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The relationship between IGF1 and the expression spectrum of miRNA in the placenta of preeclampsia patients

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ABSTRACT

Objectives: Pre-eclampsia (PE) affects many women worldwide and remains the leading cause of morbidity and mortality in neonatal and maternal settings. Abnormal expression of placental microRNAs (miRNAs) may be associated with PE.

Material and methods: This study was conducted to the relationship between IGF1 and the expression spectrum of miRNA in the placenta of preeclampsia patient. The expression of miRNA in placental tissue was compared between pre-eclampsia (n = 6) and normal pregnant women (n = 5) miRNA targets were studied by computer simulation and functional assays. The role of miRNA was verified in trophoblast cell lines by apoptosis assay and invasion assay.

Results: There was a significant increase in miRNAs in the placenta of women with pre-eclampsia compared with patients with normal pregnancy. Luciferase assay confirmed direct regulation of miRNA.

Conclusions: The expression of IGF1 and miRNA was significantly increased in the placenta of patients with pre-eclampsia.

Key words: preeclampsia; IGF1; miRNA; placenta; expression spectrum

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INTRODUCTION

Preeclampsia (PE), eclampsia and gestational hypertension are unique during pregnancy diseases, which falls within the category of gestational hypertension (hypertensive disorders in pregnancy), morbidity [1–2]. It is about 10% in China, especially in preeclampsia. PE is mainly manifested within 20 weeks after pregnancy [3–4]. Hypertension, proteinuria, can cause multiple organ dysfunction as well as functional failure in the whole body. This is leading to maternal and the main cause of perinatal mortality has been the focus of pathological obstetric research, the pathogenesis is unknown.

Because most preeclampsia conditions can quickly relieve or even heal themselves after placental delivery, some scholars believe that the placenta is the root cause of its disease. In the process of embryo implantation and placenta formation, if various factors lead to trophoblastic dysfunction, it will cause uterine spiral artery recasting disorder, placental blood supply insufficientcy, which results in preeclampsia disease. With the gradual deepening of microRNA (miRNA) function research, more and more studies have confirmed that miRNA plays an important regulatory role in the development of PE. This provides molecular biology clues that reveal the pathogenesis of PE [5, 6].

MiRNAs in eukaryotic organisms, about 18-25 bases in length, by pairing with the target gene mRNA base to induce the silent Complex (RISC) to degrade mRNA or inhibit its translation, thus achieving negative regulation of the expression level of the target gene at the post-transcription level. The discovery of MiRNAs is a milestone in the field of gene expression regulation, which regulates about 30% of human protein-coded genes, and is related to assorted physiological and pathological processes. For instance, embryonic development, organ formation and the occurrence of diseases, through negative regulation of the level of target gene expression.

MATERIAL AND METHODS

Patient samples

All patients received written informed consent. The study was approved by the Institutional Review Board of the Second Affiliated Hospital of Nanchang University. Six

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patients with pre-eclampsia placenta and 5 patients who underwent caesarean section were enrolled in our study. Patients who underwent caesarean section served as the control group. The 11 patients were used for microarray analysis of miRNAs. All of the patients were further evaluated. Patients with pre-eclampsia were confirmed by clinical diagnosis by the professional doctor in our hospital. The placenta tissues from patients were immediately snap-frozen in liquid nitrogen after abscission. The detailed information of samples were shown in Table 1 and Table 2.

Table 1. Sample Description

Sample Number	Sample Name	Group
1	34	T
2	42	T
3	86	T
4	90	T
5	95	T
6	114	T
7	32	N
8	63	N
9	50	N
10	71	N
11	81	N

Table 2. Hybridizations to be performed

Slide no.	Hy3	Hy5
Slide 1	34	
Slide 2	42	
Slide 3	86	
Slide 4	90	
Slide 5	95	
Slide 6	114	
Slide 7	32	
Slide 8	63	
Slide 9	50	
Slide 10	71	
Slide 11	81	

Methods

Microarray analysis of miRNAs

The miRCURYTM LNA array (7th generation) (version 18.0, Exiqon) includes 3,100 capture probes embodying the whole annotations in miRBase 18.0. the miRNAs of mice and rats, and the miRNAs of all viruses associated with them. Furthermore, the array covers the probes for miLlusTM human miRNAs [7].

