Original Article

# Integrative bioinformatics approaches for identifying potential biomarkers and pathways involved in non-obstructive azoospermia

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**Background:** Non-obstructive azoospermia (NOA) is a disease related to spermatogenic disorders. Currently, the specific etiological mechanism of NOA is unclear. This study aimed to use integrated bioinformatics to screen biomarkers and pathways involved in NOA and reveal their potential molecular mechanisms.

**Methods:** GSE145467 and GSE108886 gene expression profiles were obtained from the Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) between NOA tissues and matched obstructive azoospermia (OA) tissues were identified using the GEO2R tool. Common DEGs in the two datasets were screened out by the VennDiagram package. For the functional annotation of common DEGs, DAVID v.6.8 was used to perform Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. In accordance with data collected from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, a protein–protein interaction (PPI) network was constructed by Cytoscape. Cytohubba in Cytoscape was used to screen the hub genes. Furthermore, the hub genes were validated based on a separate dataset, GSE9210. Finally, potential micro RNAs (miRNAs) of hub genes were predicted by miRWalk 3.0.

**Results:** A total of 816 common DEGs, including 52 common upregulated and 764 common downregulated genes in two datasets, were screened out. Some of the more important of these pathways, including focal adhesion, PI3K-Akt signaling pathway, cell cycle, oocyte meiosis, AMP-activated protein kinase (AMPK) signaling pathway, FoxO signaling pathway, and Huntington disease, were involved in spermatogenesis. We further identified the top 20 hub genes from the PPI network, including *CCNB2*, *DYNLL2*, *HMMR*, *NEK2*, *KIF15*, *DLGAP5*, *NUF2*, *TTK*, *PLK4*, *PTTG1*, *PBK*, *CEP55*, *CDKN3*, *CDC25C*, *MCM4*, *DNAI1*, *TYMS*, *PPP2R1B*, *DNAI2*, and *DYNLRB2*, which were all downregulated genes. In addition, potential miRNAs of hub genes, including hsa-miR-3666, hsa-miR-130b-3p, hsa-miR-15b-5p, hsa-miR-6838-5p, and hsa-miR-195-5p, were screened out.

**Conclusions:** Taken together, the identification of the above hub genes, miRNAs and pathways will help us better understand the mechanisms associated with NOA, and provide potential biomarkers and therapeutic targets for NOA.

**Keywords:** Non-obstructive azoospermia (NOA); expression profiling data; functional enrichment analysis; protein–protein interactions; biomarkers

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## Introduction

Infertility is defined as the inability to conceive within 1 year of unprotected intercourse (1). Studies have shown that about 10–15% of couples have fertility problems, and male factors are responsible for 50% of infertility cases (2,3). The causes of male infertility are complex. Azoospermia, which causes 10–20% of male infertility cases (4), is a type of male infertility in which sperm is absent. Types of azoospermia include obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) (5,6). OA is mainly caused by obstruction of the posterior reproductive tract of the testis, while NOA is caused by the dysfunction of spermatogenesis. NOA is the most severe form of male infertility, with an incidence rate of 10% (7).

Human spermatogenesis essentially occurs in three stages: spermatogenic mitosis, spermatogenic meiosis, and spermatogenesis (8). Problems at any of these stages can cause sperm production to fail. While it is possible to obtain sperm of through testicular aspiration or testicular sperm extraction via microdissection, this is not feasible for the vast majority of NOA patients (9). Furthermore, the primary mechanism regulating spermatogenesis in NOA patients remains unclear (10).

In the present study, we downloaded the expression profile datasets, GSE145467 and GSE108886, from the Gene Expression Omnibus (GEO) database. We then screened out common differentially expressed genes (DEGs) using combined GEO2R and VennDiagram package analyses. We performed Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of common DEGs. The protein–protein interaction (PPI) network was constructed by Cytoscape, and the hub genes were obtained using the Cytohubba plug-in of Cytoscape. In addition, potential micro RNAs (miRNAs) of hub genes were predicted by miRWalk 3.0. It is hoped the results of this study can provide insights into the molecular mechanism of NOA and identify potential biomarkers and therapeutic targets. We present our findings in accordance with the STROBE and MDAR reporting checklists (available at http://dx.doi.org/10.21037/tau-20-1029).

