Research on miRNAs of exosomes' role on porcine oocyte development by extracted from follicular fluid

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Research

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Abstract

Follicular development is crucial to oocyte maturation in these special surrounds, and the follicular size closely related to oocyte maturation. In order to clarify porcine oocyte maturation molecular mechanism, obtained exosomes miRNAs from porcine follicular fluid (PFF) are sequenced and researched among different follicular size as described in the methods.

The results firstly show that PFF exosomes can be successfully isolated, and almost all valid reads of sequencing data of PFF exosomes can be successfully mapped to the porcine genome database. Using hierarchical clustering methods, it secondly finds that significantly expressed miRNAs can also be clustered in A, B C and D groups in heatmap according to different size follicles, which possibly to target mRNAs genes related to porcine oocyte development. Thirdly, through choosing ten significantly expressed miRNAs to predict the targeting genes for further GO analysis, the results show that the expression of neurotransmitter secretion genes changes greatly, and many targeting genes involves in regulation of FSH secretion, which is very closely related to oocyte maturation in growing follicles. Further, taking Pathway analysis for these targeting genes this ten choosing miRNAs, the results show that the pathways mainly related to the biosynthesis pathway of TGF-beta signaling pathway, which is very closely related to reproductive system function.

Finally, these miRNAs in PFF EXs may provide a valuable addition about the mechanism of porcine oocyte maturation, which could be chosen as some molecular biomarkers to choose the high-quality oocytes for further porcine embryo production in vitro or transgenic animals research in the future.

Introduction

As known to us, oocyte maturation is strongly related to follicular grow and development, which the process starts with the recruitment of some small follicles and progress to preovulatory follicles stage. As follicles grow, its size is closely accompanying increase with oocyte diameter indicating oocyte maturation status. Many reported papers proved that oocyte quality is highly correlated with oocyte diameter for further reconstructed embryo development¹. Moreover, some bovine oocyte maturation added with follicular fluid from big size follicles may improve oocyte nuclear and cytoplasmic maturation results compared to small size follicles fluids². With development of follicles, porcine follicular fluid (PFF) is increasing, which contained with many materials such as hormones, proteins and RNAs, and provide a unique microenvironment for the growth and maturation of oocytes. Therefore, follicles size is a good indicator to predict oocyte quality and potential development, and it is a good way to research oocyte maturation molecular mechanism by extracting some important vesicles from PFF in different size follicles.

Exosomes (EXs) are cell-derived vesicles that are present in many and perhaps all eukaryotic fluids, including of HFF, blood, and cultured medium of cell cultures, which generally size is among 30–150 nm. Moreover, the study found that it contains a large number of biologically active molecular substances

such as proteins, mRNAs and miRNAs, which can be released from the cells and released from the plasma membrane by budding and fission to exert their biological functions³. In particular, the EXs, which are formed by the invagination of multivesicular bodies in the lumen, are then fused to the plasma membrane and released to the cells in a manner that transports the carrier outward⁴. However, the EXs are separated from the vesicular structure in the endoplasmic reticulum under normal physiological conditions of the cells, which is released through the plasma membrane through transport to exert normal physiological regulation function⁵. It can be concluded that the EXs play an important role in regulation oocyte physiological functions by transporting materials, which have target cell signaling functions by containing various RNAs⁶.

It has been reported that these EXs exists in horse⁷ and human⁸ follicular fluid under normal physiological conditions, and milk follicles with reduced milk production⁹. Further studies have found that these extracellular miRNAs can have a role in external environmental influences through these protective devices packages as EXs¹⁰. These molecules play essential roles in a wide range of physiological processes, which have a critical role in reproduction functions(follicular development and oocyte maturation)¹¹. There have been many reports of miRNAs' exiting in porcine follicular fluids⁹, but there are not yet any reports about HFF exosomes non-coding RNA transcripts. Above all, it can be predicted that EXs plays a vital role in the relationship and regulation of the associated cells in the follicular fluid during oocyte growth and development by way of contained materials, such as miRNAs. Although our knowledge regarding oocyte quality and development has improved significantly, the molecular mechanisms that regulate and determine oocyte developmental competence are still unclear. Therefore, it is necessary to carry out these experiments to compare the PFF exosomes non-coding RNA transcripts expression among different size follicles.

In a word, this study substantially revises our understanding of the content of porcine follicular fluid exosomes in influence of oocyte maturation by interactions with follicular somatic cells and cumulus cells around oocytes, which lays the foundation for the future investigation of the role of PFF exosomes miRNAs in oocyte development mechanism. Therefore, the objective of this study was to identify and analyze the transcriptome profiles of PFF exosomes derived from different size follicles using RNA high-throughput sequencing technology.

