44 Single-Nucleotide Polymorphisms Expressed by Placental RNA: Assessment for Use in Noninvasive Prenatal Diagnosis of Trisomy 21

To the Editor:

For noninvasive prenatal diagnosis, markers that directly reflect changes in chromosome dosage are preferred over indirect markers that are associated with epiphenomena (1, 2). The RNA:single-nucleotide polymorphism (SNP) allelic ratio strategy was described recently as a means to directly assess fetal chromosome dosage in maternal plasma (2). Quantitative comparison of the allelic expression ratios of a placently expressed, chromosome 21-encoded gene, placentaspecific 4 (PLAC4), enabled detection in maternal plasma of the differences between 2 (normal) or 3 copies of chromosome 21 (2). The RNA:SNP ratio strategy is currently limited to a subset of the population with heterozygosity of the SNP used. Theoretically, an increase in population coverage can be obtained by inclusion of additional SNPs within PLAC4 or other chromosome 21-encoded transcripts with placental expression and detectability in maternal plasma (2).

We therefore tested 44 SNPs expressed by 7 chromosome 21-encoded, placentally expressed genes (2), PLAC4, collagen, type VI, alpha 2 (COL6A2), collagen, type VI, alpha 1 (COL6A1), BTG family, member 3 (BTG3), ADAM metalloproteinase with thrombospondin type 1 motif, 1 (ADAMTS1), chromosome 21 open reading frame 105 (C21orf105), and amyloid beta (A4) precursor protein (peptidase nixin-II, Alzheimer disease) (APP), for their potential use in noninvasive prenatal diagnosis. All SNP markers were tested for their presence in 1st-trimester plasma and their absence in nonpregnant women.

Peripheral blood samples were collected from pregnant women attending the Prenatal Diagnostic Centre of the VU University Medical Center. All participants gave informed consent before study inclusion. The study was approved by the ethics committee at our institution. We collected EDTA blood samples between weeks 9 and 14 of pregnancy, before invasive diagnostic procedures were performed. Samples were processed and RNA extracted as described previously, with automated isolation (BioRobot MDX) (1). RNA extraction from PAX gene tubes was performed using the BioRobot MDX with a standardized protocol (Qiagen). For selected genes, allele frequencies were determined by cycle sequencing with a Big Dye terminator, followed by capillary electrophoresis (ABI 3100XL).

Within the transcripts of the 7 genes of interest, 44 SNPs were identified (www.hapmap.org) (Table 1). Primers flanking these SNPs were designed with similar thermodynamic characteristics to permit RT-PCR analysis in single runs. All primers were intron spanning, except for the primers of PLAC4. Using a sensitive, 2-step, 1-tube RT-PCR assay (Superscript II RT-PCR, Invitrogen) supplemented with 1 mol/L betaine to increase reverse transcription efficiency and enzyme stability (1), the marker set was tested in placental tissue (positive control), plasma from nonpregnant women (negative control), and pregnant women (positive control). During the initial screen, we used 3 chorionic villus samples (weeks 8, 11, and 12) to test markers for placentation expression. Screening of pregnant and control plasma was done in triplicate. To minimize the effect of biological variation of marker levels in plasma, each of the 3 screens in plasma was performed on pooled RNA fractions isolated from individual females in series of 44. In practice, this process was performed by downstream pooling of the concentrated, individual RNA fractions isolated from plasma (10 μL each) after automated extraction of 44 different plasma samples. For the SNPs of most use, the final screen was done by individual analysis of 6 pregnant plasma and 6 control plasma samples. With the use of RNA isolated from EDTA plasma, 5 of 44 SNP markers were detectable in maternal plasma and absent in nonpregnant plasma: rs8130833 (PLAC4), rs99777003 (PLAC4), rs11554667 (COL6A2), rs9637170 (COL6A1), and rs2187247 (C21orf105) (Table 1). In contrast, in RNA isolated from whole blood collected in PAXgene tubes, no SNP markers fulfilled the criterion of absence in nonpregnant blood. Identical analysis of hPL RNA (3) excluded false positivity, because in RNA recovered from whole blood in PAXgene tubes, this marker was clearly present and absent, respectively, in samples obtained from pregnant and nonpregnant females (data not shown).

We conclude the following: (a) Although the PAX gene tube reagent that stabilizes RNA may be beneficial for RNA isolation from whole blood, the large contribution of intracellular RNA from maternal

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**Table 1. SNPs expressed by placental RNA and present in maternal plasma but not control plasma.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene</th>
<th>Exon</th>
<th>SNP*</th>
<th>HET FREQb</th>
<th>A1c</th>
<th>A2c</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PLAC4</td>
<td>1</td>
<td>rs8130833</td>
<td>0.448 A G</td>
<td>GGA</td>
<td>GCTCGCGGCTTGAAGGTGCTT</td>
<td>GGTGGGGATCTTTATGCTAGG</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PLAC4</td>
<td>1</td>
<td>rs9977003</td>
<td>0.339 A G</td>
<td>GGA</td>
<td>GCTCGCCAGCAGGAGATG</td>
<td>GGGCCAGTGGAAAAAACACGCAGT</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>COL6A2</td>
<td>28</td>
<td>rs11554667</td>
<td>&lt;0.1% C G</td>
<td>CAC</td>
<td>AGACAGCGTCGACCACATG</td>
<td>AACGCGCCGCGITGGT</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>COL6A1</td>
<td>23</td>
<td>rs9637170</td>
<td>&lt;0.1% A C</td>
<td>CCT</td>
<td>ATCGCGAACCTAAAGGCTAC</td>
<td>TCAAAATCTCGCATCAGTC</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>C21orf105</td>
<td>2</td>
<td>rs2187247</td>
<td>0.5 A C</td>
<td>GCC</td>
<td>GCCTCGCTTTGCCGTTCAACC</td>
<td>GGGGCCGCTTCCTTCCGGTGGTAG</td>
<td></td>
</tr>
</tbody>
</table>

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*a Selected from among 44 tested SNPs of the 7 gene selected genes. The additional SNPs tested (n = 39), and their primer sequences are available on request.

*b Heterozygote frequencies (HET FREQ) are given for white individuals only.

*c A1 and A2, variant alleles.
peripheral blood cells prevents wide-spread prenatal use. Prenatal PAX gene tube use appears to be limited to genes with high relative expression differences between placental tissue and maternal blood cells, such as hPL. (b) Our data confirm the utility and high expression of PLAC4 (2). The use of SNP markers is restricted to specific exons for genes with complex transcriptional organization, such as COL6A1 and COL6A2. (d) For the transcripts of COL6A2 and COLA1 with placental specificity (for example encompassing exon 23 in COL6A1), SNPs remain to be identified for use in RNA-SNP assays. The heterozygote frequencies of rs11554667 (COL6A2) and rs9637170 (COLA1) are <0.1% in the white population we tested. (e) Alternatively, for COL6A2 and COL6A1, the combined detection of exons with specificity (exons 28 and 23 for COL6A2 and -6A1, respectively) with additional exons carrying SNPs with high heterozygosities (rs2839114, rs1053312) might yield useful combinations. (f) The predictive power of C21orf105 (1, 4) for prenatal diagnosis should be retested with the RNA:SNP allelic ratio strategy by use of rs2187247. (g) Our data permit an evidence-based selection of target genes and markers to increase the population coverage of the allelic ratio strategy for noninvasive prenatal diagnosis of trisomy 21.

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References

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