## Methods

### *Microarray data source*

As all the data in this study were from the GEO public database (https://www.ncbi.nlm.nih.gov/geo/), the approval of the local ethics committee was not required.

We used the keywords "non-obstructive azoospermia" and "expression profiling by array" and "Homo sapiens" in the GEO database (https://www.ncbi.nlm.nih.gov/geo/) to search the mRNA expression dataset of NOA. Through retrieval, we downloaded the GSE145467 and GSE108886 expression profile datasets. The GSE145467 dataset, which was contributed by Hodžić *et al.* (11), is based on the GPL4133 platform of the Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (Feature Number version) and includes 10 NOA samples and 10 OA testicular samples. The GSE108886 dataset, which was contributed by Baksi *et al.*, is based on the GPL10558 platform of the Illumina HumanHT-12 V4.0 expression beadchip which contains eight NOA samples and four OA samples (including one testicular control sample).

### *Screening for DEGs*

The GEO2R tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to screen DEGs in NOA tissues compared with matched OA tissues. The *t*-test and Benjamini–Hochberg method were used to calculate the P value and false discovery rate (FDR), respectively. The DEGs were screened out according to FDR <0.05 and |FC| ≥2.5. The common DEGs in the two datasets were screened out by the VennDiagram package.

### *GO and KEGG enrichment analysis*

GO functional and KEGG pathway enrichment analysis was conducted to determine the functions of common DEGs using the Database for Annotation Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/). The results of GO and KEGG pathway enrichment analyses were downloaded as a TXT file for subsequent analysis. The results were visualized using R software version 3.6.2.

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A P value <0.05 was set to represent a statistically significant difference.

### *PPI network and hub gene identification*

The Search Tool for the Retrieval of Interacting Genes database (STRING; https://string-db.org/cgi/input.pl) is an online tool for analyzing the PPI information. We constructed a PPI network of common DEGs using the STRING based on a minimum required interaction score of 0.7. We then used Cytoscape software v3.7.1 (https://cytoscape.org/) to visualize the PPI network derived from the STRING database. Using the cytoHubba plugin in Cytoscape, the nodes in the PPI network were ranked according to the degree calculation method (12) with the top 20 genes being considered the hub genes. GO and KEGG pathway analyses for the hub genes were performed using the WebGestalt (http://www.webgestalt.org/). A P value <0.05 was considered a statistically significant difference.

### *Analysis of hub genes in the NOA subgroup*

The NOA samples in the GSE108886 dataset were analyzed according to its two subgroups: the non-obstructive azoospermia with meiotic arrest (NOA-MA) subgroup contains five samples, and the non-obstructive azoospermia with pre-meiotic arrest (NOA-PreMA) subgroup contains three samples. The Wilcox test was used to determine whether there were differences in the expression of hub genes between the two subgroups. A P value <0.05 was considered statistically significant.

### *Validation of the hub genes*

To further verify the differential expression of hub genes, we downloaded the GSE9210 dataset, which contains 47 NOA samples and 11 OA samples. The expression levels of genes in this dataset have been processed by lowess-normalized natural log [Cy5/Cy3] (13). The Wilcox test was used to compare the differential expression of hub genes between the NOA and OA samples in the GSE9210 dataset. A P value <0.05 was considered a statistically significant difference.

### *Screening to regulate hub genes*

Twenty hub genes associated with NOA were imported into the miRWalk 3.0 software (http://mirwalk.umm.uni-heidelberg.de/) to screen for the miRNAs that regulate target genes. The miRWalk 3.0 software integrated the prediction results of TargetScan, and a score >0.8 was used as the cutoff criterion. Following this, a miRNA-gene regulatory network was constructed and visualized by Cytoscape. Moreover, miRNAs which targeted more than two genes were selected.

### *Statistical analysis*

We performed R software version 3.6.2 for statistical analysis. The Wilcox test was used to compare the two groups. A P value <0.05 was considered a statistically significant difference.

## Results

### *Identification of DEGs in NOA*

For the GSE145467 dataset, 3,549 DEGs were identified, including 951 upregulated and 2,598 downregulated genes. For the GSE108886 dataset, 1,063 DEGs were identified, including 155 upregulated and 908 downregulated genes. VennDiagram analysis was performed to determine the intersection of the two datasets of DEGs. A total of 816 common DEGs were identified, including 52 common upregulated and 764 common downregulated genes (*Figure 1* and Table S1).