Materials And Methods

Porcine Follicular fluid samples collection

Firstly, all experimental porcine ovaries were collected from a local abattoir (QinYangMuYe Limited Responsibility Company, LouDi city, HuNan Province, China) and transported to our lab in a thermo-flask with Penicillin and streptomycin within 2hr of their slaughter. Moreover, all designed experiments are allowed by the Institutional Ethics Committee of Hunan University of Humanities, Science and Technology. Ovaries were at least washed three times with saline solution. Porcine follicular fluid was collected by separately aspirating with a 23-Gauge needle attached to a 5ml syringe from small follicles

(group A and B: <3mm and 3-5mm) or a 20-Gauge needle attached to a 10ml syringe from large follicles (group C and D: 5–8mm and >8mm). The follicle diameter was measured as previously reported¹². The follicular fluid samples were centrifuged at 1300 X g for 15 minutes to remove the cells, the blood and the other material was used stored at -80°C for further experiments.

Exosomes Purification and Characterization

PFF exosomes were purified and characterized according to previously published protocols with some modifications¹³. The isolation methods were performed: a volume of 15 mL of pooled samples from each woman was centrifuged at 3,500 rpm for 15 minutes at 4 °C to pellet debris. The supernatant was firstly transferred to 15 mL ultracentrifuge tubes and ultracentrifuged at 16,500 X g for 30 minutes at 4 °C, and secondly filtrated through a 0.2 mm syringe filter to obtain the medium contained exosomes. Finally, exosomes were pelleted by ultracentrifugation at 120,000 X g for 70 minutes at 4 °C and stored at -80°C for further analysis.

Exosomes pellets were suspended in PBS for electron microscopy analysis. Firstly, the resuspended EXs sample 5-10ul is added to the copper mesh and precipitated for 3 min, and the filter paper absorbs the volatile liquid from the edge. Secondly, negative dying of phosphotungstic acid was carried out after PBS rinsing, and was dry at room temperature for imaging on the machine (electron microscopy operating voltage 80-120kv) after 2 min.

EXs Small RNA library construction

EXs pellets firstly were resuspended in Trizol (Invitrogen) for RNA isolation. The small RNA library of EXs was constructed using the total RNA from 15 mL pooled follicular fluid. Small RNA cloning, sequencing, and analysis were carried out as described previously using QIAseq® miRNA Library Kit¹⁴. Briefly, 20ng of total RNA underwent rRNA depletion and DNase digestion using a NEBNext rRNA Depletion Kit (NEB) according to the manufacturer's protocol. A nucleoMag NGS Clean-up and Size Select Kit (MACHER- EY- NAGEL) was used for sample purification and library size selection. RNA samples were purified, fragmented at 94°C for 15min, and primed with random primers. Samples were converted into double-stranded cDNA, purified, and adapters were ligated to the 3' and 5' ends. The cDNA samples were amplified by PCR (14 cycles) using indexing forward primers and a universal reverse primer. After PCR amplification, RNA libraries were purified and quality control of RNA was carried out by using an Agilent 2100 Bio-analyzer (Agilent Technologies Sweden AB).

RNA-Seq data analysis of EXs from Porcine follicular fluids

Initially, the sequencing adaptors and low-complexity reads were removed in an initial data filtering step, and the quality of reads was estimated with the FASTQC program. The alignment of the reads against the swine reference genome (Sscrofa11.1, January 2017) was downloaded from the Swine Genome Sequencing Consortium project (<http://www.ensembl.org/>) and used for all subsequent bioinformatic analyses. Finally, we applied DEBseq-counts algorithm to filter the deferentially expressed genes after

significant analysis and FDR analysis under the following criteria ($\text{Log}_2\text{FC} > 1$ or $\text{FDR} < 0.05$) to choose the significantly expressed genes for further research. Functional annotation was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (<http://david.ncifcrf.gov>). Pathway analysis was carried out using the download annotation data from KEGG (<http://www.genome.jp/kegg/>) and Cytoscape (<https://cytoscape.org/>) software.

