Hypermethylated RASSF1A in Maternal Plasma: A Universal Fetal DNA Marker that Improves the Reliability of Noninvasive Prenatal Diagnosis


Background: We recently demonstrated that the promoter of the RASSF1A gene is hypermethylated in the placenta and hypomethylated in maternal blood cells. This methylation pattern allows the use of methylation-sensitive restriction enzyme digestion for detecting the placental-derived hypermethylated RASSF1A sequences in maternal plasma.

Methods: We performed real-time PCR after methylation-sensitive restriction enzyme digestion to detect placental-derived RASSF1A sequences in the plasma of 28 1st-trimester and 43 3rd-trimester pregnant women. We used maternal plasma to perform prenatal fetal rhesus D (RhD) blood group typing for 54 early-gestation RhD-negative women, with hypermethylated RASSF1A as the positive control for fetal DNA detection.

Results: Hypermethylated RASSF1A sequences were detectable in the plasma of all 71 pregnant women. The genotype of plasma RASSF1A after enzyme digestion was identical to the fetal genotype in each case, thus confirming its fetal origin. Nineteen of the 54 pregnant women undergoing prenatal fetal RhD genotyping showed undetectable RhD sequences in their plasma DNA samples. The fetal DNA control, RASSF1A, was not detectable in 4 of the 19 women. Subsequent chorionic villus sample analysis revealed that 2 of these 4 women with negative RhD and RASSF1A signals were in fact carrying RhD-positive fetuses.

Conclusions: Hypermethylated RASSF1A is a universal marker for fetal DNA and is readily detectable in maternal plasma. When applied to prenatal RhD genotyping, this marker allows the detection of false-negative results caused by low fetal DNA concentrations in maternal plasma. This new marker can also be applied to many other prenatal diagnostic and monitoring scenarios.

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Circulating fetal DNA analysis in maternal plasma is useful in the prenatal diagnosis of sex-linked disorders (1), fetal rhesus D (RhD) status (2), and β-thalassemia (3). Many of these applications focus on the detection of paternally inherited disease-causing sequences in maternal plasma. Whether the fetus has inherited such a sequence is inferred by the presence or absence of the target sequence in maternal plasma, but the absence of such a sequence in maternal plasma could alternatively be a result of low circulating fetal DNA concentrations or fetal DNA loss during sample processing. Thus, the availability of a positive control confirming the presence of fetal DNA in the sample would be a useful analytical safeguard measure (4). In addition, the quantitative measurement of a fetal DNA target that could be similarly detected in pregnancies with male or female fetuses is useful for monitoring and predicting pregnancy-related conditions associated with aberrant fetal DNA concentrations, including preeclampsia (5–7) and certain fetal chromosomal aneuploidies (8, 9).

Existing fetal DNA markers, such as Y-chromosomal sequences, cannot serve adequately as positive controls.

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1 Nonstandard abbreviations: RhD, rhesus D; CVS, chorionic villus sample.
because they are applicable only to pregnancies carrying a male fetus. To develop a positive control system for fetal DNA detection, genetic variations that differ between the mother and the fetus could be used. Such genetic variation–based approaches, however, would require the use of multiple markers to ensure that at least one would be informative and therefore may increase the complexity of the analysis (10). Alternatively, an epigenetic approach could be explored (11). We recently reported that the maspin (SERPINB5) gene promoter was heavily methylated in maternal blood cells and hypomethylated in the placenta (11). We showed that placental-derived hypomethylated maspin was detectable in maternal plasma regardless of the fetal sex and genetic variations and thus could serve as a universal fetal DNA marker (11). Operationally, the detection of unmethylated maspin sequences among a background of methylated maternal sequences requires the relatively tedious and labor-intensive procedure of bisulfite conversion (11). Bisulfite treatment of a DNA sample could also lead to DNA degradation of up to 96% (12), thus greatly decreasing the ease and efficiency of the use of hypomethylated maspin detection as a positive control for fetal DNA in a routine clinical setting.

