Detection of the placental epigenetic signature of the *maspin* gene in maternal plasma


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Edited by Yuet Wai Kan, University of California School of Medicine, San Francisco, CA, and approved August 29, 2005 (received for review April 22, 2005)

The discovery of fetal DNA in the plasma of pregnant women has opened up new approaches for noninvasive prenatal diagnosis and monitoring. Up to now, the lack of a fetal DNA marker that can be universally detected in maternal plasma has limited the clinical application of this technology. We hypothesized that epigenetic differences between the placenta and maternal blood cells could be used for developing such a marker. By using bisulfite DNA sequencing, the methylation status of the *maspin* gene promoter in placental tissues and paired maternal blood cells from pregnant women was analyzed. The *maspin* gene promoter was found to be hypomethylated in placental tissues and densely methylated in maternal blood cells. Genotyping of a single nucleotide polymorphism within the unmethylated *maspin* sequences in maternal plasma demonstrated that these sequences were derived from the fetus. By using real-time quantitative methylation-specific PCR, unmethylated *maspin* sequences were detected in maternal plasma in all three trimesters of pregnancy and were cleared within 24 h after delivery. The maternal plasma concentration of unmethylated *maspin* sequences was elevated by a median of 5.7 times in preeclamptic pregnancies compared with nonpreeclamptic pregnancies. Hypomethylated *maspin* DNA is the first universal marker for fetal DNA in maternal plasma, thus allowing the measurement of fetal DNA concentrations in pregnancy-associated disorders, irrespective of fetal gender and genetic polymorphisms. Differential DNA methylation between the placenta and maternal blood cells may be exploited to develop further markers for noninvasive prenatal assessment.

DNA methylation | noninvasive prenatal diagnosis | circulating nucleic acids

The discovery of circulating fetal DNA in the plasma of pregnant women has opened up new possibilities for noninvasive prenatal diagnosis and monitoring (1). This noninvasive source of fetal DNA has been used for the prenatal diagnosis of sex-linked diseases (2), fetal rhesus D blood group status (3), and β-thalassemia (4). In addition, quantitative aberrations in the concentrations of fetal DNA in maternal plasma have been described in a number of pregnancy-associated disorders, including fetal trisomy 21 (5, 6), preeclampsia (7, 8), and preterm labor (9). These developments underscore the clinical impact that maternal plasma fetal DNA may play in the future development of prenatal diagnosis and monitoring.

Fetal DNA circulates in maternal plasma among a high background of maternal DNA sequences. Thus, an important obstacle that has to date limited the widespread clinical application of this promising prenatal assessment approach is the lack of a fetal-specific DNA marker that can be detected in maternal plasma in all pregnancies, regardless of fetal gender and genotype. Such a universal marker is important for the quantitative and qualitative applications of circulating fetal DNA analysis. Because Y-chromosomal DNA sequences are readily distinguishable from the background maternal plasma DNA, most investigators have thus far relied on the use of such sequences as fetal-specific markers and have reported the association of abnormal concentrations of circulating fetal Y-chromosome DNA with a number of pregnancy-related complications (5–10). Studies have further demonstrated that such quantitative aberrations are detectable in maternal plasma before symptom onset (7, 11). These observations have led to the proposal that quantitative assessment of circulating fetal DNA may represent a means for predicting fetomaternal complications (12). However, the current approach based on Y-chromosomal DNA analysis cannot be used for the 50% of pregnancies involving female fetuses, thus hindering the translation of these promising observations into the realms of clinical use.

Besides quantitative fetal DNA assessments, the availability of a universal fetal DNA marker would also be beneficial to the noninvasive qualitative detection of fetal genetic traits and mutations in maternal plasma. For these applications, such as fetal rhesus D status determination (3) and paternal mutation detection (4), therapeutic management of the pregnancy is based critically on whether the fetus is shown to have inherited the relevant paternal trait or mutation, as reflected by the presence or absence of the corresponding fetal genetic sequence in maternal plasma. However, a lack of sequence amplification could also be due to low fetal DNA concentration or fetal DNA loss during sample processing. These situations could consequently be falsely interpreted as the lack of fetal inheritance of the paternal trait or mutation sought for. Thus, investigators have advocated the concurrent testing of additional fetal-specific markers to safeguard against such possibilities (13). Because Y-chromosomal markers are fetal-gender-specific, investigators have explored the use of genetic polymorphisms (14). However, exploring genetic polymorphisms greatly increases the complexity of the resulting tests, because multiple genetic markers would be needed to ensure that the fetus and mother are informative for at least one of the markers used.