GO and Pathway analysis

Based on the hierarchical structure of GO, the mutual control and subordinate relationships between all GO are organized into a database. By constructing a functional relationship network, it is easy to summarize the functional groups affected by the experiment and the intrinsic subordinate relationships of significant functions. Functional regulation analysis was performed using the significant GO-Term (p -value < 0.01) in GO-Analysis by differential genes to construct a functional regulatory network. In order to accurately perform functional classification of grape genes, GO Analysis assigns significantly different genes to different functional classifications. GO classification can describe the function of genes from various aspects, and GO can be divided into three main groups, Biological Process (BP), Molecular Function (MF) and Cellular Component.

Pathway-Analysis, based on gene annotation database, is a means to detect significant pathway of differential genes. Therefore, the key to Pathway-Analysis is to have a complete database and more complete Pathway annotations. The selected differential genes were annotated with Pathway based on the KEGG database, and all Pathway Term in which the differential genes and target genes participated were obtained. The Fisher test was used to calculate the significance level (P -Value) of Pathway, and the differential genes and negatively related genes were screened Set saliency Pathway Term. Significant P -Values < 0.05 are shown in a gray and white table.

Statistical analysis

Both the back-spliced junction reads and linear mapped reads were combined and scaled to reads per kilobase per million mapped reads (RPKM) to quantify the expression levels of miRNA. Differences in these miRNA expression profiles between A, B, C and D group were analyzed using Student's t -test. A $P < 0.05$ was considered to indicate statistically significant differences.

Results

Exosomes Isolation and Quality control of the RNA-Sequence data

The PFF exosomes are successfully separated and further verified according to the above methods using transmission electron microscope (JEM-1200EX) with operating voltage of electron microscope 80-120kv. Exosomes pellets were resuspended in phosphate-buffered saline (PBS) for electron microscopy analysis to further verify exosomes characterization according to the published paper¹⁵. The FastQC software (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used to perform the full evaluation

of sequenced data, including length distribution; quality score, and the GC content. The quality control of raw sequencing data aims to give a quick impression of whether these data have any problem, which they should be aware before doing any further analysis according the published method¹⁶.

miRNAs expression significant analysis and heat map analysis

RNA sequencing and analysis were carried out using 16 samples (Group A: A3, A5 and A6; Group B: B3, B5 and B6; Group C: C3, C4 and C5; Group D: D1 ,D5 and D6; three replicates for each sample in one group) prepared from collecting different size follicles according to above methods. On average, the total number of valid reads is roughly 6.50 million with group A, 4.30 million with group B, 6.90 million with group C and 9.30 million with group D obtained from the exosomes of PFF, as shown in table 1. Overall, about 99% of all valid reads are with group A and B, and about 99% of all valid reads are with group C and D from four six samples could be successfully mapped to the porcine non-coding RNAs database as indicated in table 1 for further research.

These different expressed miRNAs are further analyzed using hierarchical clustering methods to form the heatmap between the group B compared to group A (small size follicles: Control group) as shown in Figure 1A and choosing significantly different expressed miRNAs(miR-10a-5p, miR-200b, miR-429,miR-192, miR-141, miR-221-3p, miR-425-5p, miR-7-5p and miR-92a) of the group A compared to the control for further research as shown in Figure 1B. Secondly, These different expressed miRNAs are further analyzed using hierarchical clustering methods to form the heatmap between the group C compared to group A (the Control group) as shown in Figure 2A and choosing significantly different expressed miRNAs (miR-194a-5p, miR-7137-3p,miR-182, miR-146b, miR-4332 and miR-9793-5p) of the group A compared to the control for further research as shown in Figure 2B. Thirdly, these different expressed miRNAs are further analyzed using hierarchical clustering methods to form the heatmap between the group D compared to group A (the Control group) as shown in Figure 3A and choosing significantly different expressed miRNAs(miR-21-5p, miR-26b-5p, miR-26a, miR-146a-5p, miR-202-5p, miR-29c, miR-24-3p, miR-199a-5p and miR-10b) of the group A compared to the control for further research as shown in Figure 3B.

Northerly, it is carried out that these different expressed miRNAs are further analyzed using hierarchical clustering methods to form the heatmap between the group B compared to group C as shown in Figure 4A and choosing significantly different expressed miRNAs(miRNA-10a-5p, miRNA-200b, miRNA-95, miRNA-141, miRNA-92a, miRNA-221-3p, miRNA-7-5p, miRNA-192, miRNA-20a-5p and let-7d-5p) of the group B compared to the group C for further research as shown in Figure 4B. And it is also carried out that these different expressed miRNAs are further analyzed using hierarchical clustering methods to form the heatmap between the group B compared to group D as shown in Figure 5A and choosing significantly different expressed miRNAs (miR-200b, miR-10a-5p, miR-141, miR-221-3p, miR-20a-5p and miR-92a)of the group B compared to the group D for further research as shown in Figure 5B. Finally, it is carried out that these different expressed miRNAs are further analyzed using hierarchical clustering methods to form the heatmap between the group C compared to group D as shown in Figure 6A and choosing significantly different expressed miRNAs(miR-335, miR-497, miR-19b, miR-130a, miR-29a-3p, miR-676-3p, miR-128,

miR-125b, miR-99a-5p and miR-202-5p) of the group C compared to the group D for further research as shown in Figure 6B.

