Identification of Potential miRNA-mRNA Regulatory Network in Tetralogy of Fallot: A Network Biology Approach

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Abstract

Background

Tetralogy of Fallot (ToF) is one of the most prevalent and fatal birth defects. Its pathogenesis remains unknown and it's not easily diagnosed at early stage. Therefore, it's necessary to explore the critical regulators and molecular mechanism in ToF to find out novel molecular markers for early diagnosis.

Methods

Three ToF datasets (GSE35490, GSE40128, GSE36761) were downloaded from the GEO database. The differentially expressed microRNAs (DEMs) and mRNAs (DEGs) were identified between ToF infants and normal infants after data preprocessing, followed by GO and KEGG analysis of DEGs. Then, PPI network and modular analysis were performed to identify the hub genes. Ultimately, potential miRNA-mRNA pathways in ToF were constructed based on the integrated data.

Results

22 overlapping DEMs were found in the GES35490 and GSE40128. Simultaneous, 181 intersected genes were found and identified as the significant DEGs including 84 down-regulated DEGs and 97 up-regulated DEGs. Further, 20 hub genes ranked by top 10% with high connectivity, including STAT3, FBN1, RUNX2, were found by PPT network and modular analysis. Furthermore, GO and KEGG analysis showed these DEGs were linked to cell cycle, apoptotic process, transcription activity and FOX signaling pathway. Additionally, we establish three potential miRNA-mRNA pathway, including miR-22-STAT3, miR-336/miR486-FBN1, miR-222-RUNX2, which can participate in the formation of ToF by cell cycle and transcription activity.

Conclusions

Three miRNA-mRNA pathways were established and indicated cell cycle and transcription activity may be involved in the pathogenesis of ToF. Our study provided a novel bio-marker for early diagnosis of ToF.

Background

Tetralogy of Fallot (ToF), a cyanotic congenital heart disease (CHD), is one of the most prevalent and fatal birth defects. ToF comprise overriding aorta, ventricular septal defect, right ventricular hypertrophy and right ventricular outflow tract obstruction, with an occurrence of 1 in 3600 births and accounting 8-11% in infants suffering with CHD [1]. With the development of Surgical procedure, infants with ToF avail excellent long-term survival, however, early intervention is the key to reduce complications and improve prognosis [2]. Currently, the prenatal diagnosis of ToF mainly relies on echocardiography. Since the accuracy of echocardiography is associated with experience of examiners, fetal position and instrument quality, additional technologies are urgently needed to improve the detection rate of TOF. In recent years,

transcriptomics has been used to explore the dynamic changes of proteins and RNAs in embryonic development and diseases, expecting to be novel biomarkers for the diagnosis of ToF [3].

MicroRNAs (miRNAs), containing 19-24 nucleotides, are a class of single-strand non-coding RNAs that play a critical role in formation of the circulation system and organ development [4]. since the regulation of miRNAs in the epigenetic inheritance of mRNA expression, it has become a target of research the development of CHD. Liu et al. [5] showed miR-133a-1 and miR-133a-2 double-knockout will lead to Ventricular septal defect and chamber dilatation in mice with the abnormal expression of CCND2 and SPF. Li D et al. [6] found out 36 differentially expressed microRNAs (DEMs) between infants with VSD and normal infants and verified the target genes of DEMs were related with cardiac right ventricle morphogenesis. Based on these findings, we hypothesized that miRNAs act as regulators that regulate the expression of specific genes during fetal heart development.

To explore the critical regulators and molecular mechanism in ToF, we downloaded three ToF datasets (GSE35490, GSE40128, GSE36761) from the GEO database. We successively performed differential expression of miRNAs and mRNAs, Target prediction, GO annotation, KEGG enrichment, PPI network and construction of potential miRNA-mRNA pathways.

Methods

Microarray data

MiRNAs and mRNAs expression profile of ToF patients were obtained from the Gene Express Omnibus (GEO) website(https://www.ncbi.nlm.nih.gov/geo/). In the course of seeking datasets, the following common terminology were used: Tetralogy of Fallot, series, homo sapiens. Consequently, a total of 23 series were found. Among them, the GSE35490, GSE40128, GSE36761 datasets were selected because their specimens were derived from myocardium. The GSE35490 and GSE40128 were miRNAs expression profile which based on platform GPL8786 Affymetrix Multispecies miRNA-1 Array. While, the GSE36761 was mRNAs expression profile which based on platform GPL9052 Illumina Genome Analyzer (Homo sapiens). The GSE35490, GSE40128 and GSE36761 including 16 ToF infants and 8 normal infants, 5 ToF infants and 3 normal infants, 22 ToF infants and 4 normal infants, respectively.

Identify differentially expressed miRNAs and mRNAs

For differentially expressed miRNAs (DEMs) and differentially expressed mRNAs (DEGs) analysis between ToF and control groups. Several appropriate R software packages were used including Affy, Lumi, GEOquery, Bioconductor, Limma and VennDiagram [7,8]. The raw microarray data (CEL files) were converted into expression value and normalized using the Robust Multichip Averaging (RMA). The array of Illumina gene expression datasets was normalized using the quantile method. The paired T-test based on the R-based limma software package was used to analyzed DEMs and DEGs between ToF infants and normal infants. $|log2FC|>1$ and Adjusted P value (Adj.P value) <0.05 were considered as critical values for DEMs and DEGs.

Target prediction of DEMs and identify significant DEGs

First, the overlapping DEMs in the GES35490 and GSE40128 were identified by VennDiagram. Then, target genes of overlapping DEMs were performed by using the database of miRTarbase (v8.0), miRDB and Targetscan. Last, we extracted the intersection gene of target genes and DEGs as the significant DEGs.

