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Placental miR-1301 is dysregulated in early-onset preeclampsia and inversely correlated with maternal circulating leptin

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ABSTRACT

Introduction: miRNAs are small non-coding RNAs important for the regulation of mRNA in many organs including placenta. Adipokines and specifically leptin are known to be dysregulated in preeclampsia, but little is known regarding their regulation by miRNAs during pregnancy.
Methods: We performed high-throughput sequencing of small RNAs in placenta from 72 well-defined patients: 23 early-onset preeclampsia (PE), 26 late-onset PE and 23 controls. The regulation of some miRNAs was confirmed on qRT-PCR. Maternal circulating levels and placental mRNA of leptin, resistin and adiponectin were measured using Bio-Plex and qRT-PCR.
Results: We found that miR-1301, miR-223 and miR-224 expression was downregulated in early-onset PE, but not in late-onset PE, compared to controls. In silico analysis predicted the leptin gene (*LEP*) to be a target for all three miRNAs. Indeed, we found significant correlation between maternal circulating levels of leptin and placental *LEP* expression. In addition, we found a significant inverse correlation between maternal circulating leptin/placental *LEP* expression and placental miR-1301 expression levels. Interestingly, placental expression of miR-1301 was also correlated with newborn weight percentile and inversely correlated with both maternal systolic and diastolic blood pressure prior to delivery.
Discussion: Our results confirm that placenta is a major site of LEP expression during pregnancy. It further suggests that miR-1301 could be involved in the regulation of leptin during pregnancy and may play a role in early-onset PE.
Conclusions: miR-1301 is dysregulated in early-onset preeclampsia and could possibly play a role in the regulation of leptin during pregnancy.

1. Introduction

Preeclampsia is a pregnancy-specific disorder affecting 3–10% of all pregnancies and a significant cause of maternal and neonatal morbidity and mortality, especially dangerous for many women and their offspring in developing economies. Several studies have suggested that early- and late-onset PE are two distinct subtypes of the disease but with overlapping features [1,2]. While early-onset is regarded to often represent a more

Abbreviations: miRNA, micro RNA; *LEP*, leptin gene; early-onset PE, preeclamptic pregnancies delivered prior to week 34; late-onset PE, preeclamptic pregnancies delivered after or including week 34; RIN, RNA integrity number; TAP, tobacco acid pyrophosphatase; qRT-PCR, quantitative reverse-transcription PCR; *YWHAZ*, 14-3-3 protein zeta/delta; RT, room temperature; *RETN*, resistin gene; *ADIPOQ*, adiponektin gene; BMI, body mass index.

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severe type of the disease with a dysfunctional placentation origin, late-onset is less often associated with fetal growth restriction and remote maternal cardiovascular disease risk [3]. Also, early- and late-onset PE have been shown to have distinct placental genetic profiles [4], but distinct miRNA profiles from placental tissues for these two important categories of preeclampsia have to our knowledge not been identified. Placenta is also among the tissues with the highest concentration of miRNA, suggesting an important role for miRNA in this tissue and in pregnancy [5].

MicroRNAs (miRNAs) are short (~22 nucleotides) noncoding RNAs that play a critical role in posttranscriptional gene regulation by pairing with the 3' UTRs of protein-coding gene mRNAs. miRNA binding generally results in gene silencing, either by translational repression or target degradation. miRNAs are important for fine-tuning of gene expression in diverse developmental and physiological settings. Typically, several miRNAs regulate one mRNA and one miRNA regulate several mRNAs [6]. Consistent with this notion, a growing number of pathological processes are associated with mis-expression of certain types of miRNAs. The importance of these miRNAs in regulating placental function has only recently been uncovered. miRNA-mediated regulation in pregnancy remains poorly investigated although several independent processes associated with placental development have been shown to be regulated by miRNAs [7].

Pregnancy is a state of maternal hyperlipidemia, as compared to non-pregnancy. The hyperlipidemia is even more exaggerated in a pregnancy complicated by preeclampsia. Excessive generalized maternal (vascular) inflammation, caused by placental oxidative stress and dysfunction, with release of placenta-associated factors, has been proposed to mediate this excessive hyperlipidemia of preeclampsia [8].

The three adipokines leptin, resistin and adiponectin have been shown to be expressed not only in adipose tissue but also in placenta [9]. Leptin is a key hormone in the regulation of energy intake and energy expenditure and is the most studied of these three adipokines in pregnancy [9]. Placental leptin gene (*LEP*) expression is elevated in preeclampsia, but maternal circulating leptin levels are highly elevated even before the onset of clinical signs of disease [10,11]. In addition to its involvement in energy homeostasis, increasing evidence suggest that leptin is a proinflammatory factor [12]. Increased leptin has also been speculated to be involved in the pathophysiology of preeclampsia [9], by representing an inflammatory stimulus [8] or stimulating hypertension and proteinuria by other mechanisms [13]. Although many studies have shown a strongly increased concentration of circulating leptin and placental upregulation of *LEP* expression during pregnancy [14–16], current knowledge on how placental *LEP* expression is regulated is limited. The placental and adipose form of *LEP* mRNA is the same, but placental-specific enhancers have been identified. Both hypoxia and insulin can probably induce expression of leptin in placenta [17].

The aim of this study was to investigate the global expression of miRNAs in early- and late-onset PE compared to normal pregnancies without complications (named controls). Further, we wanted to increase the understanding of how miRNAs could contribute to the regulation of the most investigated adipokines during pregnancy, by studying if some miRNAs were inversely associated with their respective predicted adipokine targets.

