The pathogenic role and expression profile of microRNAs in preeclampsia

PhD thesis

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Introduction

Preeclampsia is of the leading causes of maternal and fetal morbidity and mortality, affecting 3–8% of all pregnancies around the world. It is a multisystemic pregnancy-specific disorder, characterized by the development of hypertension and proteinuria after 20 weeks of gestation. Although the pathophysiology of the disease is still elusive, it is believed that placental malperfusion is the main cause of the disease, which hinders maternal blood flow to the placenta and also leads to high perfusion pressure constituting shear stress to the trophoblast layer. This phenomenon results in injured trophoblast and subsequent release of possibly harmful materials including cell fragments and extracellular vesicles into the maternal circulation, that are capable of activating the maternal endothelium.

Extracellular vesicles are lipid bilayer structures that are released from cells into the extracellular environment. After secretion from cells, extracellular vesicles may modulate the activity of neighboring cells or travel to distal regions serving a non-hormonal way of intercellular communication. Exosomes are the smallest of extracellular vesicles. In the course of physiologically normal pregnancy, exosomes of trophoblast origin can be detected from the 6th week of gestation in the maternal circulation. The number of these vesicles increases until term and returns to non-pregnant levels in 48 hours after delivery. The levels of placenta-specific exosomes and their content may serve information of placental health. In preeclampsia, placental dysfunction causes the enhanced shedding of trophoblast-derived extracellular vesicles into the maternal circulation and subsequent release of toxic material including microRNAs (miRNAs).

miRNAs are small, noncoding RNA molecules, which negatively regulate gene expression at the post-transcriptional level, by either repressing protein translation of target genes or inducing target mRNA degradation. One particular miRNA is able to control multiple genes, and a single gene can be a target of several miRNAs. They play pivotal role in the main physiological processes, including differentiation, proliferation, apoptosis and other mechanisms

Genome-wide screening studies identified several hundreds of miRNA species expressed by human placenta. The function of placenta-specific miRNAs is poorly understood, but is clear that they take part in the regulation of placental development and are essential for normal physiology. Abnormally expressed miRNAs contribute to pregnancy complications such as

preeclampsia. miRNAs are released from trophoblast cells to maternal blood by active sorting or passive release mechanisms. Circulating miRNAs are encapsulated inside extracellular vesicles (e.g. exosomes) or bound to stabilizing proteins, which are mainly Argonaute proteins. In either way, extracellular miRNAs have been shown to be stable and protected from RNase degradation, which implicates their use as potential biomarkers. Cells seem to selectively release certain non-coding RNAs, thereby affecting the function of surrounding and distant target cells. Exosomal miRNAs can be internalized by recipient cells, where they mediate functional effects by altering host gene expression. Non-vesicular miRNAs are believed to be non-specific by-products of general physiological processes and cell death.

In preeclampsia, altered placental development and spiral artery remodeling leads to placental hypoxia, which can change the number and content of secreted extracellular vesicles. *hsa-miR-210* is one of the so-called "hypoxamiRs", which are sensitive to the hypoxic environment. It is specifically activated by HIF-1α and considered as one of the hallmarks of hypoxic responses in several cell types. *hsa-miR-210* was found to be upregulated both in the placenta and in the circulation of women with preeclampsia, although it is questionable whether it is the cause or the consequence of the disease. The mode of placental miRNA secretion (vesicular or non-vesicular) and the significance of the different forms are not interpreted either.

Aims

I. The function of placenta-specific miRNAs is poorly understood, but is clear that they take part in the regulation of placental development and are essential for normal physiology. Abnormally expressed miRNAs contribute to pregnancy complications such as preeclampsia. In this study, we created miRNA regulated interaction network by integration of available miRNA and gene expression profiles in preeclampsia. Our aim was to find possible connections with the pathogenesis of the disease by bioinformatics approaches.

II. miRNAs are released from trophoblast cells to maternal blood by active sorting or passive release mechanisms. In preeclampsia, altered placental development and spiral artery remodeling leads to placental hypoxia, which can change the number and miRNA content of secreted extracellular vesicles. In this study, our aims were to quantify exosomal total-miRNA concentration and to perform expression analysis of exosomal *hsa-miR-210* in the circulation of women affected by chronic hypertension (CHT), gestational hypertension (GHT), and preeclampsia (PE). In the latter group, we also studied whether these factors change with the severity of the disease.