Extraction of total RNA

Total RNA of patients was extracted by using total RNA isolation kits (Invitrogen) and miRNeasy mini kits (QIAGEN) under the conditions recommended by the manufacturer. The purity of extracted RNA was determined by using the spectrophotometer (ND-1000, Nanodrop Technologies). In addition, we evaluated the RNA integrity by gel electrophoresis [8].

RNA labeling

RNA labeling was performed by using the miRCURYTM Hy3TM / Hy5TM Power Labeling Kits (Exiqon, Vedbaek, Denmark). Furthermore, the sample (1 μg) was performed 3'-end-labeling by using a Hy3TM fluorescent label. We also mixed 2.0 μL of RNA in water with 1.0 μL LCIP buffer and CIP (Exiqon). The mixture was incubated at 37°C for 30 min and at 95°C for 5 min. In addition, we added labeling buffer (3.0 μL), labeling enzyme (2.0 μL), fluorescent label (Hy3TM) (1.5 μL), and DMSO (2.0 μL) into the mixture, which was incubated at 16°C for 1 h and at 65°C for 15 min [9].

Array hybridization

The miRCURYTM LNA array (Section 18.0) (Exiqon) was used for array hybridization. Firstly, hybridization buffer [25 (mu)L] was added into the above sample [25 (mu)L]. Next, the mixture was incubated at 95 (deg.) C for 2 min in order to denaturate, followed by incubation at 4°C for 2 min. After that, the mixture was hybridized to the microarray at 56 (deg.) C. for 16–20 h. Bay Hybridization Systems (Hybridization System — Nimblegen Systems, Inc., Madison, WI, USA) provided effective mixing and invariant incubation temperature. Finally, slides were prepared and washed three times with a wash buffer kit (Exiqon). After washed, slides were dried by centrifugation (400 rpm/min) for 5 min. Finally, slides were scanned using Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA) [10].

Array Information

Exiqon's miRNA arrays feature Tm-standardized and LNATM enhanced capture probes, which have outstanding specificity and sensitivity even for AT-rich miRNAs. They also have excellent repeatability. The correlation between arrays was 99%. The dynamic range was more than 5 orders of magnitude.

miRCURY LNA™ microRNA Array, 7th gen — hsa, mmu & rno

The 7th generation miRNA array included 3100 capture probes, which covered the whole of miRNAs in humans, mice and rats annotated in miRBase 18.0, and viral miR-

NAs. Moreover, there were capture probes for miLlusTM human microRNAs in the array, which were proprietary microRNAs not found in miRBase [11].

Data analysis

The scanned image was imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. The replicated miRNAs were averaged and the miRNAs more than 50 intensities were selected for calculation of normalization factors. The expressed data was normalized by using median normalization. Moreover, miRNAs, with obviously different expression, were discerned by using Volcano Plot filtration. MEV software (v4.6,TIGR) was used for hierarchical clustering.

RESULTS

Low intensity filtering and data normalization

The scanned image was imputed into GenePix Pro 6.0 software (Axon) to do grid alignment and to extract data. The repetitive miRNAs were average while miRNAs which were more than 50 intensities were chosen to calculate querynormalization. The median normalization was used to normalize the data of miRNA. The miRNAs were selected for differential expression. The following list represents only a subset of the overall results. The entire results can be found in the expression "expression matrix" in the miRNA expression profiling Data.xls file (Tab. 3).

miRNA purity

The box plot is an intuitive method in order to effectively visualize the level of disaggregation of data sets. It is an effective way to compare the distribution of miRNAs. In addition, the log2 ratio distribution of each sample was basically identical (Left: data with non-normalized log2-ratio distribution; right: data with median normalized log2-ratio distribution) (Fig. 1).

