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Differential DNA Methylation between Fetus and Mother as a Strategy for Detecting Fetal DNA in Maternal Plasma

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Background: **Fetal DNA has been detected in maternal plasma by the use of genetic differences between mother and fetus. We explore the possibility of using epigenetic markers for the specific detection of fetal DNA in maternal plasma.**

Methods: **A differentially methylated region in the human *IGF2-H19* locus and a single-nucleotide polymorphism in this region were chosen for the study. The methylation status in this region is maintained in such a way that the paternal allele is methylated and the maternal allele is unmethylated. The single-nucleotide polymorphism was typed by direct sequencing of PCR products. The methylation status of this region was ascertained by bisulfite conversion and methylation-specific PCR. Differentially methylated fetal alleles were detected in maternal plasma by direct sequencing and a primer-extension assay.**

Results: **Women in the second (n = 21; 17–21 weeks) and third (n = 18; 37–42 weeks) trimesters of pregnancy were recruited. Among these 39 volunteers, the 16 who were heterozygous for the single-nucleotide polymorphism were chosen for further analysis. In 11 of these 16 cases, paternally inherited methylated fetal alleles were different from the methylated alleles of the respective mothers. Using direct sequencing, we detected paternally inherited methylated fetal DNA in 6 of 11 (55%) cases. In 8 of the 16 heterozygous cases, the fetuses possessed an unmethylated maternally inherited allele that was different from the unmethylated allele of the mother. Using a primer-extension assay, we detected fetal-derived maternally inherited alleles in maternal plasma of four of eight (50%) cases.**

Conclusions: **These results represent the first use of fetal epigenetic markers in noninvasive prenatal analysis. These data may also have implications for the investigation of other types of chimerism.**

Recently, much interest has been generated on the biology and diagnostic applications of circulating nucleic acids *(1)*. The demonstration of tumor-derived DNA in the plasma of cancer patients *(2)* has led to the finding of a similar phenomenon in pregnant women, namely that fetal DNA circulates in maternal plasma *(3)*. This latter discovery has opened up new possibilities for noninvasive prenatal diagnosis *(3, 4)*. The clinical application of this phenomenon has been helped by the relatively high absolute and relative concentrations of such circulating fetal DNA in maternal plasma and serum *(5)*. With this approach, noninvasive prenatal detection of several conditions has been achieved, including fetal rhesus D status *(6)*, myotonic dystrophy *(7)*, achondroplasia *(8)*, and certain chromosomal translocations *(9, 10)*.

However, it is important to note that in all of the above situations, investigators have been restricted to detecting genes or mutations that the fetus has inherited from the father, which are genetically distinguishable from the DNA sequences of the mother. This limitation exists because fetal DNA in maternal plasma and serum is present in an excess background of maternal DNA *(4)*. This concept has been explored previously in detail for the detection of fetal nucleated cells in the cellular fraction of maternal blood *(11)*. This principle has led to the following two limitations: (*a*) for a biallelic polymorphism, fetal DNA cannot be conclusively demonstrated in mothers who are heterozygous for the polymorphism;

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and (*b*) the detection of an allele that the fetus has inherited from the mother is not thought to be possible.

Recently, much interest has been focused on the biology of epigenetic phenomena, namely processes that alter the phenotype but are not associated with changes in the DNA sequence *(12, 13)*. One of the best characterized epigenetic processes is DNA methylation *(12, 13)*. We reasoned that, using DNA methylation differences between the mother and fetus, we could overcome the above-mentioned limitations in the detection of fetal DNA in maternal plasma. For this investigation, we chose the human *IGF2-H19* locus *(14)* as a model system. The methylation status of this region is maintained in such a way that the paternal allele is methylated and the maternal allele is unmethylated *(15)*. Because the methylation status differs depending on the parental origin, this region has been called a differentially methylated region (DMR).[3] We attempted to show that we could overcome both of the limitations stated above by differential methylation between the mother and fetus. Our strategy is illustrated in Fig. 1.