Gene annotation and enrichment analysis of significant DEGs

DAVID, a visualized, annotated and integrated discovery online database, was used to analysis gene ontology (GO) annotation and KEGG pathway of significant DEGs. GO annotation includes biological process (BP), cellular component (CC), molecular function (MF). P-value <0.05 was considered as statistically significant.

PPI network construction, modular analysis and identification of hub genes

STRING is a database of predicted protein-protein interactions which include physical(direct) and functional(indirect) associations. In our study, STRING database was used to construct the PPI network of significant DEGs. All PPI networks were selected which the combined score was more than 0.4. In addition, Cytoscape, a software of biological pathways and visualizing molecular interaction network, was used to visualize the PPI network. The MCODE plugin of Cytoscape was used to identify the central module of significant DEGs. While, the CYTOHUBBA plugin of Cytoscape was used to calculate the importance ranking of significant DEGs. The top 10% ranked genes were defined as hub genes.

Identification and prediction of the targeted miRNAs of hub genes

To study the relationship between miRNAs and hub genes in ToF, three databases (miRmap, miRanda, TargetScan) were used to predict the targeted miRNAs of hub genes. Further, intersection miRNAs were extracted in targeted miRNAs and overlapping DEMs.

Results

Identification of DEMs and DEGs in ToF

To identify the DEMs, miRNA expression profiles (GSE35490 and GSE40128) were filtered by affy and limma software packages of R. 81 DEMs were obtained in GSE35490 including 77 down-regulated DEMs and 4 up-regulated DEMs. While, 248 DEMs were obtained in GSE40128 including 82 down-regulated DEMs and 166 up-regulated DEMs. To identify the DEGs, we performed normalization and background correction of mRNA expression profile (GSE36761), then 1576 DEGs were obtained including 652 down-regulated DEGs and 924 up-regulated DEGs(Table S1). The heatmap and volcano of DEMs and DEGs in those three datasets were shown in Fig.1, respectively.

Target prediction of overlapping DEMs and Identify significant DEGs

Based on the criteria, a total of 22 overlapping DEMs were found in the GES35490 and GSE40128 (Fig.2a). To understand the functions of overlapping DEMs in ToF, the database of miRTarbase (v8.0), miRDB and Targetscan were used to calculate the target genes. 5557, 6908 and 15424 target genes were detected in these three databases, respectively. The VennDiagram was used to identify the intersected genes between target genes of overlapping DEMs and DEGs. Then, 181 intersected genes were found and identified as the significant DEGs including 84 down-regulated DEGs and 97 up-regulated DEGs (Table.1) (Fig.2b).

GO annotation and KEGG pathway analysis of significant DEGs

To identify the function of DEMs and DEGs in ToF, DAVID were used to analyze the GO annotation and KEGG pathway of significant DEGs. Biological meaning and systematic features of significant DEGs were fist annotated by GO ontology. The enriched BP, CC and MF terms were showed in Fig.3, respectively. BP analysis indicated the significant DEGs were enriched in cell cycle, cell proliferation, apoptotic process, cell differentiation and movement of cell. CC analysis enriched in cell surface, proteinaceous extracellular matrix, basement membrane, extracellular region and integral component of plasma membrane. MF analysis enriched in transcription factor activity, transcription regulatory region, antiporter activity, cAMP binding and core promoter binding. In addition, the KEGG pathway was used to analyze the wiring diagrams of molecular interactions, reactions and relations in significant DEGs. Five enriched pathways were found including miRNAs in cancer, small cell lung cancer, type II diabetes mellitus, pancreatic cancer. Unfortunately, in the KEGG analysis, the enriched pathways were mainly related to cancer and diabetes, but not to CHD.

PPI network construction, modular analysis and identification of hub gene

To analyze the interactions of significant DEGs, PPI network of 181 significant DEGs was constructed by STRING database. As shown in Fig.4, a total of 113 nodes and 145 combined edges were found. To identify the ranking of significant DEGs, we used the CYTOHUBBA plugin of Cytoscape to calculate the “degree” of significant DEGs. Twenty genes ranked by top 10% genes with high connectivity were defined as hub genes for ToF (table.2). Further GO and KEGG analysis of 20 hub genes were performed. The result of GO annotation showed that hub genes were enriched in cell cycle regulation and apoptotic process regulation in biological process, cell surface and plasma membrane in cellular component, protein binding and transcription factor activity in molecular function. The KEGG analysis showed hub genes were enriched in Fox0 signaling pathway and Jak-STAT signaling pathway (Fig.5). The MCODE plugin of Cytoscape was used to identify the central module of PPI network. 4 central modules and 33 MCODE-related genes were identified which contained the 13 hub genes (Fig.6). GO and KEGG showed these modules were significant enriched in regulation of cell cycle and transcription factor activity.

MiRNA-mRNA network construct

Based on the above analysis, we believed that 13 hub genes play an important role in the development of ToF. By matching miRNA-mRNA pair in three databases and taking intersection with DEMs of GES35490

and GSE40128, we establish three potential miRNA-mRNA network including miR-22-STAT3, miR-336/miR486-FBN1, miR-222-RUNX2.