Correlation Matrix and scatter plot

A scatter plot is a visualization that can be used to evaluate the repetitiveness of chips. Its axis was the normalized signal value (scale scale) (Fig. 2).

Differentially expressed miRNAs screening

Differentially expressed miRNAs with statistically significant differences were identified. We performed Volcano Plot filtration on both groups in the experiment. The threshold, a fold change more than 1.5 and a P value less than 0.05, was used to screen for up-regulation or down-regulation of miRNA. The following list represents only a portion of the differentially expressed miRNAs in the T to N results (Tab. 4).

Heat map and hierarchical clustering

It was displayed the bidirectional hierarchical clustering of samples and miRNAs in the heat map. The abscissa represented miRNA and the ordinate represented samples. On the

Table 3. The results of miRNA Expression

ID	Name	Fold Change	P-value	ForeGround-BackGround		Normalized	
		T vs N	T vs N	...	Mean of N group	...	Mean of N group
13138	Hy3	0.996336378	0.989417	...	333.4	...	1.148126
42638	hsa-miR-23a-5p	1.568977638	0.07266	...	117.3	...	0.390772
42888		1.14788641	0.665814	...	1586.9	...	5.350498
17519	ebv-miR-BART1-3p	1.097571346	0.730312	...	17.2	...	0.059658
17278		1.160911656	0.5042	...	19.4	...	0.066363
46507	hsa-miR-921	1.586768402	0.200918	...	42.2	...	0.140007
17928	hsa-miR-181a-2-3p	1.510173499	0.312414	...	17.2	...	0.059737
42826		0.783954977	0.240246	...	590.1	...	2.016165
17537		1.008976924	0.970497	...	171.2	...	0.585387
42722		1.739150162	0.078576	...	17	...	0.058376
42645		0.560113342	0.294479	...	22.2	...	0.081096
46636		1.195298862	0.508479	...	31.1	...	0.104871
11134	hsa-miR-502-5p	0.931910856	0.855179	...	252.6	...	0.832847
17295	hsa-miR-583	0.927580386	0.667943	...	503.8	...	1.70745
32825	hsa-miR-620	1.204907579	0.413182	...	40.6	...	0.14099
46276		1.87952483	0.05826	...	6	...	0.021253

ID — Contains the miRNA ID number constituted by Exiqon; Name — miRNA name; P-value — T-test result between samples in different groups; Foldchange — Ratio of normalized intensities between two conditions; ForeGround-BackGround — The intensities of the miRNAs before Median normalization; Normalized — The normalized values of the miRNAs after Median normalization