In search of other fetal epigenetic markers, we recently demonstrated that the promoter of the RASSF1A tumor suppressor gene is hypermethylated in the placenta but hypomethylated in maternal blood cells, a methylation pattern that is exactly opposite that of the maspin promoter (unpublished data). Consequently, the background maternally-derived hypomethylated RASSF1A sequences could potentially be removed by methylation-sensitive restriction enzyme digestion, whereas the hypermethylated placental (fetal) RASSF1A sequences are expected to be resistant to methylation-sensitive restriction enzyme digestion and thereby should be detectable and quantifiable by real-time PCR.

To illustrate the clinical application of such a universal fetal DNA positive control in noninvasive prenatal diagnosis, we incorporated the use of this new RASSF1A system and the existing SRY system (13) as positive controls for fetal DNA to perform prenatal RhD blood group typing for a cohort of pregnant women. Our aim was to investigate whether the new RASSF1A system would be more effective than the existing SRY system for detecting cases falsely negative for RhD.

**Materials and Methods**

**Patient Recruitment and Sample Collection**

For the module investigating the noninvasive prenatal detection of RhD blood group, pregnant women were recruited from the King’s College Hospital, London, United Kingdom. These patients were undergoing 1st-trimester Down syndrome screening, but all fetuses were later confirmed to be karyotypically normal. For other modules, patients were recruited from the Department of Obstetrics and Gynaecology, Prince of Wales Hospital, Hong Kong. The study was approved by the institutional review boards of the participating clinical units. Informed consent was obtained from all participating women.

Chorionic villus samples (CVSs) were collected primarily for Down syndrome prenatal diagnosis. Third-trimester placental tissues were collected after elective cesarean delivery of uncomplicated pregnancies. Maternal peripheral blood samples were collected just before the performance of obstetric procedures and at 24 h after delivery.

**Sample Processing and DNA Digestion by Methylation-Sensitive Restriction Enzyme**

**Placental tissue and blood cells.** DNA was extracted from 200 µL of maternal blood cells and 0.2 g of placental tissue with the QIAamp DNA Blood Minikit (Qiagen) and the QIAamp DNA Minikit (Qiagen), respectively, and 100 ng of placental and maternal blood cell DNA were digested with 100 U of BstU I, a methylation-sensitive restriction enzyme, at 60 °C for 16 h.

**Plasma samples.** Plasma was harvested (14) and DNA was extracted from 800 µL (RhD blood typing module) or 1.6 mL (other modules) of plasma with the QIAamp Minikit and eluted with 50 µL of H2O, and 35 µL of plasma DNA were digested with 100 U of BstU I enzyme at 60 °C for 16 h.

**Bisulfite Sequencing of the Beta-actin Gene in Placenta and Maternal Blood Cells**

We bisulfite-converted 1 µg of each extracted DNA sample with the CpGenome Universal DNA Modification Kit (Chemicon) according to the manufacturer’s recommendations. Each bisulfite-converted DNA sample was subjected to PCR amplification with the primers 5’-TTATTTGGYGGYGGGTGGA-3’ and 5’-TCCCTCCT-CCTTCTCTCAATCTC-3’. The PCR product was cloned and sequenced. After confirming the completeness of bisulfite conversion, the CpG sites sequenced as cytosine or thymine residues were scored as methylated or unmethylated, respectively.

**Real-time Detection of SRY, RASSF1A, and Beta-actin Sequences**

Real-time PCR assays were established for the detection of SRY, RASSF1A, and beta-actin (ACTB) sequences. The sequences of the primers and probes are listed in Table 1. The contents and thermal profiles of the 3 assays were identical apart from the primers and probes used. Each reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nmol/L each primer, and 85 nmol/L probes. We used 5 µL of non–enzyme-treated plasma DNA or 7.15 µL of enzyme-digested plasma DNA mixture (equivalent to 5 µL of undigested plasma DNA)

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7 Human genes: maspin (SERPINB5), serpin peptidase inhibitor, clade B (ovalbumin), member 5; beta-actin (ACTB), actin, beta; RASSF1A, Ras association (RalGDS/AF-6) domain family 1A; RhD, Rh blood group, D antigen.
Table 1. Primers and probes for the real-time PCRs for RHD, RASSF1A, beta-actin, and SRY.