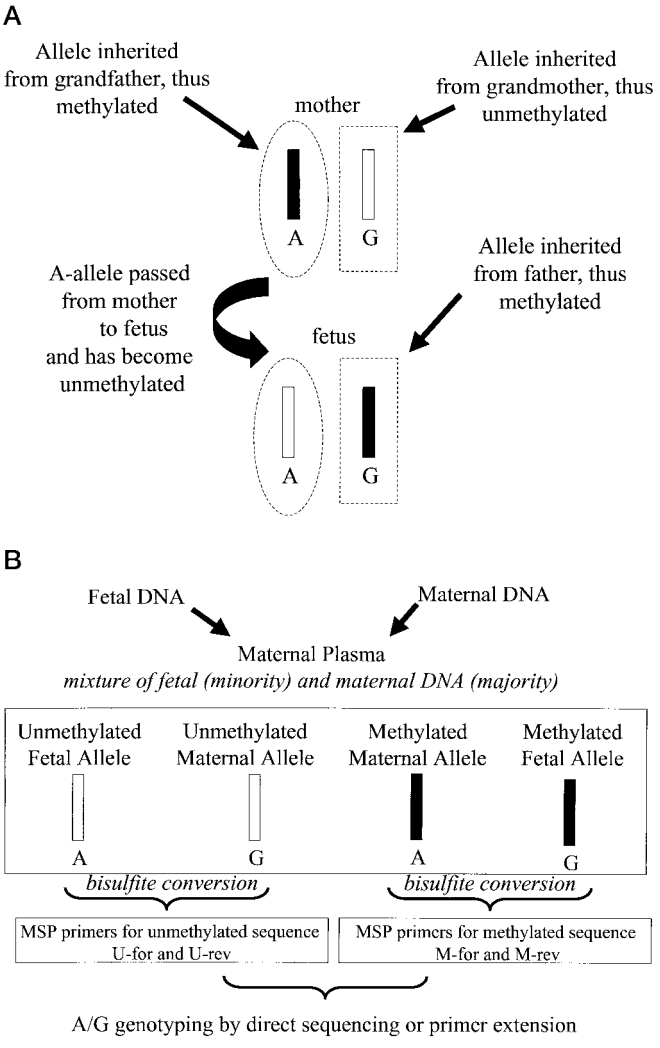

Fig. 1. Strategy for the epigenetic detection of fetal DNA.

(*A*), schematic diagram showing the difference in methylation status of the studied region in the *IGF2-H19* locus between the sexes of the parents of origin. This region is methylated when inherited from the father and unmethylated when inherited from the mother. Methylated sequences are denoted by *filled bars*, whereas unmethylated sequences are denoted by *open bars*. *A/G* denotes the respective allele of the SNP. In this study, only mothers heterozygous for the polymorphism, i.e., A/G heterozygotes, were chosen for the detection of fetal DNA in maternal plasma. The illustrated scenario represents only one of the possibilities. (*B*), schematic diagram showing the detection of differentially methylated fetal alleles from maternal plasma. The MSP primers for the methylated sequence were used for detecting paternally inherited fetal alleles from maternal plasma. The MSP primers for the unmethylated sequence were used for detecting maternally inherited fetal alleles from maternal plasma.

Materials and Methods

STUDY PARTICIPANTS AND SAMPLES

We obtained ethics approval from the Clinical Research Ethical Committee of the Chinese University of Hong Kong. Samples were collected from pregnant women with informed consent. Women in the second (n = 21; 17–21 weeks) and third (n = 18; 37–42 weeks) trimesters of pregnancy were recruited for this study. These volunteers were randomly selected from a large cohort of women recruited for an ongoing project on noninvasive prenatal diagnosis from 1997 to 2001. None of the recruits for the present study had preeclampsia or preterm labor in the current pregnancy. EDTA-maternal blood and fetal amniotic fluid samples were collected from the second trimester cases as described previously *(5)*. For the third trimester cases, we collected EDTA-maternal blood samples at 2–3 h before normal vaginal delivery. EDTA-fetal cord blood samples were also collected immediately after delivery as described *(5)*. Plasma and buffy coat from all recruited blood samples were harvested and stored at −20 °C as described *(5)*, except plasma samples were recentrifuged at 16 000*g*. Amniotic fluid samples were stored at 4 °C.