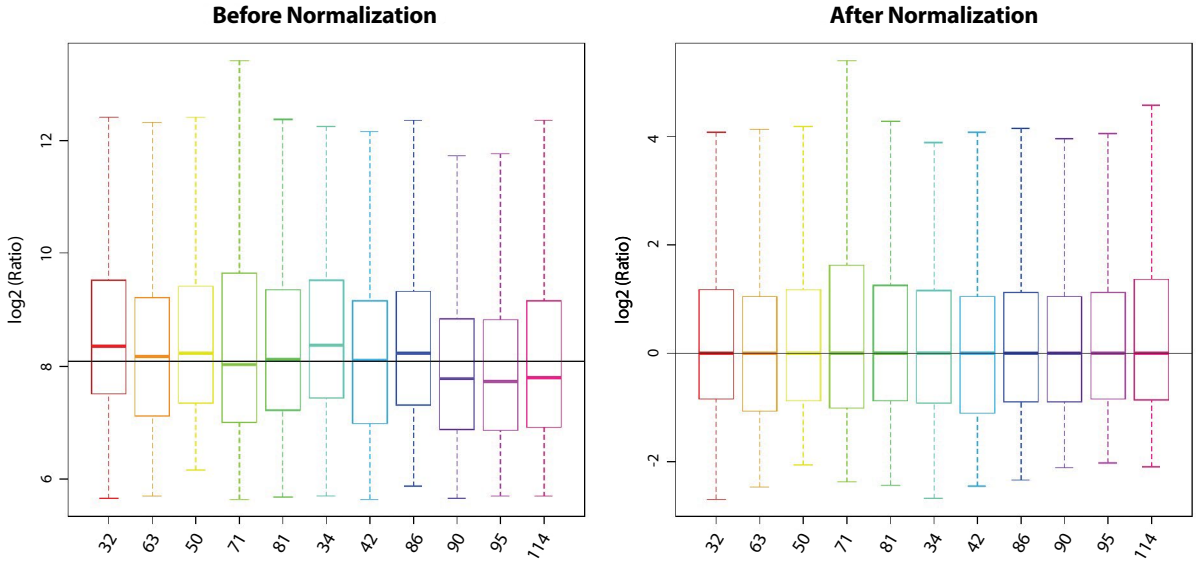

Figure 1. The box plots of before and after normalization

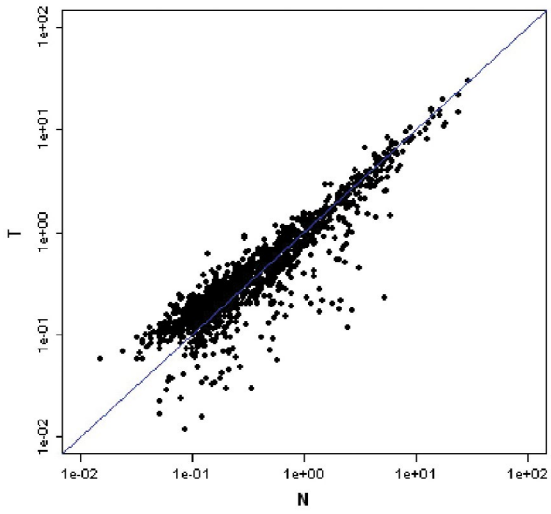

Figure 2. Scatter-plot is for Group-T vs Group-N

left is the miRNA cluster tree, and on the top is the sample cluster tree. According to their expression levels, samples and miRNAs were divided into different groups by cluster analysis, which help us to evaluate their correlation.

Hierarchical clustering based on "Differentially expressed miRNAs in T vs N passed Volcano Plot" was carried out. The results of hierarchical clustering showed a distinguishable miRNA expression profile between samples. The relationship between IGF1 and the expression spectrum of miRNA was showed in Figure 3 and Figure 4.

DISCUSSION

The MicroRNA (miRNA) gene is a new class of non-coding single-stranded RNA molecules 19 to 25 nucleotides in length that are widely found in eukaryotes and have no open reading frame (ORF) [12]. The miRNAs are characterized by diversity of species expression, high conservation, tissue specificity, and clustering of genes. The miRNAs do not directly encode proteins. It is believed that mature miRNAs cause complete or incomplete complementary pairing with targeted messenger RNAs (mRNAs), causing degradation or translational inhibition of target mRNAs and regulating expression of target genes. More and more researches believe

Table 4. T vs N 1.5 fold up regulated miRNAs

ID	Name	Fold change	P-value	ForeGround-BackGround			Normalized		
		T vs N	T vs N	32	...	...	32	...	...
148635	hsa-miR-933	1.52065878	0.0283	34	...	...	0.103976	...	...
168910	hsa-miR-4735-5p	1.63602691	0.029116	52	...	...	0.159021	...	...
168569	hsa-miR-5088	3.22345834	0.038633	39	...	...	0.119266	...	...