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Sequence</th>
<th>Primer/probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>RASSF1A</td>
<td>RSF-b151F</td>
<td>5'-AGC CTG AGC TCA TTG AGC TG3'-FAM-AGC TAC GCA (TAMRA)-3'</td>
<td>Primer</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>RSF-dsgnR</td>
<td>5'-ACC AGC TGC CGT GTG G-3'</td>
<td>Primer</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>RSF-dsgnT</td>
<td>5'-FAM-CCA AGG CGG TGC GCA T(MGB)-3'-FAM-ACC GCC GAG ACC GCG TC(MGB)-3'</td>
<td>Probe</td>
</tr>
<tr>
<td>beta-actin</td>
<td>Actin-163F</td>
<td>5'-GGC CGG TTC CGA AAG TT-3'</td>
<td>Primer</td>
</tr>
<tr>
<td>beta-actin</td>
<td>Actin-298R</td>
<td>5'-GGG CGG ATC GGC AAA-3'</td>
<td>Primer</td>
</tr>
<tr>
<td>beta-actin</td>
<td>Actin-243T</td>
<td>5'-FAM-ACC GCC GAG ACC GCG TC(MGB)-3'</td>
<td>Probe</td>
</tr>
<tr>
<td>SRY</td>
<td>SRY-109F</td>
<td>5'-TGG CGA TTA AGT CAA ATT CGC-3'</td>
<td>Primer</td>
</tr>
<tr>
<td>SRY</td>
<td>SRY-243R</td>
<td>5'-CCC CCT AGT ACC CTG ACA ATG TAT T-3'</td>
<td>Primer</td>
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<tr>
<td>SRY</td>
<td>SRY-142T</td>
<td>5'-FAM-AGCAGT AGA GCA GTC AGG GAG GCA GA(TAMRA)-3'</td>
<td>Probe</td>
</tr>
</tbody>
</table>

* MGB, minor-groove binding.

as the template for each PCR. The thermal profile was 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. All reactions were run in duplicate, and the mean quantity was taken. A DNA construct containing 1 copy each of the RASSF1A, SRY, and beta-actin amplicons was established as the quantitative standard of the 3 assays. The detection limit for all the 3 assays was 1 copy per reaction.

**ENZYME DIGESTION EFFICIENCIES FOR UNMETHYLATED RASSF1A AND BETA-ACTIN SEQUENCES**

To investigate the similarity of the enzyme digestion efficiencies for unmethylated RASSF1A and beta-actin sequences, 1-μg aliquots of maternal buffy coat were digested with 100 U of BstUI enzyme for different time intervals (from 15 min to 16 h). The concentrations of RASSF1A and beta-actin sequences were measured in each sample after the enzyme digestion.

**REAL-TIME DETECTION OF RHD SEQUENCES**

The RhD genotype was determined by real-time amplification of 2 regions located in exon 7 and exon 10 of the RHD gene as previously described (2, 15). The results of the 2 assays were identical in all cases. Therefore, the results in the subsequent sections will be presented as RHD-positive or RHD-negative only.

**DUPLEX REAL-TIME PCR ASSAYS FOR RASSF1A AND BETA-ACTIN**

A duplex assay for the simultaneous amplification of RASSF1A and beta-actin was developed for the RhD blood group typing module. The reaction mix, thermal profile, primers, and probes used in this assay were identical to those used in the monoplex assays, apart from changing the fluorescent reporter dye of the beta-actin TaqMan probe to VIC™ (Applied Biosystems). The final concentrations of the beta-actin primers and probe were increased to 450 nmol/L and 126 nmol/L, respectively.

