Identification of Pregnancy-Associated MicroRNAs in Maternal Plasma

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BACKGROUND: Several placental microRNAs (miRNAs) have been identified as pregnancy-associated molecules with the potential for use in estimating the condition of the placenta. Our understanding of these novel molecules is still limited, however. The aim of this study was to isolate and characterize pregnancy-associated miRNAs in maternal plasma.

METHODS: By microarray-based screening of 723 human miRNAs, we selected miRNAs that exhibited signal intensities >100 times higher in placental tissues than in the corresponding whole blood samples. Subsequent quantitative real-time reverse-transcription PCR revealed miRNAs produced predominantly in the placenta that showed significantly decreased concentrations in maternal plasma after delivery. These miRNAs were identified as pregnancy-associated miRNAs.

RESULTS: We selected 82 miRNAs produced predominantly in the placenta and identified 24 as pregnancy-associated miRNAs. The genes encoding these miRNAs included 16 that are clustered on 19q13.42 and 5 clustered on 14q32. As the pregnancy progressed into the third trimester, the plasma concentrations of cell-free chromosome 19–derived miRNAs (has-miR-515-3p, has-miR-517a, has-miR-517c, has-miR-518b, and has-miR-526b) increased significantly \((P = 0.0284, 0.0069, 0.0125, 0.0284, \text{ and } 0.0093, \text{ respectively, Wilcoxon signed rank test})\), whereas that of cell-free has-miR-323-3p on chromosome 14q32.31 showed no change \((P = 0.2026)\).

CONCLUSIONS: In addition to the known pregnancy-associated miRNAs, we identified new pregnancy-associated miRNAs with our microarray-based approach. Most of the genes encoding these miRNAs were clustered on 19q13.42 or 14q32, which are critical regions for placental and embryonic development. These new pregnancy-associated miRNAs may be useful molecular markers for monitoring pregnancy-associated diseases.
Slides were washed for 10 min at room temperature in phosphate-buffered saline (PBS) containing 0.1% Tween 20. After a first centrifugation at 3000g for 10 min, we centrifuged the supernatant at 16 000g for 10 min to remove blood cells. Total RNA containing small RNA molecules was extracted from 5.0 mL maternal blood and 3.0 mL maternal plasma with a mirVana miRNA Isolation Kit (Ambion/Applied Biosystems) according to the manufacturer’s instructions.

Quality assessment and concentration measurements of total RNA, including small RNAs, were performed with a Bioanalyzer (Agilent Technologies) and a NanoDrop spectrophotometer (Thermo Fisher Scientific), respectively. We subjected 100 ng total RNA from the placenta or from the corresponding maternal blood sample of each trimester to microarray analysis. For each sample, 1 µg total RNA (which included small RNAs) was labeled with cyanine 3–cytidine bisphosphate (pCp–Cy3). The miRNA Labeling Reagent and Hybridization Kit (Agilent Technologies) were then used according to the manufacturer’s instructions to hybridize the labeled RNA for 20 h to Human miRNA Microarray Version 2 (Agilent Technologies), which included 723 miRNAs encoded by genes located across all chromosomes except chromosome Y. The number of miRNAs located on each chromosome in the Human miRNA Microarray Version 2 varied from 7 to 86, and the numbers of miRNAs located on chromosomes 19 (n = 86), 14 (n = 75), and X (n = 86) were relatively larger than the numbers for the other chromosomes.

Slides were washed for 10 min at room temperature in 6× standard saline citrate (0.9 mol/L NaCl and 0.09 mol/L sodium citrate) containing 50 µL/L Triton X-102 and then for 5 min in 0.1× standard saline citrate (0.015 mol/L NaCl and 0.0015 mol/L sodium citrate) containing 50 µL/L Triton X-102. The slides were scanned on an Agilent microarray scanner (model G2565A) at 100% and 5% sensitivity settings. Agilent Feature Extraction software (version 9.5) was used for image analysis. miRNAs exhibiting signal intensities in placental samples >100 times those of the corresponding whole blood samples were selected as placenta-produced miRNAs. These miRNAs were considered candidate pregnancy-associated miRNAs (see Fig. 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol56/issue11).

For quantitative real-time reverse-transcription PCR analysis, we used a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions and 2.5 µL total RNA in a total reaction volume of 25 µL. We then performed quantitative PCR with the TaqMan Universal PCR Master Mix (Applied Biosystems). For each miRNA assay, we prepared a calibration curve by 10-fold serial dilution of single-stranded cDNA oligonucleotides corresponding to each miRNA sequence over the concentration interval of 1.0×10^3 to 1.0×10^7 copies/mL. Each sample and each calibration dilution were analyzed in triplicate. Each assay could detect the RNA concentration down to 1000 copies/mL of plasma. Every batch of amplifications included 3 water blanks as negative controls for each of the reverse-transcription and PCR steps. All data were collected and analyzed with an ABI Prism 7900 Sequence Detection System (Applied Biosystems). miRNAs were selected as pregnancy-associated miRNAs when circulating placenta-produced miRNAs were detected (>1000 copies/mL) in all 10 maternal plasma samples before pregnancy termination and showed a significantly decreased concentration after the termination of the pregnancy (P < 0.05, Wilcoxon signed rank test; see Fig. 1 in the online Data Supplement).