Discussion

ToF is a common and lethal birth defects. The survival rate and prognosis of infant with ToF depend on timing of intervention [9]. while, the detection rate of infant with ToF has greatly reduced because of fetal position, complexity of cardiac structure and fat layer thickness in pregnant women. therefore, it is necessary to find out more effective and specific bio-markers in infant with ToF. With the rapidly development of bio-informatics, Transcriptomics has become an important method of exploring early embryonic development and diagnosing congenital diseases [10]. This study aims to identify the DEMs and DEGs between infant with ToF and normal infant by analyzing three datasets (GSE35490, GSE40128, GSE36761), to further provide bio-markers for the diagnosis of ToF and explore its pathogenesis.

To decrease the false positive and false negative rates, we used multiple cohorts to analyze DEMs and DEGs. Then, the intersection genes were adopted for the next analysis. First, 22 intersection DEMs were identified between GSE35490 and GSE40128. Second, 181 overlapping genes were identified between target genes of 22 DEMs and DEGs of GSE36761. Last, to study the role of 181 overlapping genes in ToF, GO annotation, KEGG pathway and PPI network were performed on these genes.

GO annotation showed that the expression of 181 overlapping DEGs were mainly related to BP including cell cycle, cell proliferation, apoptotic process, cell differentiation and movement of cell. As we known, the cell cycle plays a crucial role in regulating gene expression during cardiac development. abnormal cell cycle is a major source of transcriptional variation which can cause abnormal cell proliferation [11]. the balance of cell proliferation and apoptotic is a key step in the development of heart [12]. an increase in apoptotic cells and decrease in myocardial cells will lead to heart defects in all stages of embryogenesis [13]. this is consistent with our results. In CC analysis, it mainly enriched on cell surface, proteinaceous extracellular matrix, basement membrane and extracellular region. Kota P et al. [14] pointed out that cell surface molecules can dynamically coordinate cell motility, and this process contributes to various biological processes in embryonic development. In addition, Li Y et al. [15] pointed out a network of multiple interacting proteins on cell surface may be responsible for the development of CHD. In MF analysis, it mainly involves transcription factor activity and protein dimerization activity. Studies have shown that transcription factors play the role of specificity and precise quantitative outputs in a developing heart. The interactions of heterotypic transcription factors will cause congenital heart defects [16]. Gong J et al. [17] pointed out transcription factor contributed to the formation of CHD by regulating DNA methylation, which supports our findings. Unfortunately, in the KEGG analysis, the enriched pathways were mainly related to cancer and diabetes, but not to CHD. Therefore, we further identified hub genes by using PPI network and Cytoscape. Then, GO annotation and KEGG of hub genes were performed.

A PPI network was constructed for the 181 overlapping DEGs, and 145 combined edges were found. The core genes include STAT3, FBN1, RUX2, PIK3R1, IL6R, BCL6, IL1R1, EPHA2, SOCS2, EGR1. Most of them have been confirmed to be associated with the development of CHD [18,19]. According to “degree” algorithm in Cytoscape, we identified 20 hub genes in network and the highest degree gene is STAT3. the GO annotation showed that the hub genes were mainly enriched in cell cycle regulation and apoptotic process regulation. This is compatible with our previous study. While, the KEGG analysis of hub genes were mainly enriched in FoxO signaling pathway and JAK-STAT signaling pathway. both of them are associated with the development of CHD. FoxO, a transcription factor, mediates cell cycle arrest at the post-translational level by up-regulating cell cycle inhibitors [20]. some studies showed that FoxO are significant components of signaling pathways that instruct cardiac development and angiogenesis. Deletion of FoxO will lead to a series of severe cardiac defects and embryonic death [21,22]. Ripoll C et al. [23] found out a specific enrichment in JAK-STAT signaling pathway among VSD+ASD patient. All of the above studies confirmed that these two pathways are associated with the development of CHD.

Through GO, KEGG and PPI, multiple pathways and functional enrichment had found in infants with ToF. Therefore, these genes or related pathways may be associated with cardiogenesis and cardiac malformations. MiRTrabase, miRDB and Targetscan predicated that hub genes would target miRNAs, and only miR-22-STAT3, miR-336/miR486-FBN1, miR-222-RUNX2 remained after intersection with DEMs of GSE35490 and GSE40128. Therefore, we inferred that miR-22-STAT3, miR-336/miR486-FBN1, miR-222-RUNX2 play an important role in the development of ToF and could be used as a bio-marker for the diagnosis of ToF. The PPI network showed that STAT3 was the most central and the highest scoring gene. STAT3, a key signaling protein, was often regarded as a bio-marker to study the occurrence and development of cancer [24]. In recent years, some studies showed activation of the STAT3 protects cardiomyocytes from injury by decreasing apoptosis and increasing cell viability [25,26]. in the present study, the expression of STAT3 in infants with ToF was down-regulated. This suggests that STAT3 can participate in the formation of ToF by regulating apoptosis and cellular proliferation in the fetal heart. There have no reports about the relationship between miR-21 and CHD. While, some studied pointed out that the expression of miR-21 was significantly inhibited by the overexpression of STST3 [27,28]. Che X et al. [29] pointed out FBN1 directly binds to FBXO2 can inactivating the cell cycle by polyubiquitination. MIR-486 can regulate cell cycle and DNA repair by participating in controlling G1/S transition after DNA damage [30]. miR-222 and RUNX2 were certificated to play an important role in DNA damage and cell cycle response [31,32]. With the further deepening of research, we believe miR-22-STAT3, miR-336/miR486-FBN1, miR-222-RUNX2 are expected to be potential markers for early detection and diagnosis of CHD.

Although we conducted a comprehensive analysis of the DEMs and DEGs between infants with ToF and normal infants and successfully identify several GO annotations and KEGG pathway which may be linked to development of ToF, several limitations should be discussed in this study: (1) The identification of DEMs and DEGs was performed on public databases rather than clinical samples. (2) The multilayers of assumption and the lack of validation in vitro and in vivo. Therefore, the improvement of clinical samples and corresponding experiments needs to be performed in the future and emergency initiation is required.