**GENOTYPING OF THE RASSF1A GENE**

A single nucleotide polymorphism (id rs4688725) is located within the RASSF1A amplicon. We determined RASSF1A genotypes for maternal blood cells, the placenta, and maternal plasma DNA with and without BstUI I enzyme digestion. PCR amplification of the RASSF1A sequence was performed with the primers RSF-b151F and RSF-dsgnR. After PCR amplification, we set up a primer extension reaction, based on the homogeneous MassEXTEND protocol (Sequenom), and each 14-μL reaction contained 10 μL of PCR products, 0.77 μmol/L extension primer Rsf-R17 5'-CAG CCG GGT GGG CCC TdT-3', 1.15 U of thermosequenase, and a mixture of dideoxynucleotides (ddATP, ddCTP, and ddTTP) and the dideoxynucleotide dGTP (64 μmol/L each). For a RASSF1A sequence with an allelotype A, the primer would be extended to produce 5'-CAG CCG GGT GGG CCC TdT-3' with a molecular mass of 5476.6 Da. For a RASSF1A sequence with an allelotype C, the primer would be extended to produce 5'-CAG CCG GGT GGG CCC TdGdC-3' with a molecular mass of 5790.8 Da. The final base extension products were analyzed by the MassARRAY compact matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Sequenom). The genotype of the RASSF1A was determined by the TyperAnalyzer software (Sequenom).

**Results**

**DETECTION OF RASSF1A AND BETA-ACTIN SEQUENCES IN PLACENTAL TISSUES AND MATERNAL BLOOD CELLS WITH AND WITHOUT BstUI I ENZYME DIGECTION**

Because BstUI I is a methylation-sensitive restriction enzyme, we would expect hypomethylated DNA sequences, such as the RASSF1A molecules derived from maternal blood cells, to be digestible and not detectable after enzyme digestion. Without enzyme digestion, RASSF1A sequences were detectable in both the placental tissues and maternal blood cells. After enzyme digestion, only RASSF1A molecules from the placenta were detected. In contrast, beta-actin molecules were detectable only without enzyme digestion, regardless of their origins. This finding is in line with the bisulfite sequencing results that all CpG sites in the beta-actin amplicon were unmethylated in both the placenta and the maternal blood cells (see Fig. 1 in the Data Supplement that accompanies the online
version of this article at http://www.clinchem.org/content/vol52/issue12).

COMPARISON OF THE ENZYME DIGESTION EFFICIENCIES FOR UNMETHYLATED RASSF1A AND BETA-ACTIN SEQUENCES
The concentrations of RASSF1A and beta-actin sequences showed a positive correlation for any given extent of incomplete enzyme digestion ($r = 0.986; P < 0.0001$; Pearson correlation; see Fig. 2 in the online Data Supplement). These data suggest that the enzyme digestion efficiencies for unmethylated RASSF1A and beta-actin sequences were similar, thus justifying the use of beta-actin as a control for the completeness of the enzyme digestion of unmethylated RASSF1A sequences.

DETECTION OF RASSF1A IN MATERNAL PLASMA AFTER ENZYME DIGESTION
We analyzed the plasma samples of 71 pregnant women (28 in the 1st trimester and 43 in 3rd trimester) and 25 nonpregnant women for RASSF1A after enzyme digestion. The median fetal gestational ages for 1st-trimester and 3rd-trimester pregnant women were 12 weeks and 38 weeks, respectively. RASSF1A sequences were detectable in all pregnant women but not in those who were not pregnant (Fig. 1). The median plasma RASSF1A concentrations of the 1st-trimester and 3rd-trimester pregnant women were 40 copies/mL and 245 copies/mL, respectively. The beta-actin digestion control was analyzed for all the samples. In 4 plasma samples, beta-actin sequences were detectable after enzyme digestion (3 from pregnant women and 1 from a nonpregnant woman). There was no statistically significant difference in the rate of incomplete enzyme digestion between the pregnant and nonpregnant women ($4.2\%$ vs $4.0\%$; $P = 1.0$; Fisher exact test).