ID — Contains the miRNA ID number constituted by Exiqon; Name — miRNA name; Foldchange — Ratio of normalized intensities between two conditions; P-value — P-value calculated from t-test; ForeGround-BackGround — Intensities of the miRNAs in each sample before Median normalization; Normalized — Normalized values of the miRNAs in each sample after Median normalization

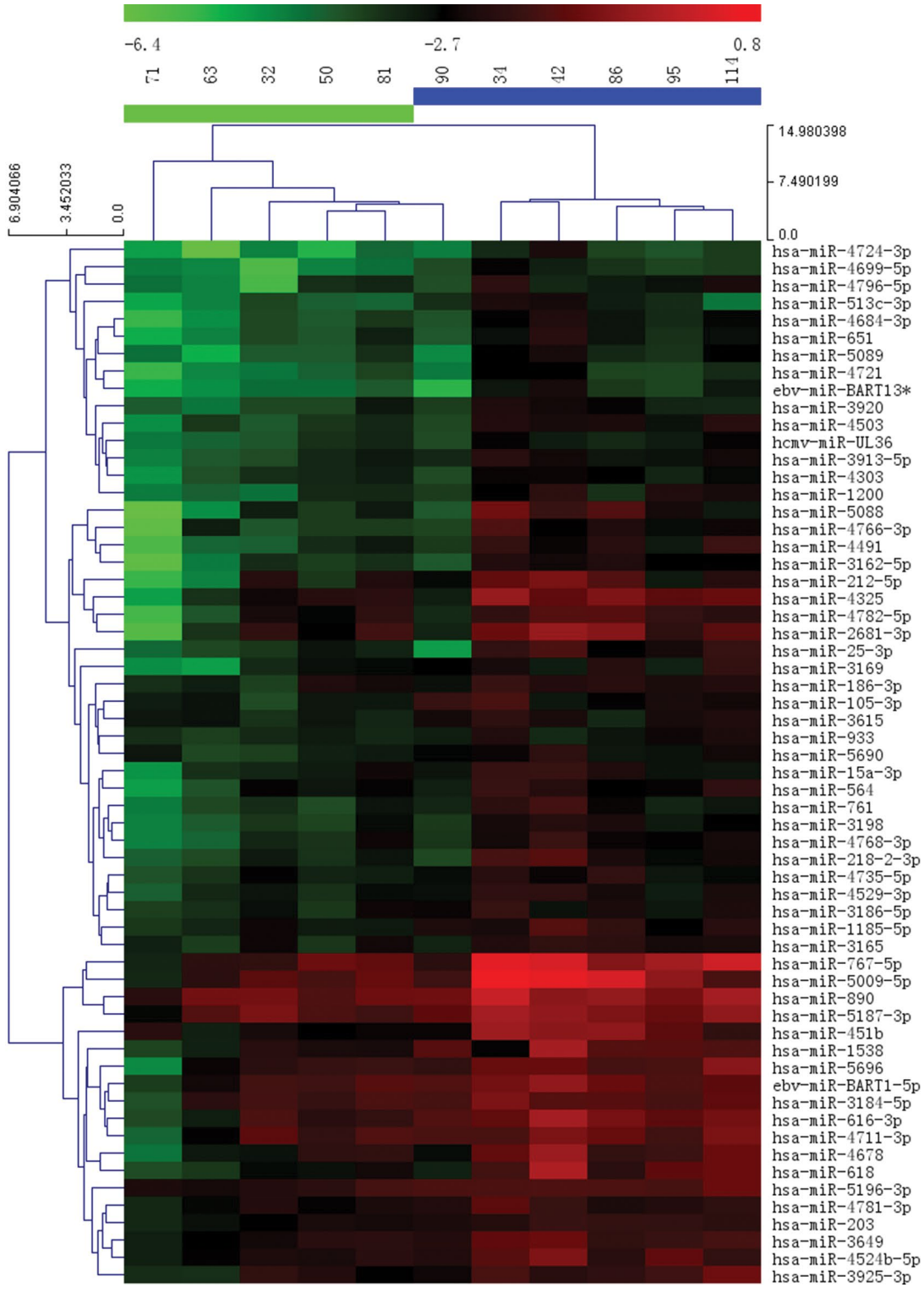

Figure 3. Hierarchical clustering for Differentially expressed miRNAs in T vs N passed Volcano Plot. Red indicates high relative expression, and green indicates low relative expression

that miRNAs play a role in all levels of life. They play an important role in development, cell differentiation, apoptosis, proliferation and tumorigenesis. They have become one of the new research hotspots in life sciences in recent years.