Conclusions

In summary, our present analysis indicated several DEMs and DEGs between infants with ToF and normal infants, further revealed DEMs and DEGs contributing to the development of ToF by cell cycle apoptotic process, transcription activity and FOX signaling pathway. Last, the mechanism of miR-22-STAT3, miR-336/miR486-FBN1 and miR-222-RUNX2 pathway in the development of CHD was presented. We hope that there information we found will contribute to in-depth studies in the future and provide novel bio-marker for the early diagnosis of ToF.

Abbreviations

ToF: Tetralogy of Fallot; CHD : congenital heart disease; miRNAs: MicroRNAs; GEO: Gene Express Omnibus; DEMs: differentially expressed miRNAs; DEGs: differentially expressed mRNAs; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; PPIs: Protein-protein interactions; STRING: Search tool for the retrieval of interacting genes

Declarations

Ethics approval

This research study was conducted retrospectively from data obtained for clinical purposes. We consulted extensively with the IRB of The Second Affiliated Hospital of Fujian Medical University who determined that our study did not need ethical approval. An IRB official waiver of ethical approval was granted from the IRB of The Second Affiliated Hospital of Fujian Medical University.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated for this study can be found in the Gene Expression Omnibus (GEO): GSE35490, GSE40128, GSE36761. The data underlying this article are available in the article and in its online supplementary material.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HW was agreed to be accountable for all aspects of the work and drafted the manuscript. SH and XL revised the manuscript critically for important intellectual content. BD made substantial contributions to analysis and interpretation of data. YY and HY made substantial contributions to acquisition of data; GL given final approval of the version to be published. All authors read and approved the final manuscript.

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Code Availability All code used during the study are available from the corresponding author by request.

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Tables

Table.1 Top 10 up-regulated and down-regulated significant DEGs

Up-regulated DEGs	logFC	t	adj.P.Val
TNNI1	5.659459614	14.26863195	6.49E-10
SFRP1	3.892687898	8.368973533	3.52E-06
ZNF711	3.148646484	6.06496047	2.54E-04
ZNF385B	2.865064731	3.711266907	2.03E-02
IGF2BP1	2.697183985	3.624347084	2.33E-02
F3	2.625453734	9.066957248	1.12E-06
NPNT	2.590512503	5.674580002	5.51E-04
FREM2	2.518437909	4.139458589	9.09E-03
ABCG2	2.23633956	7.830112172	1.00E-05
PER3	2.056835229	5.710368901	5.12E-04
Down-regulated DEGs	logFC	t	adj.P.Val
GRIN2A	-4.568671926	-8.369791712	3.52E-06
ITGBL1	-4.414805245	-5.096151082	1.54E-03
KCNK1	-4.144716917	-7.235485064	2.91E-05
ADAMTS4	-3.915452189	-4.622334467	3.75E-03
NGFR	-3.028742552	-4.865585748	2.40E-0
NPTX2	-3.020167078	-6.208318421	1.94E-04
RGMA	-2.912814665	-3.649715917	2.25E-02
SLC7A5	-2.873814089	-4.780073184	2.80E-03
RNF157	-2.818407983	-5.502282317	7.91E-04

Table.2 Top20 significant DEGs by degree score ranking

Rank	Name	Score	Regulation
1	STAT3	19	Down
2	PIK3R1	10	Up
3	BCL6	8	Down
3	EGR1	8	Up
5	FURIN	7	Down
6	BCL2L11	6	Up
6	FPR2	6	Down
8	RUNX2	5	Up
8	ADIPOQ	5	Down
8	ARNTL	5	Down
8	NGFR	5	Down
8	GADD45A	5	Up
8	SOCS2	5	Up
8	GRIN2A	5	Down
8	GRIA1	5	Up
16	ABCG2	4	Up
16	ADAMTS4	4	Down
16	FBN1	4	Down
16	PRDM1	4	Up
16	KCNQ3	4	Up