### *GO enrichment analysis*

GO enrichment analysis of the common DEGs included the following three parts: biological process (BP), molecular function (MF), and cell component (CC). We imported the common DEGs into the DAVID online analysis tool for GO enrichment analysis. This showed that for BP, common upregulated DEGs were significantly associated with extracellular matrix (ECM) organization, negative regulation of cell proliferation and apoptotic process, cell adhesion, and cellular protein metabolic process. Common downregulated DEGs were significantly associated with spermatogenesis, multicellular organism development, cell differentiation, spermatid development, and sperm motility. Common upregulated DEGs that were significantly associated with CC included extracellular exosome, extracellular space, extracellular region, ECM, and proteinaceous ECM. Common downregulated DEGs

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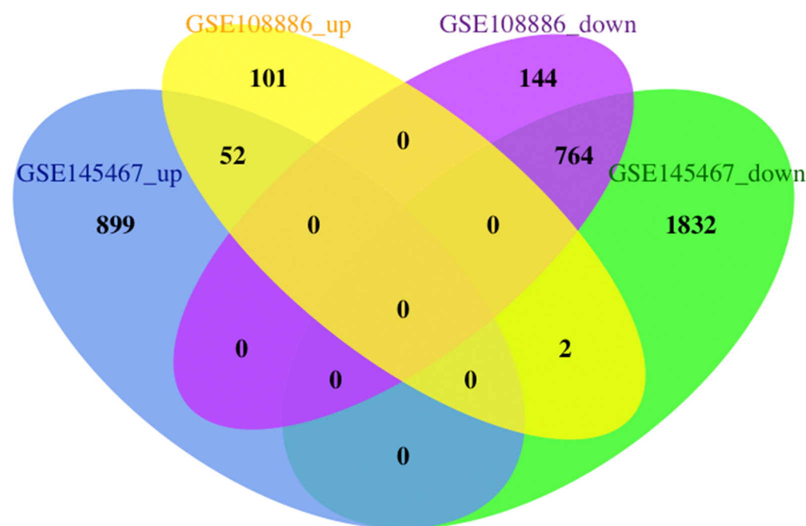


**Figure 1** Identification of 816 (52 upregulated and 764 downregulated) common differentially expression genes (DEGs) from GSE145467 and GSE108886 microarray profile datasets. The FDR <0.05 and |FC| ≥2.5 as the cut-off criterion. FDR, false discovery rate; FC, fold change.