III. *hsa-miR-210* was found to be upregulated both in the placenta and in the circulation of women with preeclampsia, although it is questionable whether it is the cause or the consequence of the disease. The mode of placental miRNA secretion (vesicular or non-vesicular) and the significance of the different forms are not interpreted either. Our aim was to analyze the expression of *hsa-miR-210* in placenta, exosome and Ago-bound fractions comparing PE and N pregnancies. We performed an in vitro analysis of extracellular miRNA secretion of different trophoblast cell cultures under normoxic and hypoxic conditions.

Methods

Bioinformatics analysis

We looked for miRNA and mRNA expression datasets in Gene Expression Omnibus database. The search criteria included preeclamptic versus normal placenta samples, which were acquired at term and were individual, not pooled together. There were no such study available when miRNA and mRNA expression data were collected from the same biological samples. We found 2 miRNA and 8 mRNA expression studies which fulfilled the criteria. We chose one miRNA and one mRNA expression dataset based on the similar experimental design. The preprocessing of the microarray datasets was implemented using the R statistical programming language. The associated Bioconductor project provides additional R packages for microarray data analysis. We used the MAGIA web tool to integrate mRNA and miRNA expression data. The analysis is based upon the idea that a miRNA and its targets are expected to be expressed in an inverse manner. MAGIA synthesize miRNA and gene expression data analysis with target predictions to identify experimentally supported regulatory relationships between miRNAs and its predicted target genes.

We selected meta-analysis for the integrated analysis of the miRNA and mRNA gene expression profiles. This method separately processes each array to identify probes significantly variable among the two groups (preeclamptic and normal). Next we chose the intersection of two target predictors, miRanda and PITA, applying the basic score filters. The two algorithms differ in their target prediction strategies, combining them we get more specific results. Finally, we uploaded the miRNA and gene expression matrices and performed the analysis.

For network visualization, we applied the Cytoscape software package. Some of the subnetworks were expanded by adding protein-protein interactions. For protein interaction research, we used the Human Protein Reference Database. Therefore, we gained miRNA regulated PPINs (protein-protein interaction networks). Only proteins which are expressed by the placenta were added to the network. The Human Protein Atlas was used for determining the tissue specific expression of each gene. DAVID Bioinformatics Database was used for functional analysis of the regulated genes. The online software annotates the genes based on the three Gene Ontology (GO) categories: biological Pathway, molecular function, and cellular component.

Laboratory analysis

Participants

Study participants had been recruited during routine prenatal care or following hospital admission between the 24-40th weeks of gestation during 2015 -2017 at 1st Department of Obstetrics and Gynecology, Semmelweis University, Budapest, Hungary. We collected plasma samples from healthy controls and from pregnant woman affected by different types of hypertension: chronic hypertension, gestational hypertension, and preeclampsia

Placenta samples were collected from C-sections. Preeclamptic pregnancies (affected group), and healthy controls were involved.

Sample collection and handling

Blood samples: Venous blood samples were drawn into 4mL EDTA tubes and kept at 4°C until processing. Plasma was separated within few hours by two-step centrifugation (1. 2500xg, 10 min, 4°C, 2. 12500xg, 10 min, 4°C) and stored in 1,5 ml Eppendorf tubes at -80°C.

Placenta samples: Four areas suitable for sampling were located on the maternal surface, damaged areas not suitable for further examination (calcification, hematoma, etc.) were excluded. 1-2 mm from the basal membrane was removed and a pea size tissue sample was taken from the placental cotyledons. The samples were washed twice in 1x PBS solution at 4 °C and placed in RNAlater stabilizing solution to avoid RNA degradation.

Cell culture and treatment

We used JAR and HTR8 placenta-derived cell lines. The former is a choriocarcinoma cell line and has the characteristics of villous trophoblasts (VT), while the latter one is an immortalized extravillous trophoblast (EVT) cell line. JAR and HTR8 cell lines were maintained under standard conditions at 37°C and 5% CO_2 in RPMI-1640 or DMEM medium respectively, supplemented with 10% FBS and 1% penicillin-streptomycin. 4 x 10^5 cells were seeded per well on six-well plates in media containing 10% exosome-depleted FBS. Cells were treated after 24 hours with hypoxia-mimetic agent (100 uM DFO), or DMSO as control and maintained under standard conditions for another 24 hours.

RNA and miRNA isolation

Exosomal miRNA isolation by precipitation method

Exosomes were isolated from 500 µl plasma using MN exosome precipitation solution and resuspended in 300 µl RNase-free water. miRNAs were extracted from the diluted exosomes with NucleoSpin miRNA Plasma Kit and dissolved in 30 µl RNase-free water.