Figures

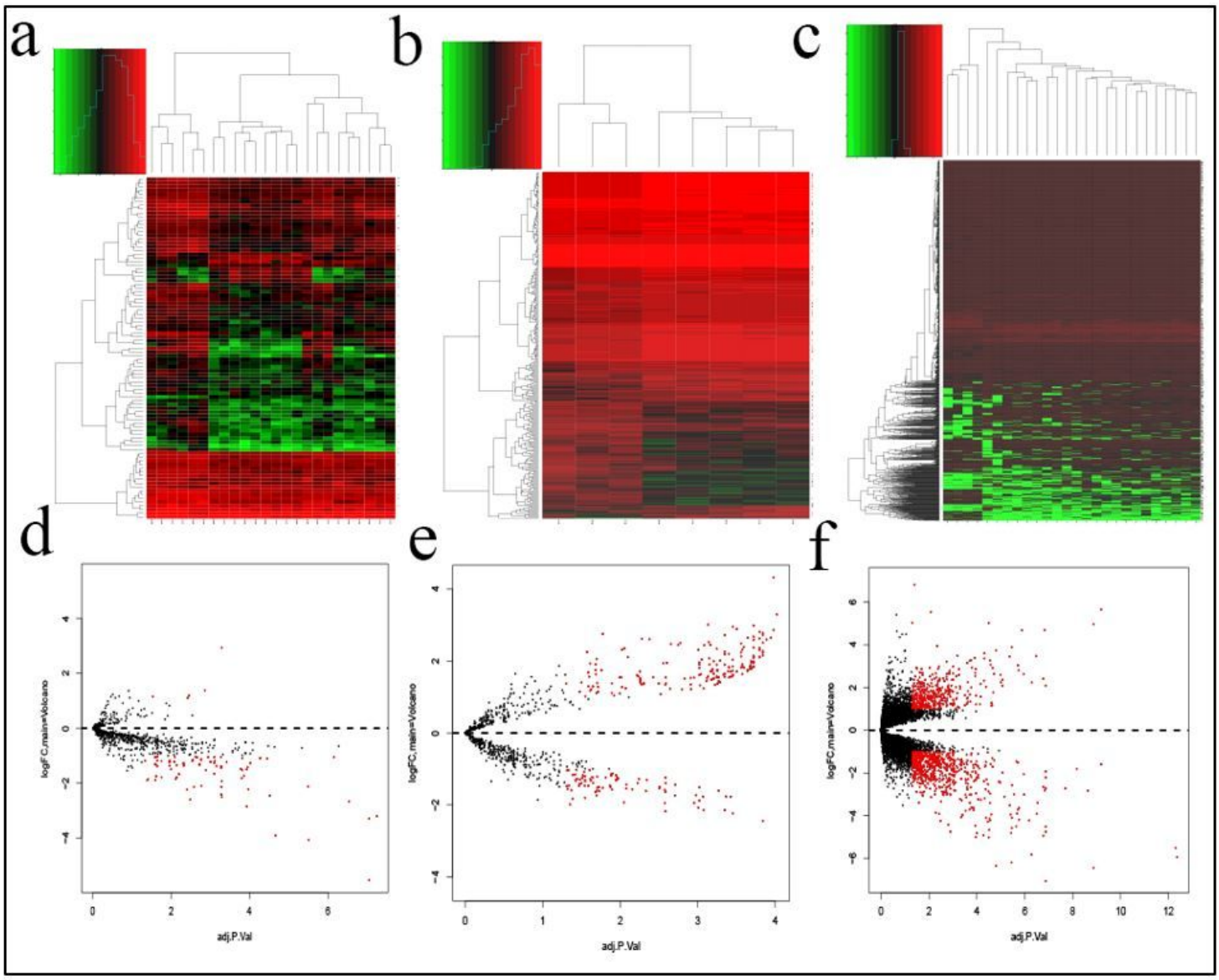

Figure 1

Heatmap and volcano of differentially expressed microRNAs and mRNAs between ToF infants and normal infants. a: GSE35490 gene expression heat map. b: GSE40128 gene expression heat map. c: GSE36761 gene expression heat map. d: GSE35490 volcano map. e: GSE40128 volcano map. f: GSE36761 volcano map.