DNA ISOLATION

We extracted DNA from plasma and amniotic fluid samples using a QIAamp Blood Kit (Qiagen). Typically, 800 μL of plasma or amniotic fluid was used for DNA extraction per column. An elution volume of 50–110 μL was used. We extracted DNA from the buffy coat using a Nucleon DNA Extraction Kit (Scotlabs) according to the manufacturer's recommendations.

GENOTYPING OF THE DMR POLYMORPHIC REGION

The DMR in the human *IGF2-H19* locus contains two 450-bp repeat and seven 400-bp repeat units *(14)* (Fig. 2). An A/G single-nucleotide polymorphism (SNP) within the DMR *(14)* was selected as a marker in our investigation (Fig. 2). PCR was used to amplify the SNP in both maternal and fetal DNA samples. We designed primers using the sequence of the *Homo sapiens H19* gene (Gen-

[3] Nonstandard abbreviations: DMR, differentially methylated region; MSP, methylation-specific PCR; SNP, single-nucleotide polymorphism; and nt, nucleotide(s).

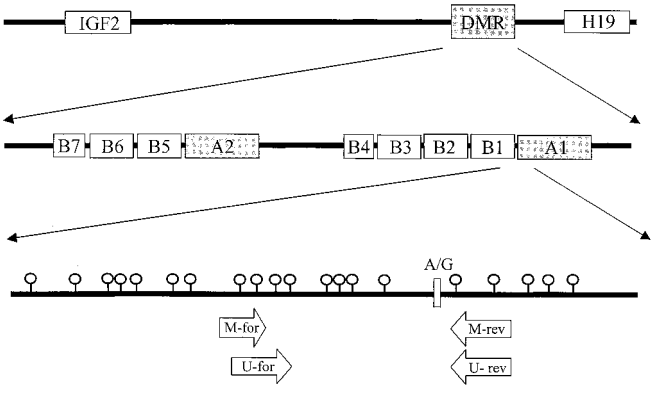

Fig. 2. Schematic representation of the DMR of the human *IGF2-H19* region.

The two 450-bp repeat (*A1* and *A2*) and seven 400-bp repeat (*B1–B7*) units are shown. The potential methylation sites on the upper-strand DNA of the studied region are represented by *open circles*. The studied SNP site (*A/G*) is indicated by an *open box*. *Open arrows* represent the location of the forward (*for*) and reverse (*rev*) primers in PCR reactions specific for the methylated (*M*) and unmethylated (*U*) alleles, respectively. Sequences of these MSP primers are shown. Sequence differences between bisulfite-treated DNA and untreated DNA are highlighted in *bold italics*, and sequence differences between methylated (paternally inherited) and unmethylated (maternally inherited) DNA are *bold underlined*.

Bank Accession No. AF125183). Typically, 2–5 μL of eluted DNA, purified from maternal buffy coat, cord buffy coat, or amniotic fluid, was added to a 25-μL PCR reaction containing 2.5 μL of 10× TaqMan buffer A (PE Applied Biosystems), 3 mM $MgCl_2$, 6.26 pmol of dNTPs, 5 pmol of primers (forward: 5′-ggACGGAATTGGTTGTAGTT-3′; reverse: 5′-AGGCAATTGTCAGTTCAGTAA-3′), and 0.625 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems). Thermocycling was carried out at 95 °C for 8 min followed by 35 cycles of 95 °C for 1 min, 56 °C for 20 s, and 72 °C for 20 s. For the forward primer, the nucleotides in upper case corresponded to positions 7927–7944 of the *H19* sequence (GenBank Accession No. AF125183). For the reverse primer, the nucleotides were complementary to positions 8309–8329 of the *H19* sequence. PCR products were then analyzed by agarose gel electrophoresis and DNA sequencing.