GO and Pathway analysis of miRNA target genes

Mature miRNAs are produced by a series of cleavage processes of nucleases of longer primary transcripts, which are then assembled into RNA-induced silencing complexes to identify target genes by base complementary pairing, and to guide the silencing complexes to degrade target genes or block the translation of target genes according to the degree of complementarity.

Based on the above research results, these predicted ten relevant miRNAs among these four groups (miR-125b, let-7d-5p, miR-200b, miR-26a, miR-92a, miR-221-3p, miR-21-5p, miR-141, miR-99a-5p and miR-10a-5p as shown in Figure 1-6) related to porcine oocyte maturation are chosen to take Miranda and RNA software to predict the targeting regulation relationship between the whole species of miRNAs (taking the intersection results of the two prediction software as the final target gene prediction results). The miR-125b, let-7d-5p and miR-200b were among the top 10 highly expressed miRNAs in four groups, which it is proved that it has potential role of miRNAs in regulating the oocyte development^{17,18}. The parameters of this analysis are set as follows: energy_miranda<-20, score_miranda>150, energy_RNAhybird <-25. The results show 67 significantly different target genes hybrid between the Miranda and RNA software, which are used to take GO further, as shown in Figure 7 and Pathway analysis, as shown in Figure 8.

The result also shows that the gene number and significance about neurotransmitter secretion changes greatly in the figure 9 as the GO enrichment scatter plot. The result also shows that the rich factor value of the regulation of follicle-stimulating hormone (FSH) secretion is almost 1 in figure 9, which indicates the functions of many targeting gene by these miRNAs mainly focus on oocyte development by FSH stimulating porcine follicular growth. In order to clarify the interaction of these targeting mRNAs and miRNAs genes (miR-125b, let-7d-5p, miR-200b, miR-26a, miR-92a and miR-221-3p), the map is shown in Figure 10, which these founding genes are closely related to reproductive system function.

Discussion

As known to us, follicular follicle in the ovary provides a unique microenvironment for oocyte development, such as interactions between follicular somatic cells and oocytes, and it is crucial to study the components of follicular fluid to elucidate the mechanism of oocyte maturation. It has been found that exosomes (EXs) are an essential carrier for signals transduction of interaction between follicular somatic cell and oocyte in follicular fluid media according to some reports^{19,20}. In this paper, HFF exosomes can be successfully isolated with an average size of 75 nm, and checked by using transmission electron microscopy, which is similar to these reports²¹⁻²³.

For understanding the molecular mechanism of oocyte maturation, it is crucial to reveal the axis of follicular somatic cells-EXs-oocyte by study the expressed genes in the PFF EXs on the RNA level. It was also reported that identified potential miRNA targets using bioinformatics analysis, which is very

important to porcine oocyte growth in follicles^{24,25}. Especially, follicle size is closely related to oocyte development according to previous reports^{26,27}, and the miRNA sequencing experiment using the total 12 PFF exosomes samples(3 repeats each group with different size follicle sample) was carried out according to above methods. The results firstly show that about 99% of all valid reads are with group A and B, and about 99% of all valid reads are with group C and D from four six samples could be successfully mapped to the porcine non-coding RNAs database as indicated in table 1 for further analysis. Secondly, these 8 miRNA genes of PFF EXs (miR-10a-5p, miR-200b, miR-429,miR-192, miR-141, miR-221-3p, miR-425-5p, miR-7-5p and miR-92a) expression is significantly enhanced with porcine follicle growth from < 3 mm to 5 mm, and miR-10a-5p expression is almost 8 times higher than group A sample as shown in Fig. 1. It is also found that miR-10a family expression is closely related to reproductive system function, which miR-10a and miR-10b, are expressed at basal levels in GCs but are highly expressed in theca and stroma cells within the ovary, and they could repress proliferation and induce apoptosis in human, mouse and rat granulosa cells, at least partly through repressing BDNF by directly binding to its 3' UTR, and the miR-10 family and the TGF- β pathway form a negative feedback loop in GC^{s28}. With porcine follicle further growth to 5–8 mm, these 6 miRNA genes of PFF EXs (miR-194a-5p, miR-7137-3p,miR-182, miR-146b, miR-4332 and miR-9793-5p) expression is significantly enhanced compared to control group(< 3 mm), and miR-194a-5p expression is almost 4 times higher than group A sample as shown in Fig. 2. Exosomes is carriers to follicles from surrounding somatic cells body blood to influence oocyte maturation, which immune stimulus could enhance vasodilatation in order to promote EXs function^{29,30}. It is also indicated that miR-194a family expression play important roles in immunology response and function, such as flounder immune response³¹ and zebrafish³².