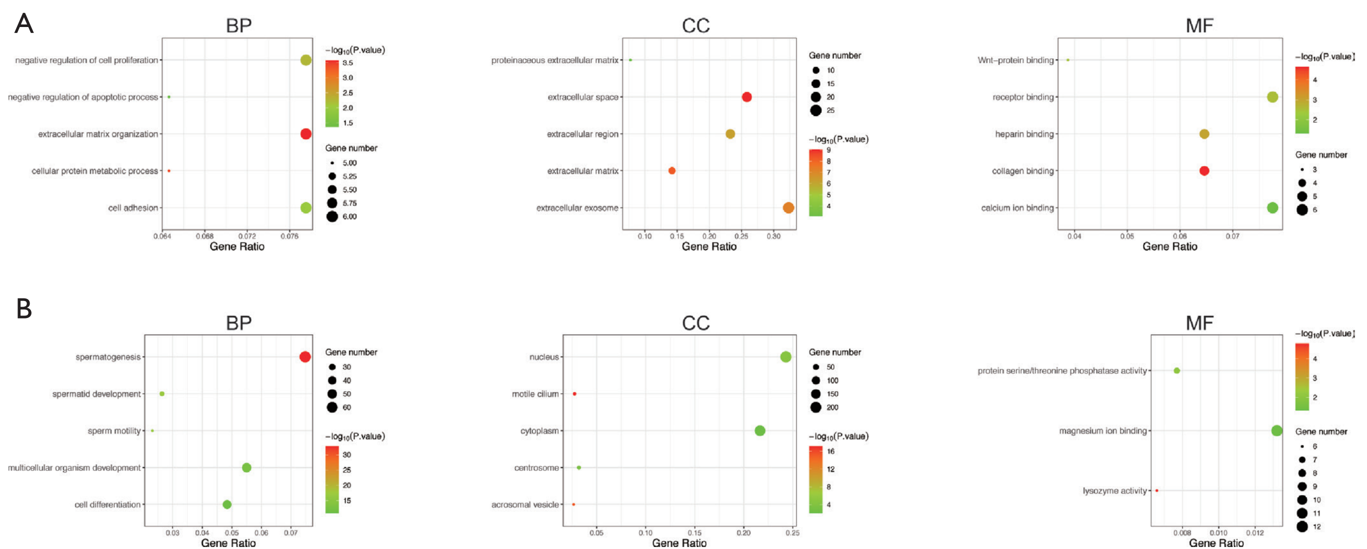


**Figure 2** Gene ontology analysis of the common DEGs. (A) Common upregulated DEGs. (B) Common downregulated DEGs. BP, biological process; CC, cellular component; MF, molecular function; DEGs, differentially expressed genes.

that were significantly associated with CC included nucleus, cytoplasm, centrosome, motile cilium, and acrosomal vesicle. GO MF showed that common upregulated DEGs were significantly associated with receptor binding, calcium ion binding, collagen binding, heparin binding, and Wnt-protein binding. Common downregulated DEGs were significantly associated with protein serine/threonine kinase activity, magnesium ion binding, protein serine/threonine phosphatase activity, lysozyme activity, and microtubule motor activity. These results are shown in *Figure 2* and *Table 1*.

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**Table 1** Gene ontology analysis of common upregulated and downregulated DEGs

| Category | Term | Count | Gene ratio | P value |
|---|---|---|---|---|
| Upregulated | | | | |
| BP | GO:0030198~extracellular matrix organization | 6 | 0.0775294 | 2.59E-04 |
| BP | GO:0008285~negative regulation of cell proliferation | 6 | 0.0775294 | 0.005787 |
| BP | GO:0007155~cell adhesion | 6 | 0.0775294 | 0.0105986 |
| BP | GO:0044267~cellular protein metabolic process | 5 | 0.0646078 | 3.85E-04 |
| BP | GO:0043066~negative regulation of apoptotic process | 5 | 0.0646078 | 0.04328502 |
| CC | GO:0070062~extracellular exosome | 25 | 0.3230392 | 5.98E-08 |
| CC | GO:0005615~extracellular space | 20 | 0.2584313 | 9.39E-10 |
| CC | GO:0005576~extracellular region | 18 | 0.2325882 | 6.52E-07 |
| CC | GO:0031012~extracellular matrix | 11 | 0.1421372 | 6.35E-09 |
| CC | GO:0005578~proteinaceous extracellular matrix | 6 | 0.0775294 | 8.18E-04 |
| MF | GO:0005102~receptor binding | 6 | 0.0775294 | 0.00290085 |
| MF | GO:0005509~calcium ion binding | 6 | 0.0775294 | 0.04816598 |
| MF | GO:0005518~collagen binding | 5 | 0.0646078 | 2.29E-05 |
| MF | GO:0008201~heparin binding | 5 | 0.0646078 | 0.00100954 |
| MF | GO:0017147~Wnt-protein binding | 3 | 0.0387647 | 0.00335112 |
| Downregulated | | | | |
| BP | GO:0007283~spermatogenesis | 68 | 0.0747565 | 1.86E-33 |
| BP | GO:0007275~multicellular organism development | 50 | 0.054968 | 2.82E-13 |
| BP | GO:0030154~cell differentiation | 44 | 0.0483718 | 1.24E-11 |
| BP | GO:0007286~spermatid development | 24 | 0.0263846 | 1.21E-17 |
| BP | GO:0030317~sperm motility | 21 | 0.0230866 | 1.32E-17 |
| CC | GO:0005634~nucleus | 221 | 0.2429586 | 3.59E-05 |
| CC | GO:0005737~cytoplasm | 197 | 0.216574 | 0.00769222 |
| CC | GO:0005813~centrosome | 29 | 0.0318814 | 3.33E-04 |
| CC | GO:0031514~motile cilium | 25 | 0.027484 | 8.08E-18 |
| CC | GO:0001669~acrosomal vesicle | 24 | 0.0263846 | 8.40E-16 |
| MF | GO:0000287~magnesium ion binding | 12 | 0.0131923 | 0.0461689 |
| MF | GO:0004722~protein serine/threonine phosphatase activity | 7 | 0.0076955 | 0.00798661 |
| MF | GO:0003796~lysozyme activity | 6 | 0.0065962 | 1.66E-05 |
| MF | GO:0004674~protein serine/threonine kinase activity | 18 | 0.0197885 | 0.06625263 |
| MF | GO:0003777~microtubule motor activity | 6 | 0.0065962 | 0.09501651 |

GO, gene ontology; DEGs, differentially expressed genes; BP, biological process; CC, cellular component; MF, molecular function; Count, number of DEGs.

