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The levels of hypoxia-regulated microRNAs in plasma of pregnant women with fetal growth restriction

Jean-Francois Mouillet^a, Tianjiao Chu^a, Carl A. Hubel^a, D. Michael Nelson^b, W. Anthony Parks^c, and Yoel Sadovsky^{a,d}

Jean-Francois Mouillet: mouilletj@mwri.magee.edu; Tianjiao Chu: tchu@mwri.magee.edu; Carl A. Hubel: chubel@mwri.magee.edu; D. Michael Nelson: nelsondm@wudosis.wustl.edu; W. Anthony Parks: parkswa@upmc.edu; Yoel Sadovsky: ysadovsky@mwri.magee.edu

^aMagee-Womens Research Institute, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh, Pittsburgh, PA

^bDepartment of OBGYN, Washington University in St. Louis

^cDepartment of Pathology, University of Pittsburgh, Pittsburgh, PA

^dDepartment of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA

Abstract

MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression at the post-transcriptional level. While mostly intracellular, a portion of cellular miRNAs is released to the circulation and their level in the plasma is altered in certain pathological conditions such as cancer, and also during pregnancy. We examined the circulating levels of a set of trophoblastic miRNAs, which we recently found to be regulated by hypoxia, in the plasma of pregnant women with fetal growth restriction. As expected, pregnancy was associated with increased plasma levels of several placenta-specific miRNAs, compared to non-pregnant controls. Among pregnant women, the overall levels of miRNA species that we analyzed were increased by 1.84-fold ($p \leq 0.01$) in plasma of women with pregnancies complicated by FGR, but decreased in FGR placentas by 24% ($p \leq 0.01$) compared to values from uncomplicated pregnancies. Together, our results show that plasma concentration of miRNAs is regulated in pregnancy, and that FGR is associated with increased circulating miRNA levels, highlighting the need to explore plasma miRNAs as potential biomarkers for placental diseases.

Keywords

trophoblast; fetal growth restriction; hypoxia; plasma; microRNA

Introduction

Fetal growth restriction (FGR) is biologically characterized as failure of a fetus to reach its growth potential. Affecting 3–10% of births, FGR represents one of the leading causes of

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Corresponding author: Yoel Sadovsky, MD, Magee-Womens Research Institute, 204 Craft Avenue, Pittsburgh, PA 15213, ph 412.641.2675 / fx 412.641.3898, ysadovsky@mwri.magee.edu.

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perinatal morbidity and mortality [1]. Growth-restricted newborns that survive the intrauterine period are at a greater risk of acute neonatal diseases, childhood neuro-developmental dysfunction, and the adult metabolic syndrome. FGR is a complex disease, resulting from an array of diverse etiologies. Its diagnosis remains difficult, and it has no effective therapy except for induced delivery, often required to avert additional harm *in utero* even at the cost of prematurity.

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression at the post-transcriptional level [2–6]. More than 650 miRNA species are known thus far, and each of them can potentially target more than a hundred mRNAs [7]. While a few miRNAs are tissue-specific, they are commonly expressed in different combinations across diverse organs. Consistent with their vast regulatory potential, miRNAs are involved in many cellular processes, including cell differentiation, proliferation, apoptosis, and metabolic homeostasis, as well as pathological processes such as viral infections [8], cardiovascular diseases [9], and cancer [10–14]. Unique organ- or disease-specific miRNA expression patterns constitute miRNA signatures that may be used as diagnostic or prognostic clinical tools [11,15,16].

Recent studies showed that miRNAs are released into the circulation where they are found in a surprisingly stable form [17–21]. The precise origin of circulating plasma miRNAs is not entirely clear. At least some miRNAs are released to the plasma within exosomes, which are circulating microparticles (50–100 nm in size) of endocytic origin that may support intercellular communication [21–23]. Exosomes from different cell types exhibit potent immunostimulatory and antitumorigenic effects [review in 24]. In contrast, exosomes released by tumor cells exhibit immunosuppressive capabilities and can promote tumor progression [25].