It would therefore be a breakthrough in noninvasive prenatal assessment if one could find a fetal DNA marker that could be detected in maternal plasma in all pregnancies. The limitations of genetic markers have prompted us to explore an epigenetic solution. Epigenetics involves processes, such as DNA methylation, that alter the phenotype but are not associated with changes in the DNA sequence (15). In this study we aim to demonstrate that a universal fetal epigenetic marker can be developed for maternal plasma detection.

The central requirement for such a marker is that it should exhibit different methylation patterns depending on whether it is originating from fetal or maternal tissues. Circumstantial evidence from the

1This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: M-*maspin*, methylated *maspin*; U-*maspin*, unmethylated *maspin*; IQR, interquartile range.

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literature has suggested that the most likely source of fetal DNA in maternal plasma is the placenta (16, 17). Data from a bone marrow transplantation model of plasma DNA have prompted us to deduce that hematopoietic cells are the source of the bulk of the background maternal DNA in a pregnant woman’s plasma (18). Based on these lines of reasoning, our proposed approach involves comparing the methylation patterns between placental DNA and maternal blood cell DNA. The candidate gene that we investigated was the maspin (SERPINB5) gene. Maspin is a tumor suppressor gene expressed in the placenta (19). Data have shown that DNA methylation plays an important role in regulating the tissue-specific expression of this gene and exploring its detection in maternal plasma.

Materials and Methods

Subjects and Sample Collection. Women with singleton uncomplicated pregnancies who attended the Department of Obstetrics and Gynecology at the Prince of Wales Hospital, Hong Kong, were recruited sequentially between February and August, 2004. Informed consent had been sought from each subject, and the study was approved by the Institutional Review Board. First-, second-, and third-trimester subjects were recruited among women undergoing pregnancy termination, maternal serum biochemical screening, and elective cesarean delivery, respectively. Maternal peripheral blood samples (12 ml of EDTA) were collected just before the performance of obstetrics procedures and elective cesarean delivery, respectively. Maternal peripheral blood samples were also collected from pregnant women diagnosed with preeclampsia between January 2003 to May 2004, with criteria as described in ref. 11.

Sample Processing and DNA Extraction. Blood samples were centrifuged at 1,600 × g for 10 min at 4°C, and the plasma portion was recentrifuged at 16,000 × g for 10 min (21). The peripheral blood cell portion was recentrifuged at 2,500 × g, and any residual plasma was removed. DNA was extracted from peripheral blood cells with the Nucleon blood DNA extraction kit (GE Healthcare, Little Chalfont, U.K.) and from placental tissues with the QIAamp tissue kit (Qiagen, Hilden, Germany). DNA was extracted from 0.8 ml of plasma with the QIAamp blood kit (Qiagen) according to the manufacturer’s blood and body fluid protocol.

Bisulfite Genomic Sequencing of maspin Promoter. Extracted DNA was bisulfite-converted by using the CpGenome universal DNA modification kit (Chemicon International, Temecula, CA). Bisulfite converts unmethylated cytosine into uracil while leaving methylated cytosine unchanged (22). The methylation status of the maspin promoter in the placental tissues and maternal blood cells was determined by cloning followed by DNA sequencing of the bisulfite-converted DNA preparations (23). PCR primers were designed to interrogate eight of the CpG sites in the maspin promoter as indicated in Fig. 1. Sequences of all oligonucleotides related to the PCR assays developed in this study are listed in Table 2, which is published as supporting information on the PNAS web site. Clones were picked randomly, and the completeness of bisulfite conversion was first confirmed before scoring. The CpG sites sequenced as cytosine or thymine residues were scored as methylated or unmethylated, respectively. At least nine clones were scored for each DNA sample.