BISULFITE CONVERSION

We performed bisulfite modification of DNA samples using a CpGenome DNA Modification Kit (Intergen) as instructed by the manufacturer. With bisulfite conversion, unmethylated cytosine residues would be converted to uracil, whereas methylated cytosine residues would remain unchanged *(16)*. The sequence difference between methylated and unmethylated DNA after bisulfite conversion could then be distinguished with different PCR primers. In general, 1 μg of buffy coat DNA from maternal or cord blood or 93 μL of eluted DNA purified from maternal plasma or amniotic fluid was used in a bisulfite-conversion reaction. Bisulfite-treated DNA was then eluted in 25–50 μL of 1× Tris-EDTA.

METHYLATION-SPECIFIC PCR

Methylation-specific PCR (MSP) assays were modified from the protocol as described *(16)*. Bisulfite-treated DNA (5 μL) was added to a 50-μL PCR reaction containing 5 μL of 10× TaqMan buffer A (PE Applied Biosystems), 2.5 mM $MgCl_2$, 10 pmol of dNTPs, 20 pmol of each of the corresponding MSP primers (Fig. 2), and 1.25 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems). The primers "M-for" and "M-rev" (Fig. 2) were designed for the methylated sequence, whereas the primers "U-for" and "U-rev" (Fig. 2) were designed for the unmethylated sequence. Reaction mixtures were thermal cycled (methylated allele: 95 °C for 45 s, 55 °C for 20 s, and 72 °C for 20 s; unmethylated allele: 95 °C for 45 s, 49 °C for 20 s, and 72 °C for 20 s) for 50 (buffy coat and amniotic fluid DNA) or 56 (plasma DNA) cycles with an initial denaturing step of 8 min at 95 °C. PCR products were then analyzed by agarose gel electrophoresis. Reaction products were purified with Microspin S-300 HR columns (Amersham Pharmacia) for DNA sequencing or the primer-extension assay.

DNA SEQUENCING

We sequenced purified PCR products with an ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and the corresponding forward primers of the PCR products. We analyzed sequencing products with an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems).

PRIMER EXTENSION ASSAY

The purified MSP product (2 μL) was added to a 25-μL reaction containing 50 μM dideoxy-ATP (2′,3′-dideoxyadenine triphosphate), 50 μM dGTP, 50 μM dTTP, 0.2 pmol of Cys-5-labeled primer (5′-GGGTTATTTGGGAATAGGATATTTA-3′), 4 U of Thermo Sequenase (Amersham Pharmacia), and 1.43 μL of concentrated buffer. Reactions were thermal cycled for 40 cycles (95 °C for 30 s, 51 °C for 20 s, and 72 °C for 20 s). The Cys-5-labeled primer was 25 nucleotides (nt) in length, and the polymorphic site was 2 nt away from the 3′ end of the primer. For the A allele, the incorporation of the dideoxy-ATP at this polymorphic site would produce chain termination, thus leading to an extension product of 27 nt (i.e., 25 + 2 nt). For the G allele, chain extension would continue until the next A residue, which was 5 nt away from the 3′ end of the primer, thus leading to an extension product of 30 nt (i.e., 25 + 5 nt). Reaction products were electrophoresed with a 14% denaturing polyacrylamide gel and analyzed with an ALF Express Sequencer (Amersham Pharmacia). Data were analyzed by the AlleleLinks software program (Amersham Pharmacia).

Results

GENOTYPING OF DMR

The maternal genotype at the SNP within the DMR (Fig. 2) was determined by direct sequencing of PCR products from the buffy coat DNA. Of the 39 women with each of the possible genotypes, 17 had GG (43.6%), 16 had AG (41.0%), and 6 had AA (15.4%) genotypes.