The human placenta also produces exosomes that may play a role in the establishment of maternal immune tolerance [26–28]. Discrete types of miRNA species are found in the plasma of pregnant women [18,19,29], with the plasma level of selected miRNA species altered in a gestational age-dependent manner. These observations imply a role for miRNAs as extracellular messengers. In this study we analyzed the plasma concentration of a set of miRNAs species that we recently identified to be regulated in human placental trophoblasts exposed to hypoxia [30]. We tested the hypothesis that the level of these miRNAs is altered in the plasma of women with pregnancies complicated by FGR.

Materials and methods

Participants

Plasma samples were collected from non-pregnant women as well as from pregnant women with a singleton healthy pregnancy, or pregnancy complicated by FGR. We used archived samples from the Preeclampsia Program Project (PEPP) study of pregnant women who delivered at Magee-Womens Hospital in Pittsburgh within the years 1995–2004. The study was approved by the University of Pittsburgh's Institutional Review Board. All participants provided written informed consent for use of their samples and de-identified clinical data under the umbrella of the PEPP and related ancillary studies by the same investigators. All women aged 14–44 years were eligible to participate. Clinical diagnoses were established during monthly meetings of a jury of clinicians. FGR was defined as birth weight <10th percentile after exclusion of pregnancies complicated by systemic maternal diseases that might be associated with placental abnormalities such as preeclampsia, diabetes mellitus, fetal genetic anomalies or non-genetic congenital abnormalities. Clinical characteristics of these patients are listed in Table 1.

Specimen processing and total RNA isolation

Maternal non-fasting blood samples were collected at the usual times for clinical indications into 10 ml EDTA-containing tubes (BD Vacutainer, Franklin Lakes, NJ). Plasma was collected by centrifugation and aliquoted at -80°C . Total RNA was extracted from plasma samples using miRNeasy mini columns (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Briefly, 300 μl of plasma were thawed on ice and mixed with 700 μl of QIAzol reagent. After mixing, 140 μl of chloroform were added, and the sample was centrifuged at 12,000 g for 15 min. The upper phase was collected and transferred to a new collection tube containing 1.5 v/v ethanol. The mixture was applied to an RNeasy Mini spin column and centrifuged at 8000 g for 15 sec. After washings the RNA was eluted from the column using RNase-free water.

Quantitative RT-PCR (RT-qPCR) assays

Plasma circulating miRNAs were determined by RT-qPCR. RNA (100 ng) was reverse transcribed using the miScript Reverse Transcription kit (Qiagen) according to the manufacturer instructions. RT product was diluted by a factor of four, and 2 μl of RT product was combined with 10 μl of 2 $\times$ QuantiTect SYBR Green PCR Master Mix, 2 μl of 10 $\times$ miScript Universal Primer, 2 μl of 10 $\times$ miScript Primer Assay in a total volume of 20 μl . PCR was carried out using a 7900HT Fast RT-PCR system (Applied Biosystems Foster City, CA, USA). Data were analyzed with SDS Relative Quantification Software version 2.3 (Applied Biosystems), with automatic setting for adapting baseline and threshold for Ct determination.

Statistical analysis

All RT-qPCR measurements were performed in duplicates. Because of unequal sample sizes for different experimental groups, as well as wide range of miRNA levels, we elected to analyze these unbalanced datasets using linear mixed effect models, with the random effect representing the sample-to-sample variation. We fitted the models with and without the treatment factor as the fixed effect, and performed analysis of variance to compare the null model (without the treatment factor as the fixed effect) and the alternative model (with the treatment factor as the fixed effect). Using a log likelihood ratio test, we calculated the p values against the null hypotheses for each miRNA, assuming that the "exposure" (e.g., FGR) had no effect on its level. The p values were corrected by Benjamini and Hochberg's method to control for false discovery rate (FDR). All analyses were done using the R statistical computing software (<http://www.R-project.org>, 2009). The linear mixed effect model analysis was done using the lme4: Linear mixed-effects models lme4 packages of R (<http://www.R-project.org/package=lme4>, 2009).