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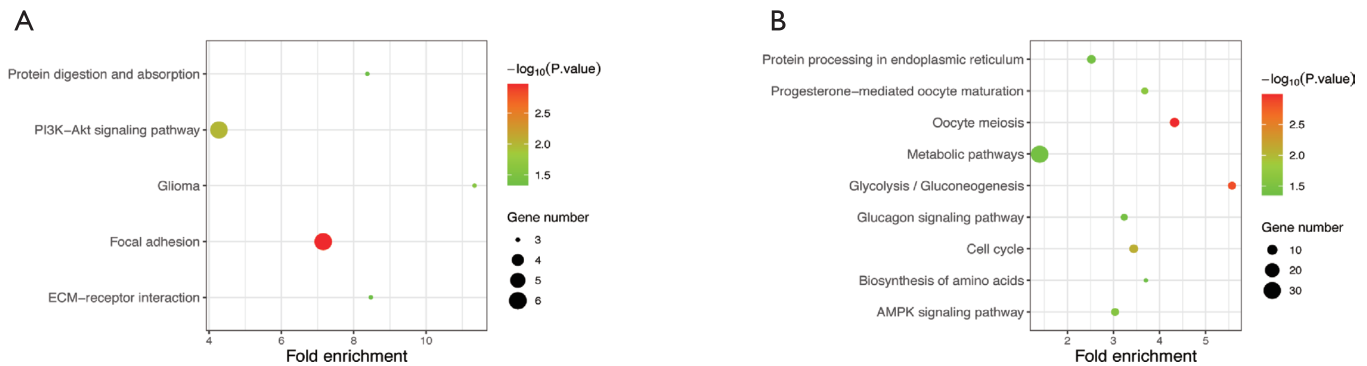


**Figure 3** KEGG pathway enrichment analysis of the common DEGs. (A) Common upregulated DEGs. (B) Common downregulated DEGs. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expression genes.

### *KEGG pathway enrichment analysis*

The KEGG pathway of the common upregulated and downregulated DEGs was analyzed by the DAVID database. The common upregulated DEGs were mainly involved in focal adhesion, PI3K-Akt signaling pathway, glioma, ECM-receptor interaction, and protein digestion and absorption. The common downregulated DEGs were mainly involved in oocyte meiosis, glycolysis/gluconeogenesis, cell cycle, progesterone-mediated oocyte maturation, AMP-activated protein kinase (AMPK) signaling pathway, glucagon signaling pathway, metabolic pathways, protein processing in the endoplasmic reticulum, the and biosynthesis of amino acids. These results are shown in *Figure 3* and *Table 2*.

### *PPI network and hub gene analysis*

According to the STRING database, the PPI network of DEGs was constructed, with 328 nodes and 604 edges being mapped as presented in *Figure 4A*. The top 20 hub genes were evaluated using the Degree algorithm of the Cytohubba plug-in, as shown in *Figure 4B* and *Table 3*. GO and KEGG enrichment analyses of the 20 hub genes were performed using WebGestalt. As shown in *Figure 5*, the GO enrichment analysis was mainly involved in metabolic process, cellular component organization, biological regulation, cytosol, cytoskeleton, membrane, and protein binding. KEGG pathway enrichment analysis was mainly involved in cell cycle, oocyte meiosis, progesterone-mediated oocyte maturation, FoxO signaling pathway, one carbon pool by folate, and Huntington disease (*Figure 6*).

### *Analysis of hub genes in the NOA subgroup*

There was no statistically significant difference in the expression of the 20 hub genes between the NOA-MA and NOA-PreMA subgroups of the GSE108886 dataset (*Figure 7*).