Fig. 1. Box plot of the plasma concentrations of RASSF1A sequence after BstU I enzyme digestion in 25 nonpregnant women and 28 1st-trimester and 43 3rd-trimester pregnant women.
The upper and lower whiskers represent the 90th and 10th percentiles, respectively. The upper, middle, and lower bars of the box represent the 75th, 50th, and 25th percentiles, respectively. The y-axis is in logarithmic scale.

Fig. 2. Correlation of the plasma concentrations of the enzyme-digestion–resistant RASSF1A and the SRY sequences in the 9 1st-trimester (A) and the 24 3rd-trimester (B) pregnant women carrying male fetuses.
The RASSF1A concentration after enzyme digestion shows a positive correlation with SRY concentration for the 1st-trimester ($r = 0.850; P < 0.0001$; Spearman correlation) and the 3rd-trimester ($r = 0.717; P < 0.0001$; Spearman correlation) pregnancies. The x-axis and y-axis represent the plasma concentrations of the RASSF1A and SRY sequences, respectively, and both axes are in logarithmic scale.

The enzyme digestion was repeated in these cases, with RASSF1A and beta-actin subsequently becoming undetectable.

CLEARANCE OF HYPERMETHYLATED RASSF1A SEQUENCE IN MATERNAL PLASMA AFTER DELIVERY
Five pregnant women each carrying a male fetus were recruited for the study of the postdelivery clearance of RASSF1A sequences in enzyme-digested maternal plasma. Both RASSF1A and SRY sequences were detectable in the plasma before delivery but were completely cleared at 24 h after delivery.
The plasma concentrations of SRY and RASSF1A (with and without enzyme digestion) were studied in the 9 1st-trimester and the 24 3rd-trimester pregnant women, each carrying a single male fetus. A positive correlation was observed between the plasma concentrations of enzyme-digestion–resistant RASSF1A and SRY sequences for the 1st-trimester (r = 0.850; P < 0.0001; Spearman correlation; Fig. 2A) and the 3rd-trimester (r = 0.717; P < 0.0001; Spearman correlation; Fig. 2B) pregnant women. Because the only source of SRY in maternal plasma was the fetal tissues, this observation further supported the fetal origin of enzyme-digestion–resistant RASSF1A. In contrast, the concentration of RASSF1A without enzyme digestion, which reflected the concentration of molecules predominantly derived from the mother, did not correlate with the concentration of SRY for the 1st-trimester (r = 0.083; P = 0.81; Spearman correlation) and the 3rd-trimester (r = 0.228; P = 0.28; Spearman correlation) pregnant women.

IDENTICAL RASSF1A GENOTYPES FOR THE ENZYME-DIGESTED PLASMA AND PLACENTA
The RASSF1A genotypes of the fetus (placentas or CVSs), mother (maternal blood cells), and enzyme-digested maternal plasma DNA were determined for 43 3rd-trimester (also with genotypes of undigested plasma DNA) and 16 1st-trimester pregnant women. The genotyping mass
spectra of a typical case (Fig. 3) show that the genotype of the maternal plasma DNA without enzyme digestion (AC) was identical to that of the maternal blood cells (AC). This is because the total circulating RASSF1A sequences were predominantly maternally derived. On the other hand, the genotype of the maternal plasma DNA after enzyme digestion (CC) was the same as the placental genotype (CC). This finding suggests that circulating enzyme-digestion–resistant RASSF1A sequences were derived from fetal tissues. The RASSF1A genotyping results of 59 pregnant women (16 1st-trimester and 43 3rd trimester) (Table 2) show that in each of the 59 cases, the genotype of the maternal plasma DNA after enzyme digestion was identical to the placental (fetal) genotype.