Real-Time Quantitative Methylation-Specific PCR. Concentrations of methylated (M-maspin) and unmethylated (U-maspin) maspin pro-
moter DNA sequences were measured in maternal plasma by real-time quantitative methylation-specific PCR assays (Fig. 1) (24) on an Applied Biosystems 7900 HT Sequence Detection System. The DNA extracted from 0.8 ml of plasma was bisulfite-converted and used as template for the assays. The PCR was performed by using a TaqMan PCR core reagent kit (Applied Biosystems). Calibration curves for each assay were prepared by serial dilutions of single-stranded synthetic DNA oligonucleotides (Proligo, Singapore) specific to the M- and U-maspin amplicons. Real-time quantitative PCR was also developed to detect the bisulfite-converted form of SRY in maternal plasma.

**Quantitative Assessment of maspin mRNA Expression.** Real-time quantitative RT-PCR was developed to determine the maspin mRNA level in placental tissues. The reactions were set up using the EZ rTth RNA PCR reagent set (Applied Biosystems). Contaminating DNA in the extracted placental tissue RNA was removed by DNase I digestion (Invitrogen, Carlsbad, CA). The calibration curve was prepared as described above. GAPDH mRNA concentration was measured for normalization as described in ref. 25.

**Genotype Analysis of maspin.** During analysis of the bisulfite sequencing data, a previously unreported SNP positioned at 156 bp upstream of the transcription start site was noted in the maspin promoter. We performed genotype analysis of this -156 SNP on placental tissues, maternal blood cells, and plasma collected from eight fetomaternal pairs. SNP genotyping was performed on genomic DNA from the placental tissues and maternal blood cells using the standard primer extension (homogenous MassEXTEND) protocol on a MassARRAY Compact system (Sequenom, San Diego), which is a MALDI-TOF MS system (26).

We then assessed the -156 SNP genotype among the U-maspin sequences in maternal plasma. Plasma (0.8 ml) was bisulfite-converted as described above. The maspin promoter was amplified using nested primers by conventional PCR. MassEXTEND assay was performed according to manufacturer’s instructions. The nested primers and extension primer were designed to anneal to U-maspin promoter sequences. The extension reaction was performed using a terminator mix consisted of ddCTP, ddGTP, ddTTP, and dATP. The extension reaction began at the SNP site (Fig. 1).

**Statistical Analysis.** Statistical analysis was performed with SIGMA-STAT 3.0 (SPSS, Chicago), except for the comparison between preeclamptic and matched control pregnancies, in which the logistic regression software LOGXACT 1.3 (Cytel, Cambridge, MA) was used.

**Results**

**Methylation Analysis of Placental Samples.** The methylation status of the maspin promoter in placental tissues and the corresponding maternal blood cells was studied among 16 normal pregnancies: eight (four male and four female fetuses) from the first trimester and eight (five male and three female fetuses) from the third trimester of pregnancy. The bisulfite sequencing data obtained from two each of the first- and third-trimester cases are shown in Fig. 2, and, for all other cases, are shown in Fig. 7, which is published as supporting information on the PNAS web site. In all cases, the studied CpG sites (Fig. 1) in the maspin promoter were almost completely methylated in the maternal blood cells but hypomethylated in the corresponding placental tissue samples (Figs. 2 and 7). We compared the proportion of unmethylated and methylated molecules among all of the placental tissue samples and that of the maternal blood cells for each CpG site by χ² analysis. The data for the statistical analysis are summarized in Table 3, which is published as supporting information on the PNAS web site. The differences in the methylation profile between the maternal peripheral blood cells and paired placental tissues in the first and third trimesters at each CpG site are statistically significant (χ² test, $P < 0.0001$) (Table 3).

**Inverse Correlation Between maspin Gene Methylation and Gene Expression.** We determined the normalized maspin expression level and the methylation indices (percent methylation) (24) for the placental tissues obtained from the 16 pregnancies described above. maspin mRNA expression was found to be inversely correlated with the maspin promoter methylation index ($r = -0.540$, $P = 0.03$, Pearson correlation) (Fig. 8, which is published as supporting information on the PNAS web site).
The median U-maspin DNA concentrations were 17.7 copies per ml (interquartile range (IQR), 6.4–27.5), 51.6 copies per ml (IQR, 29.9–71.1), and 194.5 copies per ml (IQR, 84.2–374.2), for the first, second, and third trimesters, respectively. The U-maspin concentrations observed between the three trimesters of pregnancy were statistically significantly different (Kruskal–Wallis test, \( P < 0.00091 \)).