The miRNA genes belong to a highly conserved family of non-coding genes, and various miRNAs can find homologs in other lines [13]. Therefore, miRNAs are involved in various important processes in life processes, such as early embry-

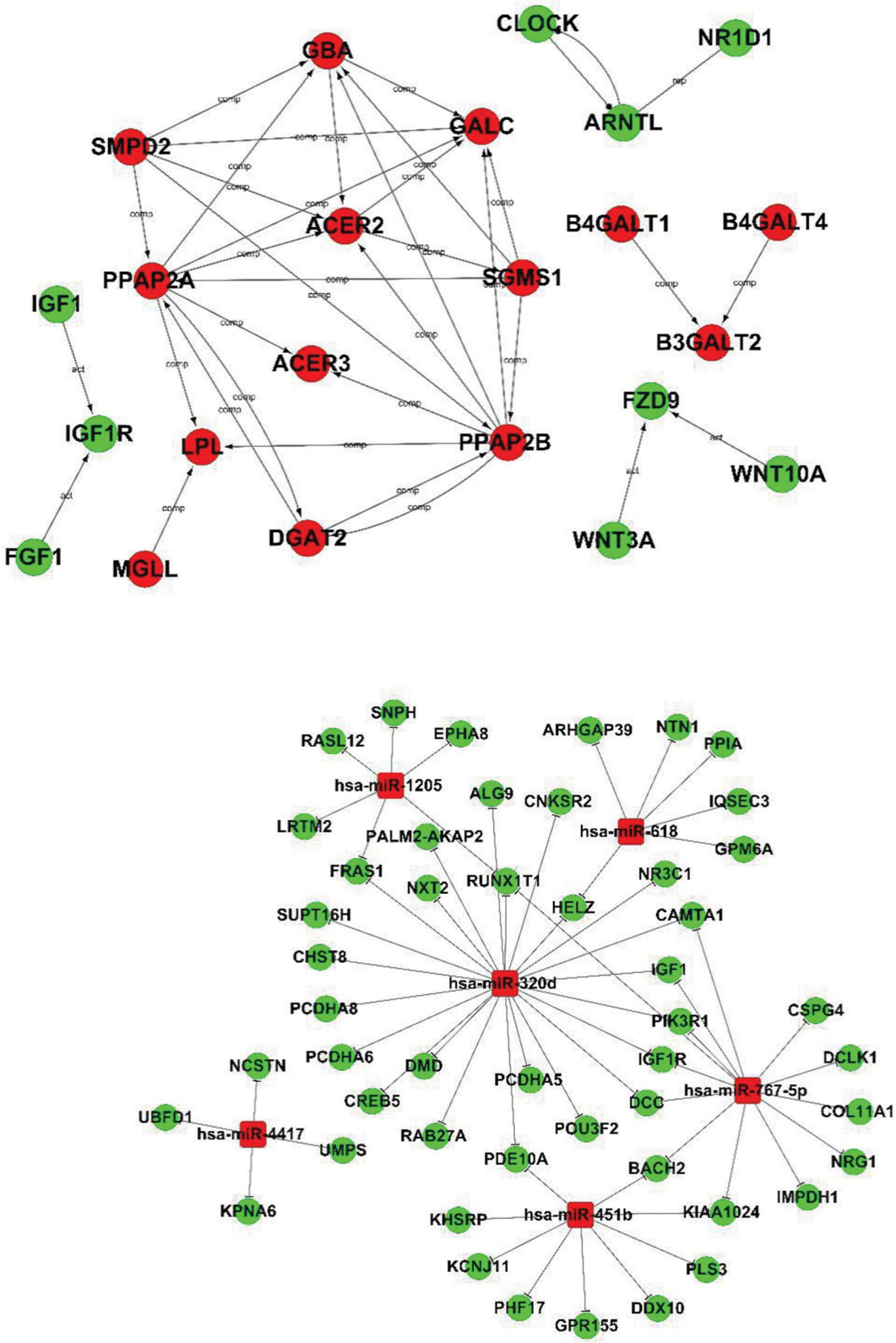

Figure 4. Signal target network diagram

onic development, cell differentiation regulation, apoptosis, cell cycle, wound healing, and the body's immune system. The diversity of a miRNA sequence structure and expression mode plays a very important role in the field of gene expression regulation.

Certain miRNAs are abnormally expressed in mouse and human placental tissues and are differentially expressed in placenta of PE patients. A growing number of studies have demonstrated partial differentially expressed miRNAs in the placenta of PE patients in cell adhesion, immunity, signaling, and cell cycle cardiovascular development. They play an important role in the pathways that play a major role in the pathogenesis of pre-eclampsia.

Insulin-like growth factor-1 (IGF-1) is located on the short arm of chromosome 12 and contains 70 amino acids with a relative molecular mass of 7.6 kD. It is a member of the insulin-like growth factor family and has a structure similar to insulin with insulin-like metabolic effects [14]. Most of the IGF-1 in the body's circulation is derived from the liver, kidneys, thymus and fat. A small amount of IGF-1 can also be detected in tissues. Studies have found that IGF-1 can also be produced in the endometrium and placenta [15]. In human and animal blood and body fluids, IGF-1 rarely exists in a free state, mainly by binding to proteins to form IGF-1 binding protein (IGFBP) to prolong its half-life and ensure its stability in vivo.The biological effects of IGF-1 are mainly exerted by binding to the IGF-1 receptor (IGF-1R), and IGF binding proteins 1 to 6 (IGFBP1 to 6) play an important regulatory role in this process. IGF-1 has a wide