Results

We recently defined a set of miRNAs (miR-93, miR-205, miR-224, miR-335, miR-424, miR-451 and miR-491) that are regulated in hypoxic trophoblasts *in vitro* [30]. We initially sought to assess the basal level of these miRNA species in plasma of a subset of healthy non-pregnant (n=11) and pregnant women (n=10), to which we added four ubiquitous species that are abundant in the placenta (miR-27a-1, miR30d, miR-141, and miR-200c). We found that while the level of the entire group of miRNAs was unchanged between pregnant and non-pregnant individuals, plasma levels of miR-141 and miR-424 was significantly higher during pregnancy. For control, we measured the levels of a subset of miRNA species that represents a miRNA cluster from chromosome 19 (C19MC), known to be almost exclusively expressed by the placenta [31,32]. As expected, the circulating levels of four members of this family (miR-517a, -518b, -518e and -524) was markedly increased in plasma samples of pregnant women (Fig. 1). Together, these data indicate that

when compared to non-pregnant women, the level of plasma miRNAs during pregnancy is miRNA species-specific.

We next compared the plasma levels of a subset of the selected miRNAs from uncomplicated pregnancies and from women with pregnancies complicated with term FGR (n=14 in each group). We found that none of the differences in the level of individual miRNA was significant between the two experimental groups (Fig. 2). In contrast, when considered as a group, the level of all tested miRNA species was elevated by 1.8-fold ($p \leq 0.01$, two sided Wilcoxon test) in plasma samples from women with FGR, compared to healthy pregnant controls. We also assessed the levels of miRNAs in RNA previously extracted from placental biopsies of women with term FGR vs. controls [33]. As shown in Fig. 3, we found no difference in the level of any of the miRNAs between the two experimental groups. Notably, as a group the level of all miRNA species was reduced by 24% ($p \leq 0.01$, two sided Wilcoxon test) in placental samples from women with FGR, compared to controls. To assess a possible correlation between the change in plasma miRNAs and placental miRNAs in pregnancies complicated by FGR vs controls, we plotted miRNA ratios from the two groups. We uncovered a significant negative correlation of -0.7 ($p \leq 0.01$, two sided t test) between plasma and placental miRNAs (Fig. 4), supporting a miRNA-specific relationship between the levels of miRNAs in the plasma and the placenta.

Discussion

We recently defined a set of miRNAs that are regulated in primary human trophoblasts exposed to hypoxia [30]. When we assessed the level of these miRNAs in plasma of healthy pregnant women, we found that the level of most of these miRNAs was unchanged during pregnancy, except for miR-424, which is increased. In agreement with previous work, we also found that plasma miR-141, which was not changed by hypoxia, is elevated in the plasma of pregnant women [18,19]. As control, we found that the level of placenta-specific miRNAs from the C19MC cluster was elevated during pregnancy, as expected. These results suggest that the placenta is an important contributor of circulating miRNAs during pregnancy. Although plasma volume is increased during pregnancy, this physiological change is relatively small (1.5-fold) and therefore unlikely to significantly influence miRNA levels. We also doubt that sample handling might have altered our results, as miRNAs are extremely stable even when stored in room temperature up to 24 h, or when undergone up to eight freeze-thaw cycles [20].

The change in miRNA species that were quantified in our study suggests an increase in total miRNA levels in the plasma of women with pregnancies complicated by FGR. This increase of circulating miRNAs in FGR was accompanied by a small diminution of miRNAs in placentas from FGR. The negative correlation between the positive fold-change of plasma miRNAs in FGR vs. the negative fold-change of placenta miRNAs in FGR is intriguing. While this relationship may represent two independent mechanisms, it may reflect related process where placental injury in FGR attenuates miRNA biogenesis and increases exosome-dependent or independent release of miRNA to the plasma. Lastly, our results do not rule out an extra-placental source of the elevated plasma miRNAs.

Elevated plasma levels of placental DNA and RNA are associated with clinical conditions related to placental dysfunction, such as preeclampsia [34–38]. A fraction of these nucleic acids, released by the placenta, originates within exosome microparticles, which are secreted from the late endosomal compartment via membrane blebbing [24]. Placental exosomes are implicated in the suppression of T-cell signaling and the promotion of immune tolerance to the developing fetus [26–28,39,40]. Exosomes from diverse tissues, including the placenta, were recently found to contain miRNAs [22,23,28,29,41]. The encapsulation of miRNAs

within membrane-limited exosomes is likely to contribute to the remarkable stability of circulating miRNAs in plasma.