Pre- and Postdelivery Correlation Between Genetic and Epigenetic Fetal DNA Markers. To determine whether there was any correlation between the fetal DNA concentration measured by using epigenetic and genetic markers, U-maspin (epigenetic) and SRY (genetic) sequences were quantified in the plasma of 12 pregnant women bearing male fetuses just before delivery by elective cesarean section. U-maspin and SRY concentrations were positively correlated (\( r = 0.668, \ P = 0.02 \), Pearson correlation) (Fig. 9, which is published as supporting information on the PNAS web site). Both assays were optimized to run under identical PCR conditions and demonstrated similar efficiencies as reflected by the slopes and \( y \)-intercepts of the two calibration curves. The slopes of the calibration curves of the U-maspin and SRY assays were \( 3.34 \) and \( 3.42 \), respectively, whereas the \( y \)-intercepts showed threshold cycle values of 44.18 and 43.52, respectively. Both assays can detect the lowest calibrator concentration (1.25 copies per \( \mu l \)) reproducibly.

We further studied the clearance of U-maspin sequences from maternal plasma after delivery. In 10 of the above cases from whom postdelivery samples were available, both U-maspin and SRY sequences were rapidly cleared from the maternal plasma after delivery to almost undetectable levels (\( P = 0.002 \) for both experiments, Wilcoxon signed-rank test) (Fig. 4A and B), demonstrating that their existence in maternal plasma was fetal-specific. As a control, the level of M-maspin (mostly representing maternal DNA) in maternal plasma did not change significantly before and after delivery (\( P = 0.16 \), Wilcoxon signed-rank test) (Fig. 4C), showing that it was not fetal-specific.

maspin −156 SNP Genotyping. The −156 SNP is an A/C polymorphism. The fetal and maternal maspin genotypes, shown in Table 1, were determined from eight third-trimester pregnancies by using genomic DNA collected from placental tissues and the corresponding maternal blood cells. Based on the methylation profile of the maspin promoter in placental tissues (Fig. 2) and the postdelivery clearance of maternal plasma U-maspin (Fig. 4A), we hypothesized that U-maspin was fetal/placental-derived. Thus, a MassEXTEND assay was designed to interrogate the −156 SNP among U-maspin promoter sequences. The A-allele of the −156 SNP would be extended by one base, and the C-allele would be extended by two bases (Fig. 1) so that the two extension products would be resolved as peaks of different masses on MS (Fig. 5). Maternal plasma samples from the eight pregnancies were bisulfite-converted, and the U-maspin −156 SNP genotype was assessed. The maternal plasma U-maspin genotypes were completely concordant with those of the placental tissues (Table 1). To demonstrate the specificity of the MassEXTEND assay toward U-maspin, bisulfite-
converted maternal blood cells, which predominantly comprised M-maspin, were also analyzed. No false-positive amplification from any of the maternal blood cell samples was noted (data not shown). MS tracings from one representative case are shown in Fig. 5, and the spectra for the remaining cases are shown in Fig. 10, which is published as supporting information on the PNAS web site.

**Elevated Maternal Plasma U-maspin Concentration in Preeclampsia.**

U-maspin DNA concentration was measured in the plasma obtained from eight preeclamptic pregnant women bearing fetuses of both sexes (median gestational age, 36.1 weeks) and from 16 gestational age-matched pregnant women without preeclampsia as controls (median gestational age, 36 weeks). The median U-maspin concentration in maternal plasma was 5.7-fold elevated in the preeclamptics group (median, 737.7 copies per ml; IQR, 306.9–1397.0) relative to the control group (median, 130.3 copies per ml; IQR, 110.7–286.2) (Fig. 6). A statistically significant difference in the maternal plasma U-maspin concentrations between the preeclamptic and control groups was observed ($P = 0.01158$; LOGXACT logistic regression by matched case-control analysis).