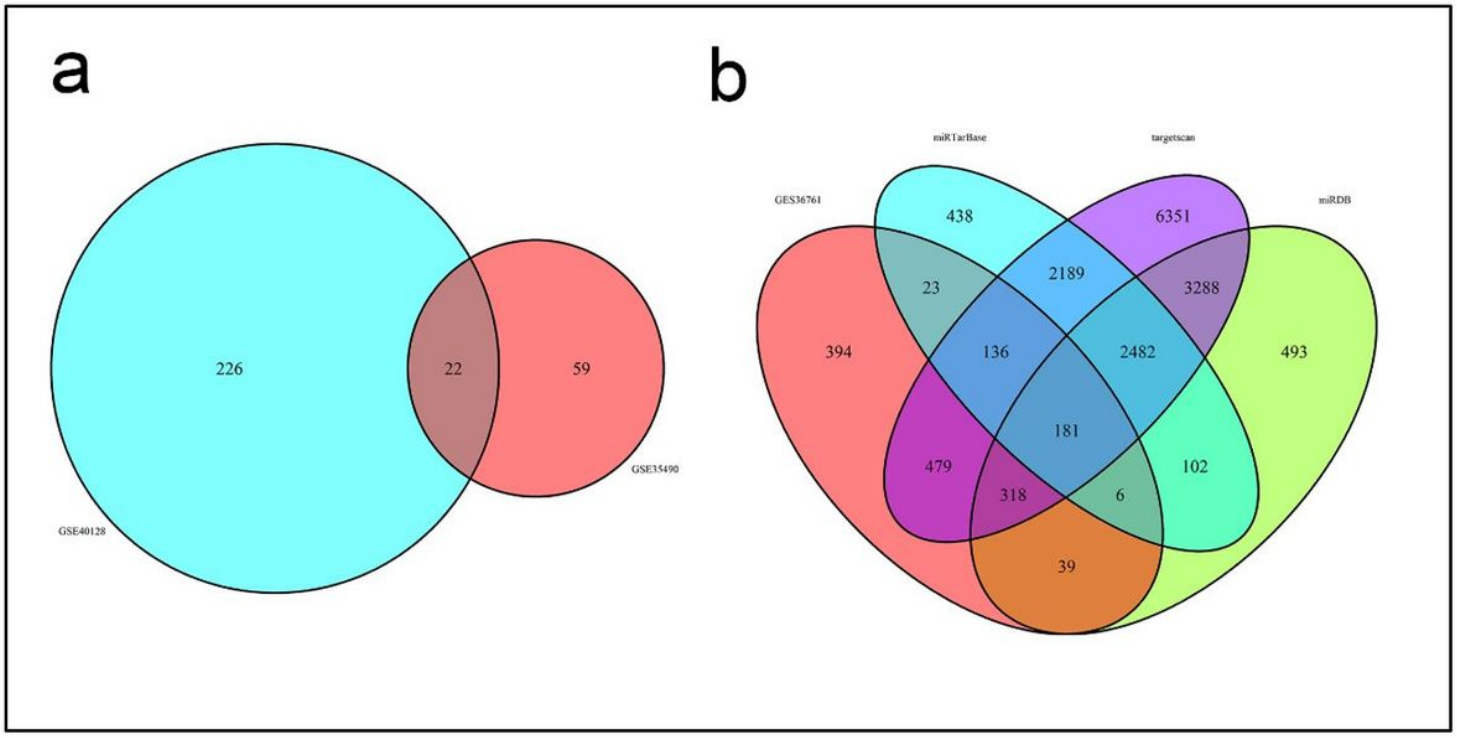

Figure 2

Venn diagram of DEMs and DEGs. a: overlapping DEMs in GES35490 and GSE40128. b: overlapping DEGs in three target gene database and GSE36761.

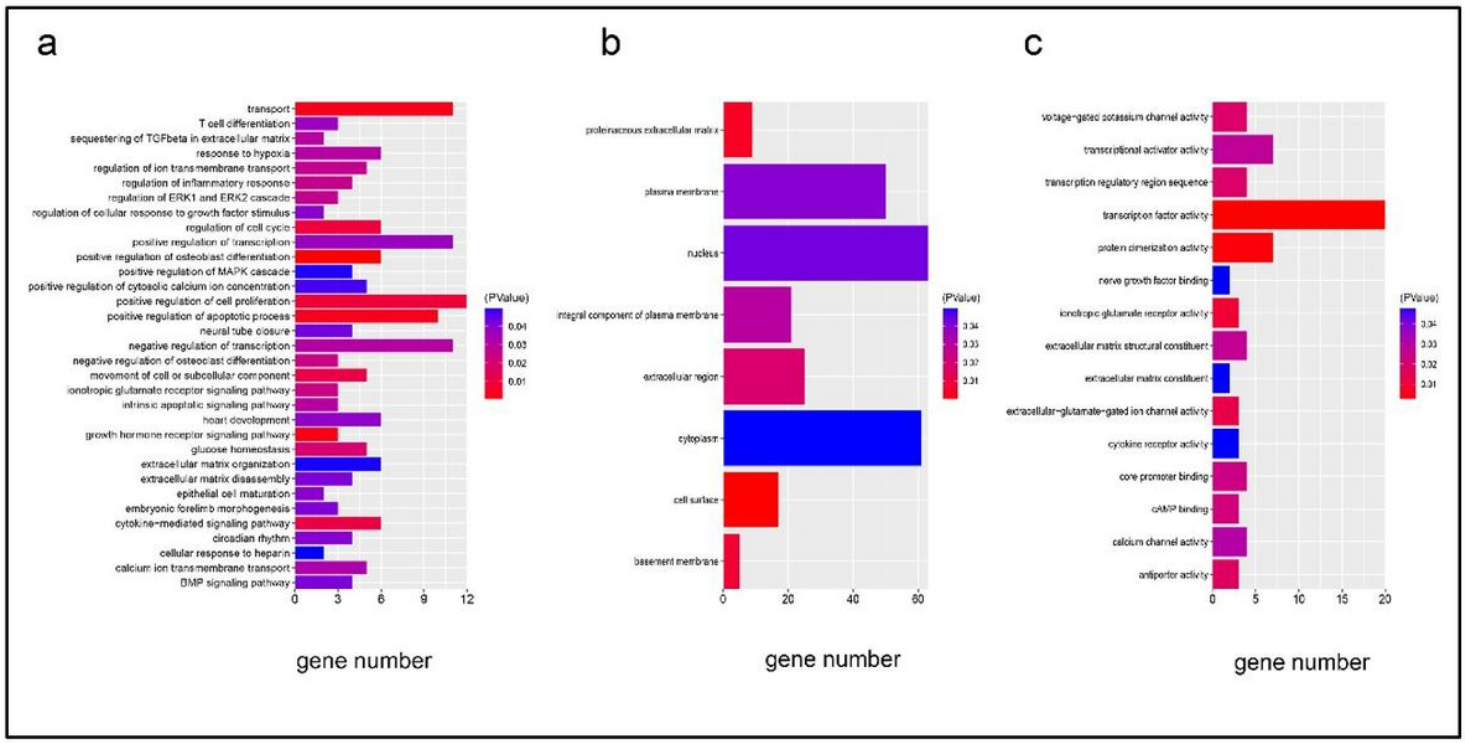

Figure 3

GO annotation of 181 significant DEGs. a: biological process(BP), b: cellular component(CC), c: molecular function(MF).