DETECTION OF FETAL DNA IN PLASMA FROM WOMEN HETEROZYGOUS FOR A BIALLELIC POLYMORPHISM

The 16 women who were heterozygous (i.e., AG) for the SNP were selected for further examination. On the basis of previous criteria *(4, 11)*, samples from these women would not be considered informative at this polymorphic locus for the detection of fetal DNA in maternal plasma because this is a biallelic polymorphism. To demonstrate that differential methylation at this genomic region would allow us to overcome this limitation, we bisulfite-treated maternal DNA and amplified it by MSP using the primers shown in Fig. 2. Similarly, fetal DNA isolated from amniotic fluid (second trimester samples) or buffy coat of cord blood (third trimester samples) was subjected to PCR and MSP to determine the imprinting status of the fetal alleles.

Among the 16 selected cases, the methylated (i.e., paternally inherited) alleles from four third trimester and seven second trimester fetal samples were different from the methylated alleles of the respective mothers (Fig. 3, a and b; compare panels 1 and 2). To test whether this differential methylation between fetus and mother would allow the fetal allele to be detected from maternal plasma, maternal plasma DNA from these cases was subjected to bisulfite conversion followed by MSP. Interestingly, the paternally inherited methylated fetal allele could be detected in two third trimester and four second trimester maternal plasma samples (Fig. 3, a and b; panel 3). To exclude the possibility that these observations were simply attributable to the existence of aberrantly methylated maternal DNA in maternal plasma, we collected a postnatal maternal plasma sample (~3.5 years after delivery) from one of the positive cases for further examination. We did not observe the additional methylated allele in this postnatal sample (Fig. 3a, panel 4), indicating that the additional methylated allele in the maternal sample during pregnancy was of fetal origin. In addition, no positive signal was observed in the plasma of noninformative cases (n = 4; data not shown), thus further demonstrating the specificity of this MSP assay. Taken together, these data indicate that the use of differential methylation between mother and fetus would allow us to detect fetal DNA in maternal plasma, even in cases that are not considered informative with existing criteria (Fig. 1).