2. Materials and methods

2.1. Patient selection

Placental tissue and maternal blood samples were obtained from an ongoing biobank recruitment of patients at Oslo University Hospital, Norway. Women with a singleton pregnancy delivered by elective caesarean section were included in this study ($n = 85$). This included 29 women with uncomplicated pregnancy (controls) delivered at term (week 37–41), 29 with preeclamptic pregnancies delivered prior to week 34 (early-onset PE), as well as 27 preeclamptic pregnancies delivering after or including week 34 (late-onset PE). No women with chronic hypertension or renal disease were included. All patients were fasting overnight (minimum 6 h), none were in labor, none had ruptured membranes or clinical signs of infection. Regional (spinal) anesthesia was used on all patients. No patients were treated with $MgSo_4$ and antihypertensive was used in 10 out of 23 of earl-onset PE, 8 out of 26 of late-onset PE and none of the control group. Preeclampsia was defined as blood pressure augmentation after 20 weeks' gestation to >140/90 on at least two occasions 6 h apart in a previously normotensive woman, combined with proteinuria. Proteinuria was defined as protein dip stick $\geq$1+ on at least two midstream urine samples 6 h apart or a 24-h urine excretion of $\geq$0.3 g protein, in the absence of urinary infection. Severe preeclampsia was defined by the American College of Obstetricians and Gynecologist's criteria [18]. The newborn birth weight percentiles were calculated according to an ultrasound-based Norwegian fetal weight percentile based on pregnancies of healthy Norwegian women [19]. The study protocol was approved by the Regional Committee of Medical Research Ethics in Eastern Norway, and informed written consent was obtained from each patient.

2.2. Biological samples

All placental tissue samples were obtained immediately after cesarean section. Placental biopsies (approx. 0.6 × 0.6 cm in size) from a macroscopically normal looking, centrally located cotyledon were collected, omitting the decidual and membrane layers. All tissues were snap-frozen in liquid nitrogen and stored at −80 °C. Blood samples were collected preoperatively from an antecubital vein before start of the intravenous infusion. Serum was centrifuged at room temperature for 10 min at 2000 *g* and stored at −70 °C until assayed.

2.3. Total and small RNA isolation from placental tissue and cDNA synthesis

The frozen tissues were pulverized in liquid nitrogen and ~50 mg of tissue was homogenized in 500 μl of lysis buffer using an ultra-Turrax homogenizer for 30 s. Total and small RNA (<200 nt) was extracted from placental tissues in the same isolation procedure using the mirVana miRNA Isolation Kit (Ambion, Life Technologies) according to the manufacturer's instructions. The quality and quantity of RNA was determined using a spectrophotometer (NanoDrop 1000, NanoDrop Technologies, Boston, MA) and capillary electrophoresis (Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico kit, Agilent Technologies, Palo Alto, CA) according to the manufacturer's protocol. Samples with RNA integrity numbers (RIN) above 5.5 where selected for further study. Of the 85 included women, 23 controls, 23 preeclamptic giving birth before week 34 and 26 preeclamptic giving birth in week 34 and later were included ($n = 72$). The average RIN value of the selected samples was 7.03 and there were no significant difference in RIN values between the 3 patient study groups. RIN value is shown to give a good indication about the expression level of specific miRNAs [20].

2.4. Small RNA library preparation and high-throughput sequencing

Small RNA library preparation was done using the ScriptMiner Small RNA-Seq Library Preparation kit (Epicentre Biotechnologies) according to the manufacturer's instructions, including the optional addition of TAP after the 3'-adapter oligo ligation. Individual small RNA libraries were made for each patient. After di-tagging, the RNA was purified using RNA Clean and Concentrator-5 (Zymo Research) eluting twice in 15 μl of nuclease free water. After cDNA synthesis the optimal number of PCR cycles was decided. 14 cycles were found to be optimal after running the samples on a 8% native TBP polyacrylamide gel (Invitrogen). PCR was amplified using 3 μl cDNA, Phusion High-Fidelity PCR Master Mix with high-fidelity Buffer (New England BioLabs) and 48 user-defined barcode-containing index primers (Supplementary Table 1), as described in the ScriptMiner library preparation manual (Epicentre Biotechnologies). The 200 μl PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instruction, eluting the samples in 35 μl 10 mM Tris-Cl, pH 8.5. The sample concentration was measured using Qubit 2.0 Fluorometer (Life technologies) according to the manufacturer's instruction, to decide the optimal amount of cDNA to use for gel extraction. cDNA in the range from 120 to 165 bp were eluted from the Pippin prep gel (Saga Sciences) according to the manufacturer's instruction using a 3% gel and Marker C. The samples were purified using Mini Elute (Qiagen) adding 10 μl Na Acetate to the sample according to the manufacturer's instruction. The samples were eluted in 10 μl elusion buffer incubated for 3 min at 65°, and then another 10 μl in addition to the first 10 μl in the same way. Sequencing library quality was checked on a Bioanalyzer using the High sensitivity DNA kit (Agilent) according to the manufacturer's instruction. Sample concentration was estimated by quantitative reverse-transcription PCR (qRT-PCR) on an Applied Biosystems 7900 HT machine, using the protocol described by Quail et al. (Quail, Swerdlow & Turner (Current Protocols in Human Genetics 18.2.1–18.2.27, July 2009). Clustering was done using the TruSeq SR Cluster Kit v3-cBot-HS GD-4013001 (Illumina). Samples were sequenced at the Norwegian Sequencing Center, Norway, using TruSeq SBS Kit v3 FC-401-3002 of 50