### *Validation of the hub genes*

With the exception of PPP2R1B, 19 of the 20 hub genes showed lower expression in the NOA samples of GSE9210 as compared to the OA samples, which was consistent with the GSE145467 and GSE108886 datasets (*Figure 8*).

### *Integrated network analysis of miRNA–mRNA interactions*

The 20 hub genes were submitted to the online tool, miRwalk 3.0. Based on the identified miRNA–mRNA pairs, we compared the interaction network containing 51 miRNA–mRNA pairs and visualized them with Cytoscape. Our analysis showed that hsa-miR-3666 and hsa-miR-130b-3p downregulated CEP55 and DYNLL2; hsa-miR-15b-5p and hsa-miR-6838-5p downregulated CEP55 and PPP2R1B; and hsa-miR-195-5p downregulated PPP2R1B and DYNLL2. The miRNA-gene regulatory network is shown in *Figure 9* and *Table 4*.

## Discussion

Spermatogenesis is a complex process, involving

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**Table 2** KEGG pathway analysis of common upregulated and downregulated DEGs

| Pathway | ID | Count | Fold Enrichment | P value | Genes |
|---|---|---|---|---|---|
| Upregulated | | | | | |
| Focal adhesion | hsa04510 | 6 | 7.155686546 | 1.08E-03 | *LAMA2, COL6A3, PDGFRA, IGF1, COL6A1, SHC1* |
| PI3K-Akt signaling pathway | hsa04151 | 6 | 4.272670807 | 9.99E-03 | *LAMA2, COL6A3, PDGFRA, IGF1, COL6A1, GNG11* |
| Glioma | hsa05214 | 3 | 11.33901099 | 2.65E-02 | *PDGFRA, IGF1, SHC1* |
| ECM-receptor interaction | hsa04512 | 3 | 8.471674877 | 4.52E-02 | *LAMA2, COL6A3, COL6A1* |
| Protein digestion and absorption | hsa04974 | 3 | 8.375405844 | 4.62E-02 | *COL6A3, CPA3, COL6A1* |
| Downregulated | | | | | |
| Oocyte meiosis | hsa04114 | 9 | 4.323695789 | 1.02E-03 | *PPP2R1B, PGR, PLCZ1, SPDYA, MAPK1, CCNB2, PPP3R2, PTTG1, CDC25C* |
| Glycolysis/ gluconeogenesis | hsa00010 | 7 | 5.5713294 | 1.46E-03 | *GAPDHS, LDHC, LDHAL6B, PFKP, PGAM2, PDHA2, PGK2* |
| Cell cycle | hsa04110 | 8 | 3.44036009 | 8.15E-03 | *CCNB2, CDC14A, DBF4, TTK, PTTG1, CCNA1, CDC25C, MCM4* |
| Progesterone-mediated oocyte maturation | hsa04914 | 6 | 3.677626303 | 2.26E-02 | *PGR, SPDYA, MAPK1, CCNB2, CCNA1, CDC25C* |
| AMPK signaling pathway | hsa04152 | 7 | 3.034789185 | 2.68E-02 | *PPP2R1B, CPT1B, CAB39L, PFKP, CCNA1, PPP2R2B, PPP2R3C* |
| Glucagon signaling pathway | hsa04922 | 6 | 3.231853418 | 3.68E-02 | *LDHC, CPT1B, LDHAL6B, PPP3R2, PGAM2, PDHA2* |
| Metabolic pathways | hsa01100 | 32 | 1.399851193 | 3.76E-02 | *INPP1, LDHC, PLCZ1, KYNU, GCNT3, SGMS2, GK2, OLAH, COX7B2, NT5C1B, ACSBG2, PGAM2, TKTL2, CERS3, AZIN2, STT3B, ALDH1A2, TYMS, GALNTL5, PHOSPHO2, PDHA2, COX6B2, PRPS1L1, PCYT2, SPAM1, ADSSL1, LDHAL6B, SI, PFKP, GAPDHS, INPP4B, PGK2* |
| Protein processing in endoplasmic reticulum | hsa04141 | 8 | 2.524287877 | 3.78E-02 | *HSPA1L, NGLY1, STT3B, DNAJC5B, UBQLNL, HSPA4L, UBQLN3, DNAJC5G* |
| Biosynthesis of amino acids | hsa01230 | 5 | 3.703165375 | 4.46E-02 | *PFKP, PGAM2, TKTL2, PRPS1L1, PGK2* |

KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; Count, number of DEGs.

spermatogonial proliferation (mitosis), spermatocyte meiosis, and spermatid differentiation (14). In this study, we integrated the GSE145467 and GSE108886 datasets and utilized bioinformatics methods to identify 816 common DEGs, including 52 common upregulated and 764 common downregulated genes in NOA. GO enrichment analysis showed that common upregulated DEGs were mainly associated with ECM organization, extracellular exosome, and receptor binding. Common downregulated DEGs were mainly associated with spermatogenesis, nucleus, and protein serine/threonine kinase activity. KEGG pathway analysis showed that common upregulated DEGs were mainly involved in focal adhesion, PI3K-Akt signaling pathway, glioma, ECM-receptor interaction, and protein

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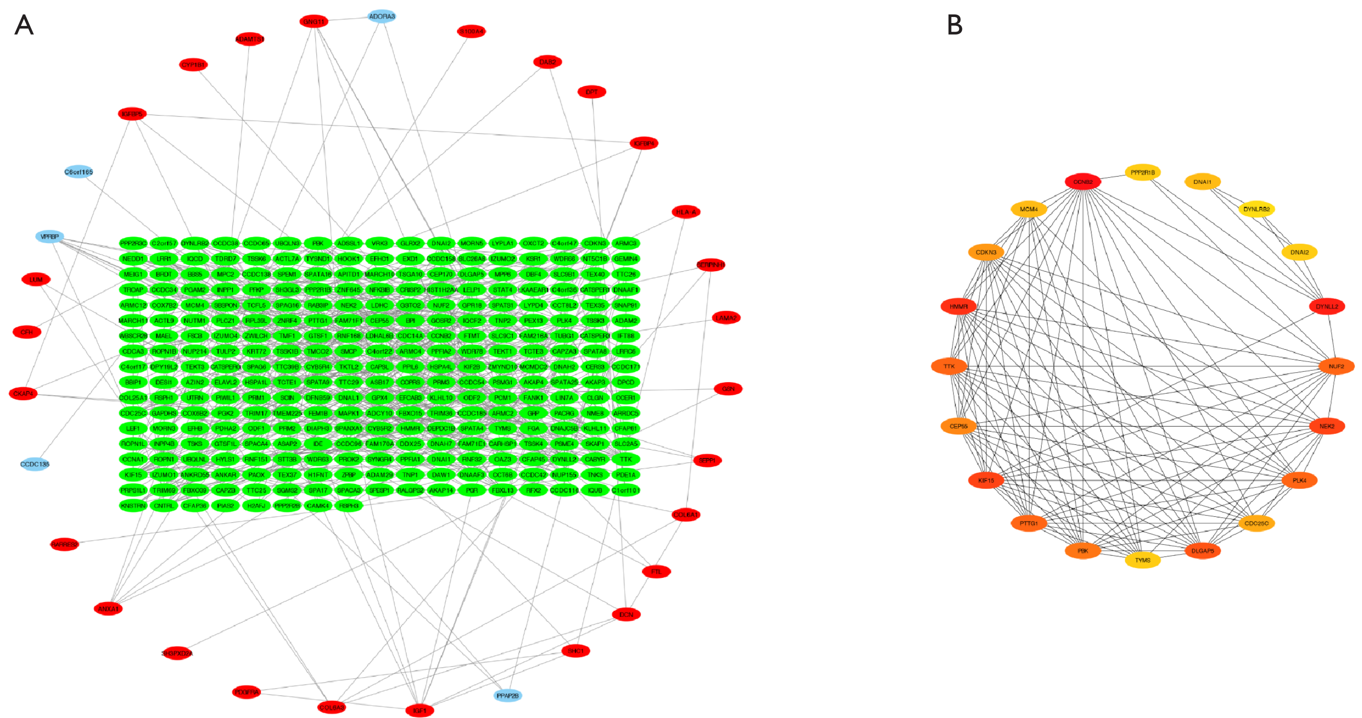


**Figure 4** PPI network analysis and hub genes in the protein–protein interaction network. (A) The PPI network for common DEGs. Red circle denotes common upregulated genes; green circle denotes common downregulated genes. (B) The top 20 hub genes in Degree score from the cytoHubba. Redder color indicates higher degree. PPI, protein–protein interaction; DEGs, differentially expressed genes.

