethylated placental SERPINB5. The “unexpected” detection of low levels of signals in predelivery maternal plasma without HpaII digestion and postdelivery maternal plasma with and without enzyme digestion can be explained by the fact that plasma DNA molecules are mainly short fragments, and the fetal-derived fragments are even shorter than the maternal ones, with 80% being shorter than 193 bp (28). Hence, the chance remains that an occasional short maternal plasma DNA fragment will terminate at the same nucleotide position that would be created by HpaII digestion and become extendable. Nonetheless, the level of detectable SERPINB5 molecules showed a statistically significant difference between the HpaII-digested and undigested predelivery maternal plasma. In addition, postpartum clearance of these enzyme-digested, stem-loop–extended SERPINB5 molecules was observed.

Further confirmation of the placental origin of the HapII-digested, stem-loop–extended SERPINB5 molecules in maternal plasma was demonstrated by the genotyping assay. The stem-loop genotyping protocol enabled consistent detection of correct placental genotypes in the placental DNA samples. Occasionally, primer extension products from the homogenous MassEXTEND reaction were detectable from maternal blood cells. This observation may occur because, as indicated by the real-time results, the SERPINB5 promoter is not 100% methylated, although it is close to 100% methylated. In addition, we used a large volume reaction for the genotyping experiments. Consequently, the nonspecific signals may be attributable to the high sensitivity of the MassARRAY platform (29). Results from the maternal plasma analysis revealed that when being subjected to the stem-loop protocol, the hypomethylated placental DNA molecules in the maternal plasma were preferentially amplified. The corresponding placental genotypes were detected in all 9 predelivery maternal plasma samples having different maternal and placental genotypes.

As a pilot study result, we demonstrated the feasibility of using a stem-loop primer to extend and amplify methylation-sensitive restriction enzyme-digested DNA. With further optimization of the current protocol to increase for sensitivity and specificity, this method would be a new approach for amplifying enzyme-digested short DNA fragments. Because the fetal DNA molecules in maternal plasma are intrinsically short, the introduction of a stem-loop extension step may facilitate more flexibility in PCR assay design, given that as few as 21 nucleotides of the target sequence are required, as in the case of the microRNA assays (25). We envision that when more of these hypomethylated placental molecules are discovered, this stem-loop technique will represent an alternative to bisulfite conversion for detection of circulating fetal epigenetic markers. The application of this technique may extend to the use of nonmethylation-sensitive restriction enzymes for the detection of cut DNA fragments for general molecular analyses.

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References