Extracellular miRNA isolation by membrane affinity spin column method

Exosomal and Ago-bound fractions were separated from plasma and cell media using the ExoEasy kit by Qiagen. Exosomes were bound to the column membrane and protein complexes were partitioned to the flow-through. Short RNAs were extracted from exosomes and vesicle-free fractions with the miRNEasy kit.

Total-RNA isolation

Total-RNA was isolated from the placenta samples and cultured trophoblast cell lines using the TRIzol lysis reagent according to the manufacturer's protocol.

RNA and miRNA quantity measurement

The RNA purity and concentration were measured by Nanodrop spectrophotometer. In order to determine total-miRNA concentration, we applied a miRNA-specific fluorometric assay using a Qubit® 2.0 Fluorometer.

Quantitative real-time PCR

The expression analysis was performed using the miRCURY LNA™ Universal RT miRNA PCR Assay (Qiagen) according to the manufacturer's instructions. Briefly, RNA samples were reverse-transcribed using the miRCURY LNA™ RT Kit. UniSp6 RNA spike-in template was added to each reaction for cDNA synthesis control. RT-PCR was carried out using miRCURY SYBR® Green master mix with specific LNA™ PCR primer sets. The relative expression of the investigated miRNAs was calculated based on the ddCT method and was normalized to *hsa-miR-103a* internal control miRNA. Melting curve analysis was carried out following each PCR run for evaluating the specificity of the assays. Along with *hsa-miR-210,*

the expression of *hsa-miR-16* and *hsa-miR-517c* was also measured, *hsa-miR-16* is one of the most abundant miRNAs in blood, found to be expressed mainly by red blood, whereas *hsa-miR-517c* is part of the primate-specific C19MC miRNA cluster, which is abundantly expressed in the placenta.

Statistics

We used the STATISTICA software package for statistical analysis. Shapiro-Wilk W test was applied to assess the normality of the dependent variables.

II. Since nor the total-miRNA concentration, neither the *hsa-miR-210* expression followed a normal distribution, nonparametric tests were used afterward. Comparisons among groups were carried out using Kruskal-Wallis ANOVA. Spearman Rank Order correlation was calculated to find a possible connection between *hsa-miR-210* expression and total-miRNA concentration. miRNA levels were given as median with interquartile ranges, and fold change values (FC) were calculated when the hypertensive and normotensive groups were compared. FC>2 and FC<0,5 was regarded as significant over- and underexpression respectively.

III/1. None of the miRNA expression values of placenta and plasma samples followed a normal distribution, thus comparisons between groups were performed using the nonparametric Mann-Whitney U test and exact probabilities were calculated. miRNA levels were given as median with interquartile ranges.

III/2. The miRNA expression values of cell culture were logarithmized and one-sided t-test was applied. miRNA levels were given as mean with standard error.

In every measurement, a p value of <0,05 considered as a statistically significant finding.

Results

I. miRNA-mRNA interaction network

Quality control measurements and normalization performed on the raw miRNA and mRNA expression datasets indicated that the obtained data were optional for the further analysis. After filtering the normalized datasets, expression information on 704 out of 1105 human mature miRNA probes and 26808 out of 40716 gene probes were remained for integration analysis. As a result of differential expression analysis, the highly over- or underexpressed miRNAs were determined. The top upregulated miRNAs (FC>2, p<0,05) included *hsa-mir-187, hsa-mir-210, hsa-mir-1181, hsa-mir-943, hsa-mir-33b*, hsa-mir-466* and hsa-mir-1238; in contrast *hsa-mir-202, hsa-mir-628-5p, hsa-mir-488, hsa-mir-548u, hsa-mir-603, hsa-mir-200b*, hsa-mir-450b* were among the most downregulated miRNAs (FC<0,5, p<0,05).

Altogether 52603 miRNA-mRNA interactions were generated by the MAGIA web tool. Adjusting the threshold, we gained 15650 interactions with q<0,1. 3564 interactions with q<0,01, 1670 interactions with q<0,001 and 80 interactions with q<0,0001. The top 250 interactions were visualized and pairs with q<0,0001 were analyzed in order to reduce FDR. The most significant hits included 85 nodes and 80 edges signalizing the connections between 52 regulated genes and 33 miRNAs. Eleven of the 52 genes were found to be associated with preeclampsia through literature search: ATG9A, BMPR2, DLG5, EMP2, FSTL3, KCNQ4, KLF6, TCF7L2, TIMP3, QSOX1, and XDH. Nine genes are targeted by multiple miRNAs: QSOX1, BAIAP2, SESTD1, PRKAB2, TIMP3, CMTM4, DAND5, EMP2 and XDH in descending degree number order. Nine of the 33 miRNAs expressions were altered in preeclampsia according to previous studies: *hsa-miR-17*, hsa-miR-18a*, hsa-miR-193b*, hsa-miR-200b*, hsa-miR-210, hsa-miR-27a*, hsa-miR-296-3p, hsa-miR-33b*,* and *hsa-miR-637*. Thirteen miRNAs regulate more than one mRNA*: hsa-mir-210, hsa-mir-1226*, hsa-mir-1263, hsa-mir-541, hsa-mir-200b*, hsa-mir-296-3p, hsa-mir-33b*, hsa-mir-18a*, hsa-mir-1181,*