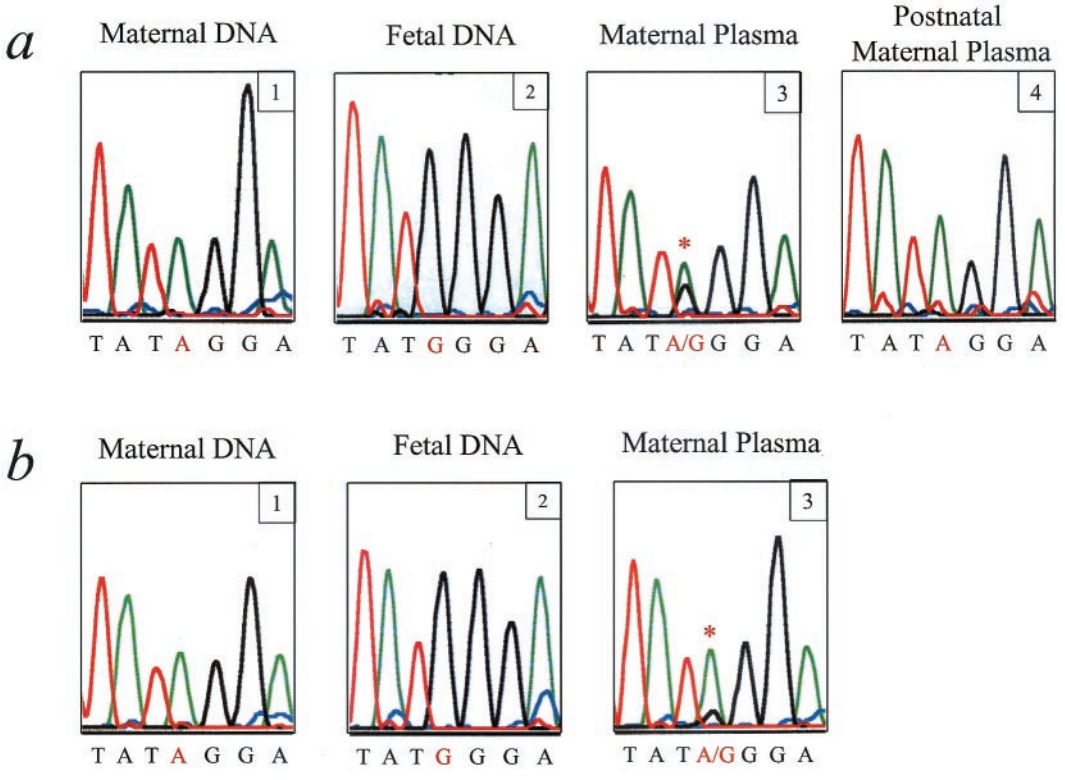

Fig. 3. Detection of methylated (paternally inherited) fetal DNA obtained from maternal plasma in the third (*a*) and second (*b*) trimesters.

DNA sequence of methylated alleles in maternal buffy coat (*a* and *b*, *panel 1*), fetal buffy coat or amniotic fluid (*a* and *b*, *panel 2*), prenatal maternal plasma (*a* and *b*, *panel 3*), and postnatal maternal plasma (*a*, *panel 4*) samples are shown. The presence of methylated fetal DNA in the prenatal maternal plasma sample is indicated by *. The polymorphic site is shown in *red letters*.