**Discussion**

In this study, we have demonstrated the feasibility of generating a new marker for prenatal assessment by studying the epigenetic differences between the placenta and maternal blood cells. We have determined that the maspin gene promoter is hypomethylated in the placenta compared with paired maternal blood cells. In general, a higher proportion of unmethylated sequences were observed in the third-trimester placental tissues compared with the first-trimester placental tissues. Although this finding could reflect a change in the methylation density of the maspin promoter as gestation progresses, other explanations should also be considered. For example, placental tissues were obtained in the form of chorionic villi from the first-trimester subjects, whereas a core biopsy from the postdelivery placenta was obtained from the third-trimester subjects. Because each of these tissues contained multiple cell types with potential variations in maspin promoter methylation, the differences in methylation density between the first and third trimester samples might partly be explained by the anatomy of the tissues obtained from different trimesters.

We hypothesized that the placenta is a source of fetal DNA release into maternal plasma (12), whereas maternal blood cells are the main contributor to the background maternal plasma DNA sequences (18). Because the maspin promoter is hypomethylated in the placenta compared with maternal blood cells, we further hypothesized that U-maspin is detectable in maternal plasma and is pregnancy-specific. Indeed, U-maspin was detected in maternal plasma from all three trimesters of pregnancy, albeit at low concentrations in some cases. Larger-scale studies could be planned to further investigate the gestation-specific reference range and degree of interindividual variation of U-maspin concentration in maternal plasma. Furthermore, U-maspin concentration was positively correlated with the conventionally used fetal DNA genetic marker, SRY. The rapid postdelivery clearance of U-maspin further supports the pregnancy-specificity of maternal plasma U-maspin.

To demonstrate that maternal plasma U-maspin is fetal/placental-derived, SNP genotyping analysis was performed. The maternal plasma U-maspin genotype was fully concordant with the placental tissue genotype for all studied pregnancies. In particular, because of the characteristic fetal and maternal genotypes for cases 300 and 310 (Table 1), the positive detection of the fetal-specific C-allele for the −156 SNP in maternal plasma samples was shown. For all mass spectra, the x axis depicts the molecular weight of the detected extension products (shown as sharp peaks), whereas the y axis depicts the intensity in arbitrary units. The expected positions of the A- and C-alleles are as marked. UEP, unextended primer.
collected from preeclamptic subjects compared with nonpreeclamptic subjects matched for gestational age. Typically, fetal Y-chromosomal DNA concentrations are measured in preeclamptic pregnancies bearing male fetuses (7, 8). Data from the present study suggest that aberrant concentrations of U-maspin are likely to be observable in the various pregnancy-associated conditions for which abnormal concentrations of circulating fetal DNA are found. Further studies are required to demonstrate whether U-maspin DNA concentration is elevated similarly to maternal plasma Y-chromosome sequences before the symptomatic onset of preeclampsia (7, 11). For applications requiring the qualitative detection of fetal DNA, such as for rhesus D blood group determination (3) and mutation detection (4), the availability of a universal fetal DNA marker would greatly enhance the confidence with which one could interpret the data regarding the detectability of the paternal trait/mutation, particularly with regard to negative results. Thus, the developments described in this paper have provided a tool for translating the detection of circulating fetal DNA into a practical system for clinical adoption.

Fig. 6. U-maspin concentrations in maternal plasma of women with preeclamptic (PET) and healthy (Normal) pregnancies. The line within each box denotes the median. Limits of the box denote the 25th and 75th percentiles. Whiskers denote the 5th and 95th percentiles. Filled circles depict the outliers.

fact, the origin of fetal DNA in maternal plasma has been a subject of debate (12). One source of circumstantial evidence comes from studying pregnancies with characteristic chromosomal aberrations confined to the placenta (16, 17). It is unclear whether such prior studies might justifiably be extrapolated to normal pregnancies without chromosomal aberrations. A second source of indirect evidence comes from work demonstrating the presence of placental-specific mRNA in maternal plasma (27). In the current study, by focusing on tissue-specific methylation changes (29), fetal DNA detection can be achieved by using a single epigenetic marker. This strategy represents a directional change in the large body of work in the field of circulating fetal DNA, which has thus far focused on exploiting genetic differences between the mother and fetus. We envision that epigenetic markers will complement genetic markers in the future development of noninvasive prenatal diagnosis.

We thank Eric Wong and Allen Chan for assistance and helpful comments regarding the data analysis. We thank Sequenom for loaning the MassARRAY Compact system, which facilitated the part of the study involving genotyping the maspin SNP. This work was supported by Hong Kong Research Grants Council Earmarked Research Grant CUHK 4279/04M.