hsa-mir-1538, hsa-mir-193b, hsa-mir-30c-1** and *hsa-mir-637* in descending degree number order. According to the resulted GO terms, differently expressed miRNAs mainly regulate genes which participate in development processes.

In the *hsa-miR-210* regulated protein-protein interaction network, from 101 interacting proteins 70 are expressed by the placenta. The network consists of 86 nodes, including one miRNA, 17 directly regulated genes and 69 indirectly regulated genes. Two nodes, BMPR2 and EIF2AK2, are outstanding regarding their number of protein interactions. In the *hsa-miR-33b** regulated PPIN, from 20 interacting proteins 17 are expressed by the placenta. The network consists of 22 nodes, including one miRNA, 4 directly regulated genes and 17 indirectly regulated genes. From the nodes, KLF4 possesses the highest number of protein interactions.

II. Exosomal total-miRNA concentration and *hsa-mir-210* expression in different types of pregnancy hypertension

The median value of total-miRNA concentration in exosome samples varied in the disease subgroups and in the control group as follows: N: 0,26 ng/µl (0,10-0,76 ng/µl); GHT: 0,28 ng/µl (0,16-0,91 ng/µl); CHT: 0,30 ng/µl (0,22-0,38 ng/µl); mPE: 0,83 ng/µl (0,42-1,85 ng/µl); sPE: 1,57 ng/µl (0,25-8,21 ng/µl). Overexpression (FC>2) was only observed in PE groups, the total-miRNA concentration was three-times higher (FC=3,19, p<0,001) in mPE and six-times higher (FC=6,03, p<0,001) in sPE groups than in the normotensive group. There was significant difference comparing CHT to mPE (p<0,05) and sPE (p<0,001), and GHT to sPE group (p<0,01). De novo and superimposed PE could not be segregated based on the total-miRNA concentration (0,96 (0,25-8,21) vs. 0,57 (0,21-4,00), p>0,05), and IUGR had no significant impact in the affected groups.

We found significant difference in the relative exosomal *hsa-miR-210* expression between the hypertension groups and the normotensive group: N: 0,13 (0,10-0,17); CHT: 0,17 (0,13-0,25); GHT: 0,23 (0,17-0,30); mPE: 0,29 (0,20-0,41); sPE: 0,37 (0,26-0,49). Overexpression only was observed in mPE (FC=2,23, p<0,001) and in sPE groups (FC=2,85, p<0,001) but not in CHT (FC= 1,31, p<0,05) and GHT groups (FC=1,77, p<0,01) in comparison to the control group. There was a significant difference comparing CHT and GHT to sPE group (p<0,05). De novo and superimposed PE could not be separated based on the

relative *hsa-miR-210* expression (0,34 (0,14-0,73) vs. 0,26 (0,16-0,64), p>0,05), and IUGR had no significant impact in the hypertensive groups. Total-miRNA concentration was positively correlated with *hsa-miR-210* expression in the hypertensive samples (R= 0,41, p<0,05) but not in the control samples.

III. Exosomal and Ago-bound *hsa-miR-210* secretion in preeclampsia

1. *hsa-miR-210* expression profile in placenta and extracellular plasma fractions

The level of placental *hsa-miR-210* was significantly upregulated in affected samples comparing to the control group (1,06 (0,84-1,62) vs. 3,26 (1,94-4,72), p<0,05), which could be related to a minor increase of circulating exosomal (1,03 (0,62-1,60) vs. 1,14 (0,68-2,36), p>0,05) and significantly elevated Ago-bound *hsa-miR-210* (1,16 (0,54-1,93) vs. 3,80 (1,61-6,50), p<0,05).

The level of placental *hsa-miR-517c* was significantly upregulated in affected samples comparing to the control group (0,82 (0,56-1,62) vs. 2,33 (1,28-4,50), p<0,05), but there was no significant difference in the level of extracellular *hsa-miR-517c* between groups (exosomal: 1,46 (0,40-2,55) vs. 1,29 (0,63-4,75); Ago-bound: 1,23 (0,35-2,50) vs. 1,45 (0,74-5,65), p>0,05). Neither the level of placental *hsa-miR-16* (0,95 (0,58-1,86) vs. 1,31 (0,76-2,00), p>0,05) nor extracellular *hsa-miR-16* was not altered due to PE (exosomal: 0,91 (0,55-1,79) vs. 1,26 (0,70-2,62), Ago-bound: 0,97 (0,52-2,13) vs. 2,08 (0,99-3,52), p>0,05).