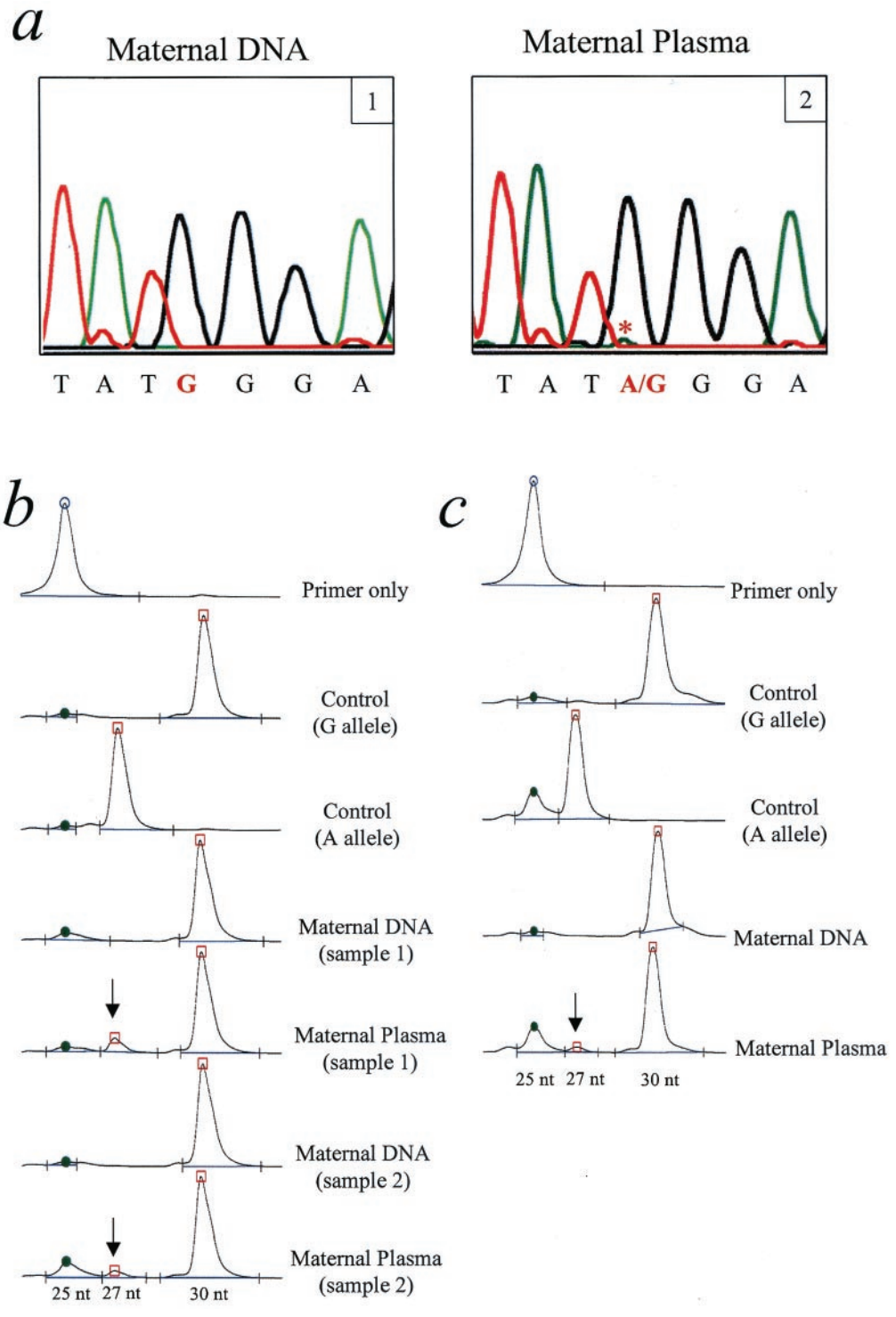

Fig. 4. Detection of unmethylated (maternally inherited) fetal DNA in maternal plasma.

(*a*), unmethylated DNA sequences were detected in maternal buffy coat (*panel 1*) and a third trimester maternal sample (*panel 2*) with direct sequencing. The presence of unmethylated fetal DNA in maternal plasma is indicated by ∗. (*b*), unmethylated fetal DNA (↓) was detected in two third trimester maternal plasma samples by the primer extension assay. (*c*), unmethylated fetal DNA (↓) was detected in a second trimester maternal plasma sample by the primer extension assay. Products from control reactions containing primer only, the unmethylated G allele, or the unmethylated A allele are shown. The sizes (*nt*) of the reaction products are shown at the *bottom*. ●, unused primer; □, detected allele.

DETECTION OF FETAL-DERIVED MATERNALLY INHERITED DNA FROM MATERNAL PLASMA

We then tested whether the use of differential methylation between mother and fetus might allow us to detect an allele that the fetus has inherited from the mother (Fig. 1). This type of analysis has previously been thought to be impossible *(4, 11)*. Because the maternally inherited allele was unmethylated, the primers "U-for" and "U-rev" (Fig. 2) were used to amplify the unmethylated allele after bisulfite conversion. Among the 16 analyzed cases, three third trimester and five second trimester maternal samples were informative. In these cases, the fetus possessed an unmethylated allele that was different from the unmethylated allele of the mother. These results implied that in these cases, the mothers had originally inherited the fetal allele from their fathers and then passed the allele on to the fetus. Of these eight informative cases, only a weak positive signal was observed in one of the third trimester samples on direct sequencing (Fig. 4a, panels 1 and 2).

We reasoned that the weak signal in this single positive case and the low detection rate of the unmethylated fetal allele from maternal plasma might be attributable to the low sensitivity of the method. To enhance the sensitivity of detection, we used a more sensitive primer-extension assay to detect the unmethylated fetal allele from the MSP reaction products. Because the SNP was an A/G polymorphism, dideoxy-ATP was used as a reaction substrate in the primer-extension assay. Extended reaction products from the A and G alleles were 27 and 30 nt long, respectively. No fetal-specific reaction product was present in the corresponding maternal buffy coat samples (Fig. 3, a and b; panel 1). Strikingly, fetal-specific extension products were observed in two third trimester (Fig. 4b, arrow) and one second trimester (Fig. 4c, arrow) maternal plasma samples, indicating the presence of unmethylated fetal DNA in maternal plasma. As controls, none of the tested noninformative cases was positive in this assay (n = 5; data not shown). These results demonstrated, for the first time, the feasibility of using epigenetic markers to detect a fetal-derived maternally inherited DNA sequence from maternal plasma.