Until follicle grow to 8 mm, these 9 miRNA genes of PFF EXs (miR-202-5p, miR-199a-5p, miR-21-5p, miR-26b-5p, miR-26a, miR-29c, miR-24-3p, miR-146a-5p, and miR-10b) expression is significantly reduced compared to control group(< 3 mm), and miR-46a-5p and miR-202a-5p gene expression is almost 3 times lower than group A sample as shown in Fig. 3. It is proved that miR-202a-5p gene is greatly expressed in bovine un-maturated oocytes³³, and miR-199a-5p gene is greatly expressed in GV stage occyte³⁴. Among these group B, C and D, It is found that these different expressed miRNAs are choose and all clustered in Figs. 4, 5 and 6 for further analysis, which is indicates some are closely related to oocyte development proved by those published papers^{28,35,36}. Based on the above miRNAs significant expression analysis, ten relevant miRNAs (miR-10a-5 ps, miR-200b, miR-141, miR-92a, miR-221-3p, miR-21-5p, miR-26a, let-7d-5p, miR-125b and miR-99a-5p) related to porcine oocyte development are choose to take for further target genes analysis. Currently, there is no gold standard for miRNA gene target analysis^{37,38}, we use Miranda and RNA software to predict the targeting regulation for further GO analysis and pathways according to reported papers^{39,40}.

Those found miRNAs(miR-125b, let-7d-5p,miR-200b, miR-26a and miR-92a) expressed in PFF exosomes from different size follicles, which is very closely related to oocyte development, which is reported by previous published papers^{17,41}. The gene number and significance about neurotransmitter secretion changes greatly as shown in Fig. 7–8, which the life activity of the hypothalamus-pituitary-ovarian

endocrine axis is very vigorous during porcine follicular growth process⁴². The result also show that many targeting genes involves in regulation of FSH secretion in Fig. 7–8, which is proved by many reports about FSH controlling the follicular development in different size follicles^{43,44}. Therefore, it can be indicated that the functions of many targeting gene by these miRNAs mainly focus on oocyte development by FSH simulating porcine follicular growth form small stage (Group A), middle stage (Group B) to matured stage (Group C and D). The results show that the pathways mainly related to the biosynthesis pathway of TGF-beta signaling pathway, Primary bile acid biosynthesis, Nicotinate and nicotinamides metabolism as shown in Fig. 9, which these potential target genes are closely related to the oocyte development and follicle growth. It was also found that many targeting genes by these miRNAs mainly involved in TGF-beta related signaling pathway, which may play a significant role during early stages of porcine oocytes nuclear and cytoplasmic maturation, and these investigated transcripts may be also recommended as the markers of porcine oocytes with high capability in further embryo development^{45,46}.

However, these potential genes GO and pathway are predicted; the associations observed in previous published papers are limited. Some research papers also show that each miRNA can have many gene targets and the gene targets may be different based on cell type⁴⁷, and the exact influence of a miRNA on gene expression may be physiologically indispensable but difficult to identify statistically⁴⁸. In conclusion, this study provides new insights into the global transcriptome changes and the abundance of specific transcripts in porcine oocytes in correlation with follicle size.

Therefore,our study has shown that differently expressed miRNAs of PFF EXs in the different size follicles with group B, C and D compared to the control (group A), which has different targeting cluster genes(miR-125b, let-7d-5p,miR-200b, miR-26a and miR-92a) in porcine oocyte maturation according to the GO and Pathway analysis, which could be used as biomarkers for understanding oocyte maturation process and choosing oocyte with high quality for further research. Moreover, there are still some questions about porcine oocyte development mechanism related to MiRNA in EXs as following. What is the relation of these different expressed miRNAs contained by exosomes and oocyte maturation in details? Which type's miRNAs can genuinely promote follicle growth and oocyte maturation and how to control the network about oocyte cytoplasmic and nuclear maturation mechanisms?