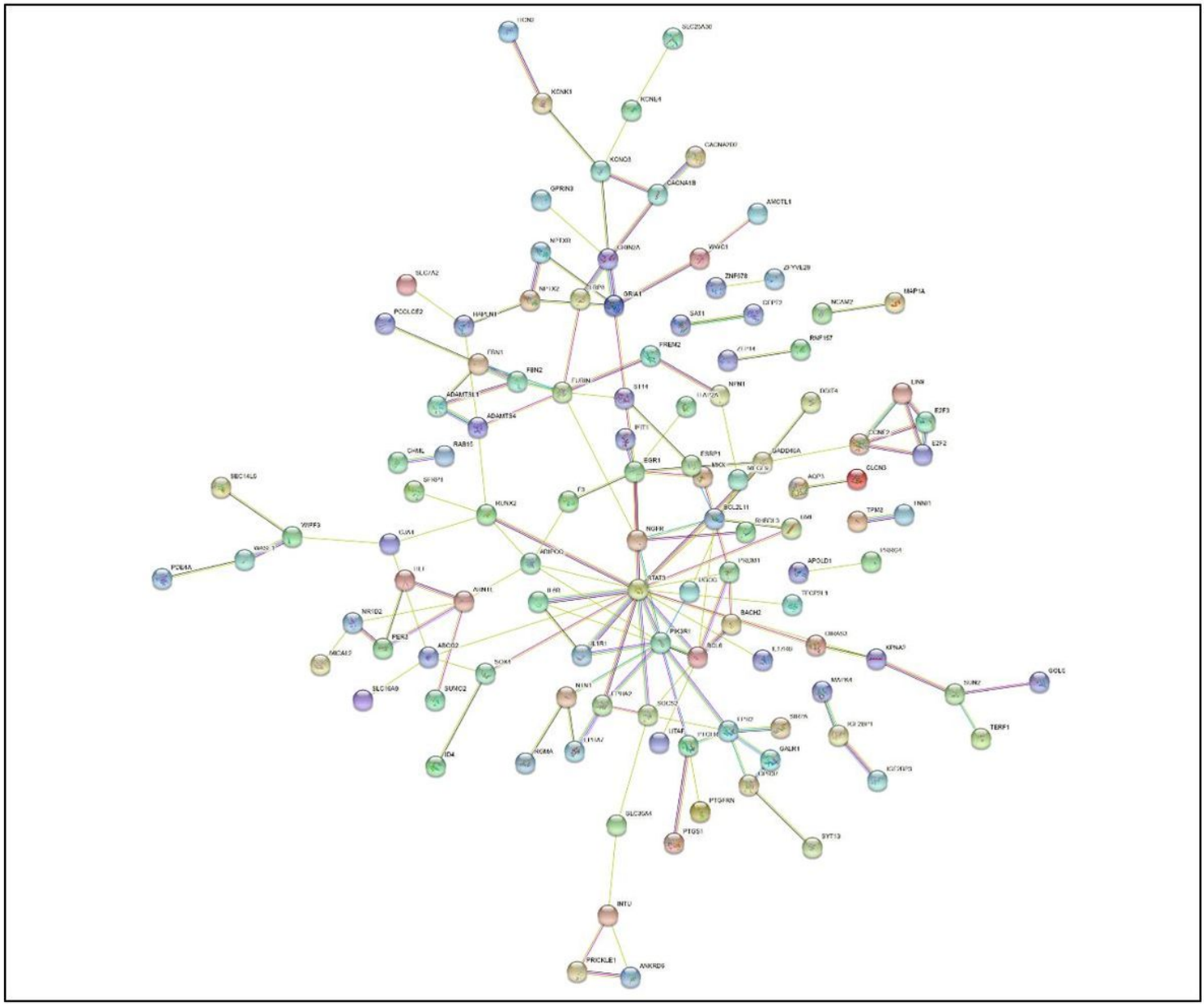

Figure 4

PPI network interaction diagram of 181 significant DEGs. PPI network interaction diagram of 181 significant DEGs.