Discussion

In this study, we used epigenetic markers to overcome the conventional limitations of detecting fetal DNA in maternal plasma. Thus, although a paternally inherited fetal allele is genetically indistinguishable from a maternal allele, we have shown that we can distinguish such an allele from the mother's plasma, using epigenetic differences between the mother and fetus. Furthermore, we have shown that it is possible to detect a maternally inherited fetal allele from maternal plasma. This novel epigenetic approach will therefore expand the repertoire of disorders in which fetal DNA in maternal plasma can be used.

In this proof-of-concept study, we were able to detect differentially methylated fetal DNA sequences from maternal plasma, even with the use of relatively insensitive methods such as direct sequencing and primer extension. We observed a lower sensitivity in the detection of the unmethylated fetal DNA in maternal plasma (Fig. 4), as compared with the analogous assay for the methylated allele (Fig. 3). This result might be attributable to the different sensitivities of the primers involved. This has necessitated the use of the more sensitive primer-extension assay, in addition to direct sequencing, for demonstrating the fetal epigenetic signature from maternal plasma. We expect that the use of more sensitive detection systems, such as allele-specific PCR *(17)* and real-time MSP *(18, 19)*, might enhance the sensitivity of plasma-based epigenetic analysis. The development of real-time MSP is particularly interesting because it opens up the possibility of quantifying fetal-specific methylation in maternal plasma, as has already been achieved for the detection of tumor DNA in circulation *(20)*.

The possible introduction of fetal DNA in maternal plasma as a routine prenatal diagnostic tool has raised questions with regard to the need of a generic marker for circulating fetal DNA *(5, 21)*. To date, most proposals for such a marker have focused on the use of genetic polymorphisms between the mother and fetus *(22, 23)*. Our demonstration of the feasibility of epigenetic markers for fetal DNA detection in maternal plasma opens up a new approach for the development of a gender- and polymorphism-independent fetal marker in maternal plasma. One way in which this can be achieved is to explore the phenomenon of tissue-specific methylation *(24)*. Biologically, the use of tissue-specific methylation markers may also allow one to directly assess what fetal cell types are responsible for releasing fetal DNA into maternal plasma.

The epigenetic analysis of maternal plasma has obvious applications to disorders associated with genomic imprinting, such as Prader-Willi syndrome *(25)*. This strategy may also have diagnostic potential for disorders, such as preeclampsia, in which imprinted genes have been hypothesized to play a role *(26)*. This approach may also have application to certain chromosomal aneuploidies that may be associated with methylation abnormalities *(27, 28)*.

Although we have focused on the use of maternal plasma as the target of methylation analysis, we envision that fetal epigenetic markers might also find applications in the analysis of fetal cells isolated from the cellular fraction of maternal blood. This possibility is particularly exciting because recent data have shown that methylation analysis could be performed in an in situ manner *(29)*.

With the recent realization that feto-maternal trafficking is a bidirectional process *(30, 31)*, we expect that epigenetic markers may also have a role in the investigation of cellular and DNA transfer from the mother to the fetus. Such an approach might also have applications in the investigation of other types of chimerism, such as posttransplantation hemopoietic chimerism *(32)* and urinary DNA chimerism *(33)*.

With our increased understanding of the human genome and the development of high-throughput, array-based technologies for methylation analysis *(34)*, we expect that the number of usable fetal epigenetic markers would rapidly increase over the next few years. Such a development would provide us with a clinically relevant panel of fetal epigenetic markers that can be used in a synergistic manner with conventional genetic markers in maternal plasma.

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