Conclusions

In a word, it firstly shows that these miRNAs of PFF exosomes of different groups (according to different size follicles) are significantly different expression from the control group (small size follicles), and some critical miRNAs genes(miR-125b, let-7d-5p,miR-200b, miR-26a and miR-92a) are clarified as potential biomarkers for molecular measure for oocyte quality and oocyte maturation molecular mechanism in the future. Nevertheless, the field of miRNAs and porcine oocyte maturation is still a long way to figure out the molecular mechanism.

Declarations

Abbreviations

EXs: Exosomes; PFF: Porcine Follicular Fluid; GC: Granulosa cell; miRNAs: MicroRNAs; TGF-beta: Transforming Growth Factor Beta ; FSH: Follicle-Stimulating Hormone

Ethics approval and consent to participate

All designed experiments are allowed by the Institutional Ethics Committee of Hunan University of Humanities, Science and Technology.

Consent for publication

The manuscript has been reviewed and approved by all authors and represents original materials that have not been published elsewhere and are not under consideration elsewhere.

Availability of supporting data

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Junhe Hu, Jinyi Dong and Zhi Zeng contributed to the initiation and discussion of the manuscript. Juan Wu, Xiansheng and Tao Tang contributed to obtain some experimental samples. Jiao Yan and Chenzhong Jin prepare for the data analysis. All authors read and approved the final manuscript.

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Table

Due to technical limitations, the table file is only available for download from the Supplementary Files section.

Figures

Figure 1

Analysis of most different expressed miRNAs of between group A and B A Heatmap of clustered genes between group A and B. B most significantly up expressed miRNA genes compare group B to A; Y-axis means log2(Fold Changes, FC) of miRNAs expression level of group B VS A.

Figure 2

Analysis of most different expressed miRNAs of between group A and C A Heatmap of clustered genes between group A and C. B most significantly expressed miRNA genes between group A and C; Y-axis means log2(Fold Changes, FC) of miRNAs expression level of group C VS A.

Figure 3

Analysis of most different expressed miRNAs of between group A and D A. Heatmap of clustered genes between group A and D. B. most significantly expressed miRNA genes between group A and D; Y-axis means log2(Fold Changes, FC) of miRNAs expression level of group D VS A.

Figure 4

Analysis of most different expressed miRNAs between group B and C A Heatmap of clustered genes between group B and C. B most significantly expressed miRNA genes between group B and C; Y-axis means log2(Fold Changes, FC) of miRNAs expression level of group CVS B.

Figure 5

Analysis of most different expressed miRNAs of group B and D A Heatmap of clustered genes between group B and D; B most significantly expressed miRNA genes between group B and D; Y-axis means log2(Fold Changes, FC) of miRNAs expression level of group D VS B.

Figure 6

Analysis of most different expressed miRNAs between group C and D A Heatmap of clustered genes between group C and D; B most significantly expressed miRNA genes between group C and D; Y-axis means log2(Fold Changes, FC) of miRNAs expression level of group D VS C.

Figure 7

GO analysis of main different expressed miRNAs among four groups GO analysis show results of all differential genes, which includes the leftmost Biological Process (BP), the middle Molecular Function (MF) and the rightmost Cellular Component (CC) in this figure. The coordinate axis Y axis is Go-Term entry name, and the Coordinate axis X axis is log10 (P-Value). The red color represents significant items, and the blue color represents non-significant items.

Figure 8

The 10 miRNAs selected from different experimental groups were compared for functional analysis of target genes, involving metabolic pathway functional analysis. The purpose of this picture is to show the first 20 entries in Pathway-Term prominently. The meaning of axis Y axis is the value of Enrichment, and X axis is Pathway-Term entry name. Red represents significant items and blue indicates non-significant items.

Figure 9

GO enrichment scatter plot of choosing 10 miRNAs targeting gene for functional analysis The scatter plot is a graphical display of GO enrichment analysis results. The degree of GO enrichment in the figure is measured by Rich factor, P_value, and the number of genes enriched on this pathway. The Rich factor refers to the ratio of the number of differential genes enriched in the GO to the number of annotated genes. The larger the Rich factor indicates the greater the degree of enrichment.

Figure 10

Functional map of target gene interaction among 7 miRNAs from experimental groups The seven different miRNA (green color) and their targeting mRNAs (gray color) are sorted by color in this image.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.doc](#)