range of biological functions, in addition to promoting insulin metabolism, such as gluconeogenesis and glycolysis, hypoglycemic and hypolipidemic. It also has the effects of relaxing blood vessels, promoting cell differentiation and mitosis.In recent years, its research on the mechanism of action in cell differentiation, apoptosis, proliferation and carcinogenesis has become a hot topic.

Because IGF-1 may be involved in the regulation of embryo implantation, revascularization as well as placental fetal growth and development. Abnormal expression of IGF-1 may lead to pathological pregnancy such as PE, which directly affects pregnancy outcomes [16–17]. Studies have confirmed that the incidence of PE is associated with decreased levels of IGF-1 expression.Some researchers have pointed out that the use of immunohistochemistry and radioimmunoassay found that IGF-1 levels in peripheral blood of patients with preeclampsia decreased compared with normal pregnancy While the expression level of IGFBP-1 in placenta of preeclampsia increased, presumed eclampsia Low levels of IGF-1 in the maternal blood and elevated expression of IGFBP-1 in the placenta may lead to placental dysplasia and fetal growth restriction. In addition, some researchers also compared the expression levels of IGF-1 in the pre-eclampsia and normal maternal serum, found that the serum levels of IGF-1 in pre-eclampsia were significantly lower than those in the control group. There was also a significant difference in blood IGF-1 levels between mild pre-eclampsia and severe pre-eclampsia.The more severe the condition, the lower the level of IGF-1 in the maternal serum. It was shown that the pre-eclampsia condition can be assessed by detecting the expression level of IGF-1 in maternal serum [18].

CONCLUSIONS

Expression of IGF1 and miRNA was significantly increased in the placentas of patients with preeclampsia.

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