Although we found that the expression of individual miRNAs is altered by hypoxia in trophoblasts [30], the plasma level of these individual miRNA species was not significantly altered in women with FGR. This can be attributed to the following factors: (a) the inherent difference between cellular levels of miRNAs in injured trophoblasts *in vitro* and release to the circulation in FGR *in vivo*, (b) a lack of confirmation of trophoblast injury in placentas of women with clinical FGR, which might have led to inclusion of women without placental dysfunction, and therefore increased data variability, (c) the relatively high inter-individual variability in plasma miRNA levels, and (d) our use of a relatively conservative statistical approach, where we adjusted the p-values using Benjamini and Hochberg's FDR method for multiple comparisons instead of more permissive approaches [42]. Notably, the use of equal amounts of plasma miRNA template in our RT-qPCR reactions was based on total extracted plasma RNA, and not on internal controls, which are not standardized at the present time. In order to normalize our results to a constant miRNA we attempted to assess the levels of two small plasma RNAs species, RNU6B and RNU5A, but detected excessive variability, which prohibited us from performing this normalization procedure. As most miRNA species trended toward a higher level in plasma samples from FGR pregnancies, a larger number of participants might have yielded significant differences.

We note that our results thus far do not support the use of the miRNA species targeted by our study as biomarkers for pregnancies complicated by placental dysfunction. Larger, high-throughput analyses of plasma miRNAs are needed prior to application of these and similar discoveries to clinical care.

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Fig. 1. The levels of miRNAs in plasma samples from non-pregnant and pregnant women

Box plots of the log-transformed relative values by RT-qPCR, normalized to the median of the levels in the control (non-pregnant women). RNA samples were extracted from plasma samples, obtained from non-pregnant women (n=10) or uncomplicated pregnancies (n=11). Base 2 logarithms of expression values are plotted. Each box shows the variance of relative values of miRNAs with the lower boundary indicating the 25th percentile, the line within the box indicating the median, and the upper edge marking the 75th percentile. Upper and lower whisker caps indicate the 95th and 5th percentiles. Outliers are indicated by circles. * denotes miRNA species that are significantly different in the plasma of non-pregnant women and pregnant women (adjusted p value ≤ 0.05).

Fig. 2. Relative miRNA levels in plasma from normal vs FGR pregnancies

Box plots of the base-2 log-transformed relative values by RT-qPCR, analyzed and plotted as described in the legend to Fig. 1. RNA samples were extracted from plasma samples obtained from uncomplicated pregnancies (n=14) or FGR pregnancies (n=14). Note that the levels of all miRNA species, considered as a single group, was higher by 1.8-fold in FGR than in control plasma samples ($p \leq 0.01$).

Fig. 3. Relative miRNA levels in the normal vs FGR placentas

Box plots of the base-2 log-transformed relative values by RT-qPCR, analyzed and plotted as described in the legend to Fig. 1, and normalized to RNU6B levels. RNA samples were extracted from placental biopsies, obtained from uncomplicated pregnancies (n=4) or FGR pregnancies (n=5). Data are presented as described in Fig. 1. Note that the levels of all miRNA species, considered as a single group, was lower by 24% in FGR than in control plasma samples ($p \leq 0.01$).

Fig. 4. The relative change of miRNA levels from plasma vs placental samples in pregnancies complicated by FGR

Plot of the log-transformed ratio of miRNA levels in plasma vs placental samples in FGR/control, measured by RT-qPCR. Base 2 logarithms of ratio are plotted. The dotted line is the regression line for the entire set of miRNAs ($r = -0.7$; $p \leq 0.01$).

Table 1

Maternal and neonatal characteristics

	Control (n=14, mean $\pm$ SD)	FGR (n=14, mean $\pm$ SD)	p value
Maternal age (yrs)	25.2 $\pm$ 5.8	25.4 $\pm$ 5.4	NS
Pre-pregnancy weight (kg)	63.15 $\pm$ 10.82	61.72 $\pm$ 11.5	NS
Gestational age at delivery (wks)	39.7 $\pm$ 1.8	39.5 $\pm$ 1.1	NS
Neonatal weight (g)	3239.0 $\pm$ 335	2539.4 $\pm$ 185	p <0.001
APGAR score < 7 at 5 min	0	0	NS