2. In vitro *hsa-miR-210* secretion from trophoblast cell-lines

The treatment of villous trophoblast cells with hypoxia-mimetic agent selectively upregulated *hsa-miR-210* as compared to the other examined miRNA species (1,02±0,11 vs. 2,27±0,16, p<0,05). In the cell media, the level of exosomal *hsa-miR-210* was significantly increased (1,04±0,15 vs. 2,53±0,35, p<0,05) and enhanced release of Ago-bound miRNAs was also observed (1,06±0,21 vs. 2,40±0,29, p<0,05). There was no significant difference in the case of *hsa-miR-16*, and *hsa-miR-517c (hsa-miR-16* intracellular: 1,10±0,26 vs. 1,04±0,26, exosomal: 1,69±0,92 vs. 2,87±1,72, Ago-bound: 1,02±0,11 vs. 0,99±0,16;. *hsa-miR-517c* intracellular:1,01+0,08 vs. 1,04±0,06, exosomal: 1,15±0,32 vs. 1,52±0,71, Ago-bound 1,31±0,54 vs. 1,57±0,51)..

The treatment of the extravillous trophoblast cells with hypoxia-mimetic agent strongly upregulated *hsa-miR-210* (1,07±0,19 vs. 3,80±0,35, p<0,05), and this phenomenon was even

more pronounced in secreted exosomal samples: only the level of exosomal *hsa-miR-210* was increased (1,03±0,14 vs. 6,80±2,46, p=0,057) and a no significant change on Ago-bound hsa-miR-210 level was observed (1,10±0,26 vs. 1,95±0,16). There was no significant difference in the case of *hsa-miR-16* (intracellular: 1,08±0,22 vs. 1,23±0,30, exosomal: 1,08±0,19 vs. 0,67±0,10, Ago-bound: 1,42±0,54 vs. 0,78±0,25) and *hsa-miR-517c* is not expressed in this cell line.

Conclusions

I. In this study, we applied bioinformatics approaches to integrate high-throughput experimental data on miRNA and mRNA expression profiles in preeclampsia. We created miRNA regulated interaction network by integration of available miRNA and gene expression profiles in preeclampsia. *hsa-mir-210* was the highest degree node in the network, and it has been associated with preeclampsia before.

II. In this study, we quantified exosomal total-miRNA concentration and to performed expression analysis of exosomal *hsa-miR-210* in the circulation of women affected by different types of pregnancy-related hypertension disorders. We found that the concentration of total-miRNA and exosomal *hsa-miR-210* was significantly higher in the circulation of women affected by preeclampsia, and it was correlated with the severity of the disease. Based on our results it can be assumed that in preeclampsia, *hsa-miR-210* is secreted via the exosomal pathway, and it may contribute to the multisystem nature of the disease.

III/1. The level of placental *hsa-miR-210* was significantly higher in case of preeclampsia, which could be related to a minor increase of circulating exosomal and highly elevated Ago-bound *hsa-miR-210*. Based on these results, we conclude that apart from exosomal secretion, there is also a large-scale release of Ago-bound *hsa-miR-210*.

III/2. The DFO treatment had a different effect on the villous and extravillous trophoblast cell lines: Intracellular *hsa-miR-210* was upregulated in both cases, however, in extravillous cell media, only the level of exosomal *hsa-miR-210* was increased and no change on Ago-bound *hsa-miR-210* level was observed. Therefore, in this case, hypoxia resulted in the specific exosomal sorting of *hsa-miR-210* – as this cell type has a role in trophoblast invasion, this phenomenon may have a role in placental dysfunction. In villous cell media, the level of exosomal *hsa-miR-210* was increased and enhanced release of Ago-bound *hsa-miR-210* was also observed. The expression profile was quite similar to that of the placenta and plasma samples – which can be explained by that in placenta this cell type is in contact with maternal circulation. Based on this data, we postulate that in preeclampsia, exosomal *hsa-miR-210* are secreted actively from the trophoblast, which may have a role in intercellular communication and causing the disease and there is also a passive release of Ago-bound *hsa-miR-210*, which is the by-product of cell-death and is a possible consequence of the disease.

Publications

Publications related to the Doctoral Theses

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