Chromosomal localization of the encoding gene and tissue production were evaluated for each miRNA by searching the microRNA.org (http://www.microrna.org/) and mirBase (http://www.mirbase.org/index.shtml) databases. Statistical analysis was performed with StatView 5.0 software (SAS Institute). Differences were evaluated with the Wilcoxon signed rank test, and statistical significance was set at P values <0.05.

Of 723 human miRNAs, 82 were produced predominantly in the placenta according to our comparisons of the miRNA profiles of placental tissues for the first and the third trimesters and those of the corresponding samples of maternal blood cells. These placental miRNAs occurred with signal intensities >100-fold higher than those in maternal blood cells. Of these 82 miRNAs (see Table 1 in the online Data Supplement), 12 were detected only in the first trimester, 25 were detected only in the third trimester, and the remaining 45 miRNAs were detected in both trimesters (see Table 1 in the online Data Supplement).

Our analysis of the chromosomal locations of the genes encoding the 82 miRNAs that were predominantly produced in the placenta showed that 44 (53.7%) were clustered on chromosome 19q13 and 13 (15.9%) were clustered on 14q32 (see Table 1 in the online Data Supplement). Thirty-six miRNAs (81.8%) encoded by genes clustered on 19q13 and 2 miRNAs (15.4%) encoded by genes clustered on 14q32 were detected in both trimesters. Of the 82 miRNAs that were produced predominantly in the placenta, 24 showed significantly decreased concentrations in the maternal plasma after de-
livery of the placenta (Table 1); we therefore identified them as pregnancy-associated miRNAs. Of the genes encoding these 24 pregnancy-associated miRNAs, 21 (87.5%) were clustered on 19q13.42 or 14q32, 16 (66.7%) were located on 19q13.42, and 5 (20.8%) were on 14q32. The mean intraassay CV, which is the ratio of the standard deviation to the mean, for the probes in the quantitative real-time reverse-transcription PCRs was 4.7% (range, 0.6%–11%) (Table 1). A search of the microRNA.org database (http://www.microrna.org/) for miRNA production in tissues indicated that 13 of the 24 genes encoding pregnancy-associated miRNAs had a “specific expression pattern from placenta tissue,” which indicated that these miRNA genes were not expressed in tissues other than placenta (Table 1). All of the genes encoding these miRNAs are located on 19q13.42. The genes encoding the remaining 11 pregnancy-associated miRNAs (3 on 19q13.42 and 8 on other chromosomes) indicated a “predominant expression pattern from placenta tissue,” which indicated miRNAs that were produced predominantly in placenta tissue rather than in blood cells (Table 1).

Of the 24 pregnancy-associated miRNAs, 6 miRNAs (has-miR-515-3p, has-miR-517a, has-miR-517c, has-miR-518b, has-miR-526b, and has-miR-323-3p) that showed the most significantly decreased concentrations in maternal plasma after pregnancy termination were selected for an analysis of time-
dependent changes in cell-free miRNA concentrations in maternal plasma during pregnancy ($P = 0.0051$, Wilcoxon signed rank test; Table 1). As the pregnancy progressed into the third trimester, the plasma concentrations of cell-free chromosome 19q13.42–derived miRNAs (has-miR-515-3p, has-miR-517a, has-miR-517c, has-miR-518b, and has-miR-526b) increased significantly ($P = 0.0284, 0.0069, 0.0125, 0.0284$, and $0.0093$, respectively, Wilcoxon signed rank test), whereas the concentration of cell-free has-miR-323-3p encoded by the corresponding miRNA gene on 14q32.31 showed no change ($P = 0.2026$) (Fig. 1).

Most of the initial 82 miRNAs with predominant production in placental tissue showed no post-delivery clearance, suggesting that maternal tissues other than blood cells contributed to their presence in maternal plasma. It is noteworthy that 21 of 24 genes encoding pregnancy-associated miRNAs present in maternal plasma were clustered on 19q13.42 or 14q32, which are critical regions for placental growth and embryonic development (12–15). The concentrations of circulating pregnancy-associated miRNAs in maternal plasma showed a time dependence as pregnancy progressed into the third trimester. Therefore, the pregnancy-associated miRNAs we identified may be useful molecular markers for the early detection or prenatal monitoring of pregnancy-associated diseases, such as pre-eclampsia, placenta accreta, intrauterine growth restriction, and hydatidiform moles. Further understanding of the clinical importance of the pregnancy-associated miRNAs we detected will re-

**Fig. 1. Time-dependent changes in pregnancy-associated miRNA concentrations in maternal plasma during pregnancy.**

First, first trimester; Third, third trimester; After, after delivery. Asterisks indicate significant differences between plasma miRNA concentrations (see text for $P$ values).
quire clarification about how the alterations in the concentrations of these miRNAs in maternal plasma affect specific gene expression in some maternal tissues.

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References


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