<table>
<thead>
<tr>
<th>Blood cells</th>
<th>Undigested plasma</th>
<th>Placenta</th>
<th>Digested plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AA</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>11</td>
<td>AC</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td>12</td>
<td>CC</td>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td>3</td>
<td>AA</td>
<td>AA</td>
<td>AC</td>
</tr>
<tr>
<td>3</td>
<td>AC</td>
<td>AC</td>
<td>AA</td>
</tr>
<tr>
<td>7</td>
<td>AC</td>
<td>AC</td>
<td>CC</td>
</tr>
<tr>
<td>2</td>
<td>CC</td>
<td>CC</td>
<td>AC</td>
</tr>
</tbody>
</table>

A. 3rd-trimester pregnant women.

<table>
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<tr>
<th>Blood cells</th>
<th>CVS</th>
<th>Digested plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
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<tr>
<td>3</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>3</td>
<td>AC</td>
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</table>

Noninvasive prenatal RHD blood typing with enzyme-digestion–resistant RASSF1A as a positive control for fetal DNA

Of 355 pregnant women screened for RhD status, 54 were RhD-negative, and their plasma and CVS were subjected to further investigation for fetal RhD status. RHD sequences were detected in the maternal plasma of 35 of these 54 pregnant women. In the plasma DNA samples of the remaining 19 women with negative maternal plasma RHD results, 15 were positive for enzyme-digestion–resistant RASSF1A sequences and the other 4 showed negative results. In the former group, the positive detection of the RASSF1A sequences in maternal plasma confirmed the presence of fetal DNA, and therefore the negative RHD results could be confidently interpreted. Indeed, CVS analysis revealed that all these 15 women were carrying RhD-negative fetuses. On the contrary, the failure to detect RASSF1A sequences in the remaining 4 cases suggested that their negative RHD results should be interpreted with caution. In fact, the CVSs were positive for RHD in 2 of these 4 cases, indicating that the maternal plasma RHD results were falsely negative because of low fetal DNA concentrations in those plasma samples. These results indicate that the RASSF1A assay has served its function in flagging potentially problematic cases for further investigation. Of the 19 plasma samples negative for RHD, only 6 were positive for SRY, thus highlighting the inadequacies of SRY as an internal control. The flow chart of the screening procedures and results is presented in Fig. 4.

Discussion

In this study, we developed a new test for the detection of fetal DNA in maternal plasma. This test is based on the detection of a hypermethylated placental (fetal) DNA sequence in the maternal circulation. In contrast to existing fetal DNA markers that detect Y-chromosomal sequences and genetic variations between the fetus and mother, this new method is applicable to all pregnancies irrespective of the sex and genetic variations of the fetus.

The methylation pattern of the RASSF1A promoter in the placenta and maternal blood cells allows the use of methylation-sensitive restriction enzyme digestion for specifically cutting the maternally derived background RASSF1A sequences while leaving the placently (fetal) derived RASSF1A sequences intact. This system is expected to be more sensitive, more reliable, and easier to use than the bisulfite-based method used for the detection of unmethylated mastin sequences (11). In this study, RASSF1A sequences were detectable in all the 1st- and 3rd-trimester maternal plasma samples after enzyme digestion.

In addition, an internal control system was devised for the detection of incomplete enzyme digestion, which could potentially lead to false-positive results. This internal control system consisted of a real-time PCR assay targeting the beta-actin gene promoter. Because, as we
have shown, the digestion efficiencies of unmethylated beta-actin and RASSF1A sequences are similar, an undetectable beta-actin signal should reflect the completeness of the enzyme digestion of unmethylated RASSF1A sequences. During the evaluation of the detectability of RASSF1A sequences in enzyme-digested plasma DNA, this digestion control system detected 4 cases of incomplete enzyme digestion. By duplexing the RASSF1A and beta-actin assays, the robustness of this new fetal DNA test is further enhanced.