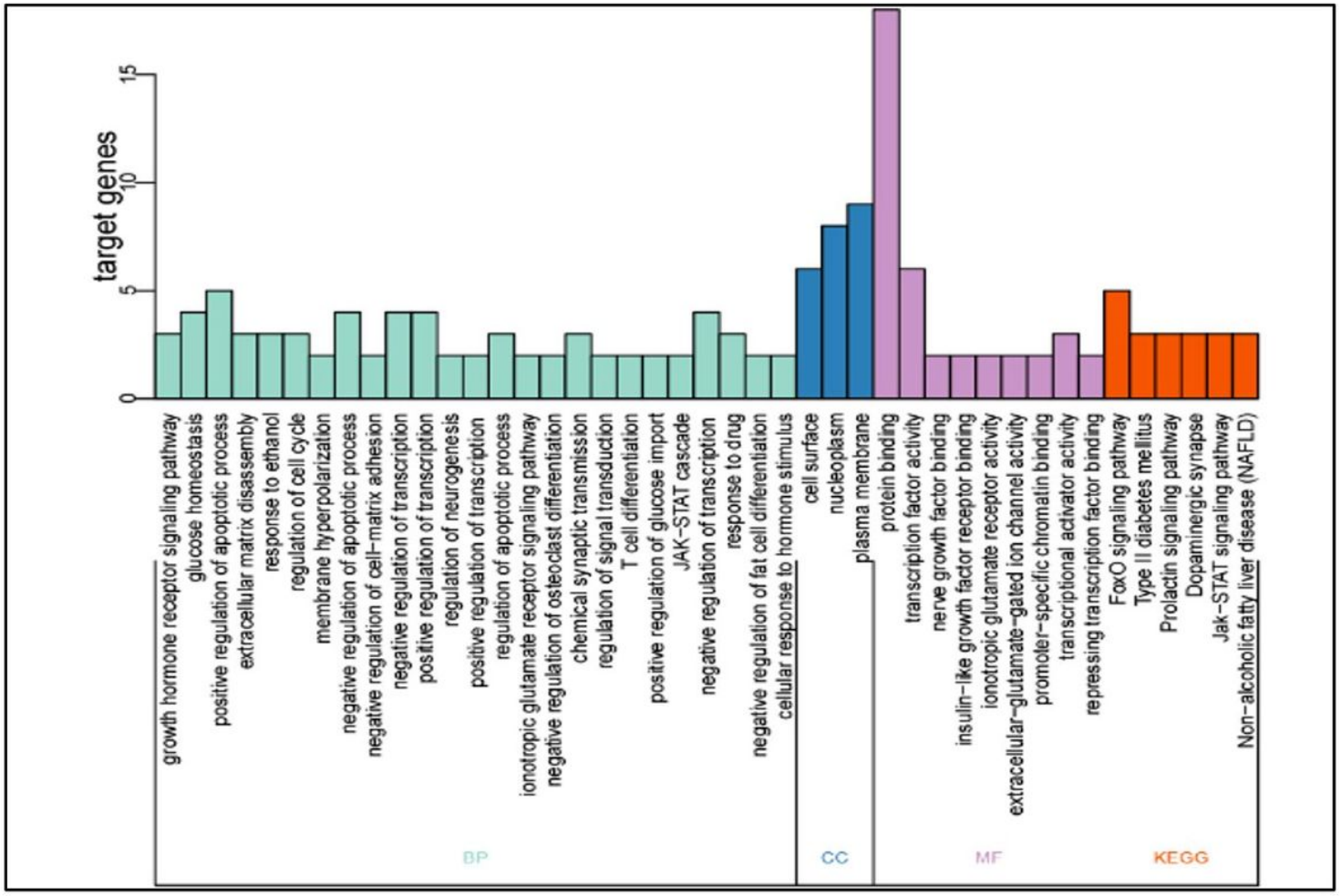

Figure 5

Functional enrichment analyses of the hub genes. The numbers on the x-axis are the names of GO terms or pathways. The numbers on the y-axis are gene counts. BP, biological progress; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

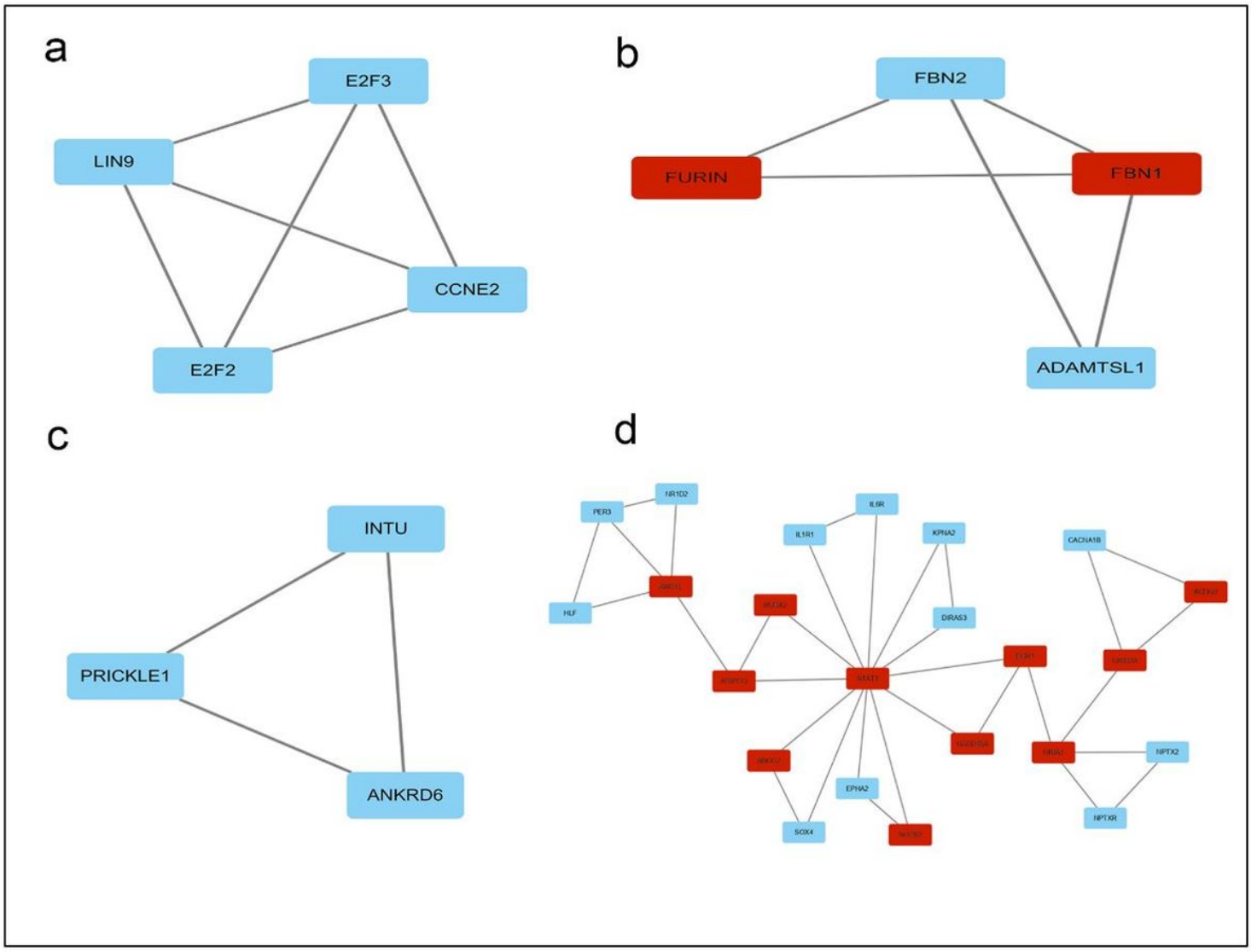

Figure 6

Topological diagram of the MCODE genes. a: Modules1, b: Modules2, c: Modules3, d: Modules4. red colors represent hub genes.

Supplementary Files

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

- TableS1.docx