Our findings demonstrate that the enzyme-digestion-resistant RASSF1A sequences that are detectable in maternal plasma are of fetal origin because (a) there was postpartum clearance of such sequences; (b) they were not detectable in the plasma of nonpregnant women; (c) a positive correlation between the maternal plasma concentration of enzyme-digestion–resistant RASSF1A and SRY sequences was observed, reaffirming their common fetal origin; and (d) in every case the plasma RASSF1A genotype was identical to the fetal genotype after enzyme digestion.

In addition to being a potentially useful generic circulating fetal DNA marker for the monitoring and assessment of pregnancy-related conditions, the new test was an ideal system to be used as a positive control for fetal DNA detection in maternal plasma. Hence, in the last part of the study, we attempted to demonstrate the clinical utility of such a positive control system in prenatal diagnosis. We recruited patients from early gestation and used a reduced volume of plasma for DNA extraction. As a result, some of the extracted plasma samples would be expected to have very low fetal DNA concentrations. Of
the 19 RhD-negative pregnant women with no detectable RhD sequence in their plasma, 15 were positive for RASSF1A, thus confirming the presence of fetal DNA in these plasma DNA samples. Therefore, the negative detection of RhD in their plasma should indicate a RhD-negative fetus. For the 4 cases that were negative for both RhD and enzyme-digestion-resistant RASSF1A, their fetal RhD typing results should be regarded as inconclusive because of the absence or low concentration of fetal DNA in their plasma samples. Through CVS analysis, 2 of these 4 cases were confirmed to be carrying RhD-positive fetuses. Thus, for these 2 cases, their falsely negative maternal plasma RhD results were correctly identified by our failure to detect the positive control, enzyme-digestion-resistant RASSF1A. Although the CVSs of the other 2 cases were negative for RhD, the absence of RhD signal could be due either to a low fetal DNA concentration in their plasma DNA samples or an absence of RhD sequence in the fetus. In other words, these 2 apparently correct RhD typing results could merely be a coincidence of the collection of maternal plasma samples with low fetal DNA concentration from pregnant women carrying RhD-negative fetuses. In real-life situations in which no CVSs would be available, any fetal RhD typing results with negative detection of enzyme-digestion-resistant RASSF1A should be regarded as inconclusive, and further testing with another plasma sample would be indicated.

To further illustrate the advantage of using a sex-independent fetal marker, the results were compared with an existing fetal DNA marker, SRY. The SRY assay would be positive only when the fetus is a male. Of the 19 women with negative detection of RhD sequence in their plasma, only 6 were positive for SRY, which further confirmed an RhD-negative fetus. In the remaining 13 cases, whether the negative detection of RhD and SRY sequences in the plasma DNA was a result of a female RhD-negative fetus or the inadequate fetal DNA in the maternal plasma could not be ascertained.

In this study, we developed a universal fetal DNA marker that can be readily detected in maternal plasma. As illustrated in the example of noninvasive fetal RhD status determination, this new test is invaluable in prenatal diagnostic procedures as a positive fetal DNA control. Larger cohorts would be necessary, however, to compare the effectiveness of this marker with that of other existing polymorphic markers. Moreover, it should be noted that aberrant methylation of the RASSF1A gene promoter can also be found in certain malignant conditions. Therefore, for patients with a known history of cancer, alternative fetal DNA markers may be necessary. In the context of a pregnant woman without a known history of cancer, however, it is unlikely that the plasma methylated RASSF1A sequences would be derived from a concurrent occult malignancy rather than from the placenta. We envision that, due to its applicability to all pregnancies and the methodologic simplicity of the assay, this new marker will be an attractive alternative to existing sex-specific and polymorphic markers as positive controls for fetal DNA detection in noninvasive prenatal diagnostic procedures using plasma DNA.

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