digestion and absorption. Common downregulated DEGs were mainly involved in oocyte meiosis, glycolysis/gluconeogenesis, cell cycle, progesterone-mediated oocyte maturation, AMPK signaling pathway, glucagon signaling pathway, metabolic pathways, protein processing in endoplasmic reticulum, and biosynthesis of amino acids. Most of the above KEGG pathways are involved in spermatogenesis (15-17). However, the two pathway names of "oocyte meiosis" and "progesterone-mediated oocyte maturation" are derived from their roles in female fertility, but genes involved in the "oocyte meiosis" pathway also play an important role in sperm meiosis (18). Similarly, we speculated that genes involved in the "progesterone-mediated oocyte maturation" pathway may also play a role in spermatogenesis. These common DEGs may, therefore, be closely related to spermatogenic disorder in NOA patients.

We then identified 20 hub genes: *CCNB2*, *DYNLL2*, *HMMR*, *NEK2*, *KIF15*, *DLGAP5*, *NUF2*, *TTK*, *PLK4*, *PTTG1*, *PBK*, *CEP55*, *CDKN3*, *CDC25C*, *MCM4*, *DNAI1*, *TYMS*, *PPP2R1B*, *DNAI2*, and *DYNLRB2*, which were all downregulated genes. Now we summarize the several hub genes that have been studied (*Table 5*), and then we will describe and discuss them in detail.

CCNB2 is a member of the cyclin B family, which contributes to G2/M transition in both mitosis and meiosis (19). Research has found CCNB2 to be continuously expressed in the medaka testis during the process of spermatogenesis (28). CDC25C is a member of the CDC25 family of protein phosphatases, which affects the G2/M phase transition of the cell cycle by activating CDC2 (26). One study reported that under the influence of selenite-induced oxidative stress, CDC25C was downregulated and p21 (a kinase inhibitor) increased (29). This resulted in the downregulation of the CDC2/cyclin B1 complex that regulates the G2/M phase checkpoint, thereby causing cell cycle arrest in male Balb/c mice (29). Lin *et al.* (30) examined the testicular messenger RNA (mRNA) transcription levels of 29 patients with NOA, including 18 patients with successful sperm extraction and 11 patients with failed sperm extraction. They found that the mRNA transcription levels of CCNB2 and CDC25C were significantly reduced in NOA patients with failed sperm extraction (30). This further indicates that CCNB2 and CDC25C might play

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**Table 3** Degree values and descriptions of the top 20 hub genes

| Rank | Gene symbol | Gene description | Degree |
|---|---|---|---|
| 1 | *CCNB2* | Cyclin B2 | 25 |
| 2 | *DYNLL2* | Dynein light chain LC8-type 2 | 22 |
| 3 | *HMMR* | Hyaluronan mediated motility receptor | 21 |
| 4 | *KIF15* | Kinesin family member 15 | 20 |
| 4 | *NEK2* | NIMA related kinase 2 | 20 |
| 6 | *DLGAP5* | DLG associated protein 5 | 19 |
| 7 | *PTTG1* | Pituitary tumor-transforming 1 | 18 |
| 7 | *PLK4* | Polo like kinase 4 | 18 |
| 7 | *TTK* | TTK protein kinase | 18 |
| 7 | *NUF2* | NUF2, NDC80 kinetochore complex component | 18 |
| 11 | *PBK* | PDZ binding kinase | 17 |
| 12 | *CEP55* | Centrosomal protein 55 | 16 |
| 13 | *CDKN3* | Cyclin dependent kinase inhibitor 3 | 15 |
| 14 | *CDC25C* | Cell division cycle 25C | 14 |
| 15 | *DNAI1* | Dynein axonemal intermediate chain 1 | 13 |
| 15 | *MCM4* | Minichromosome maintenance complex component 4 | 13 |
| 17 | *DNAI2* | Dynein axonemal intermediate chain 2 | 12 |
| 17 | *PPP2R1B* | Protein phosphatase 2 scaffold subunit Abeta | 12 |
| 17 | *TYMS* | Thymidylate synthetase | 12 |
| 20 | *DYNLRB2* | Dynein light chain roadblock-type 2 | 11 |

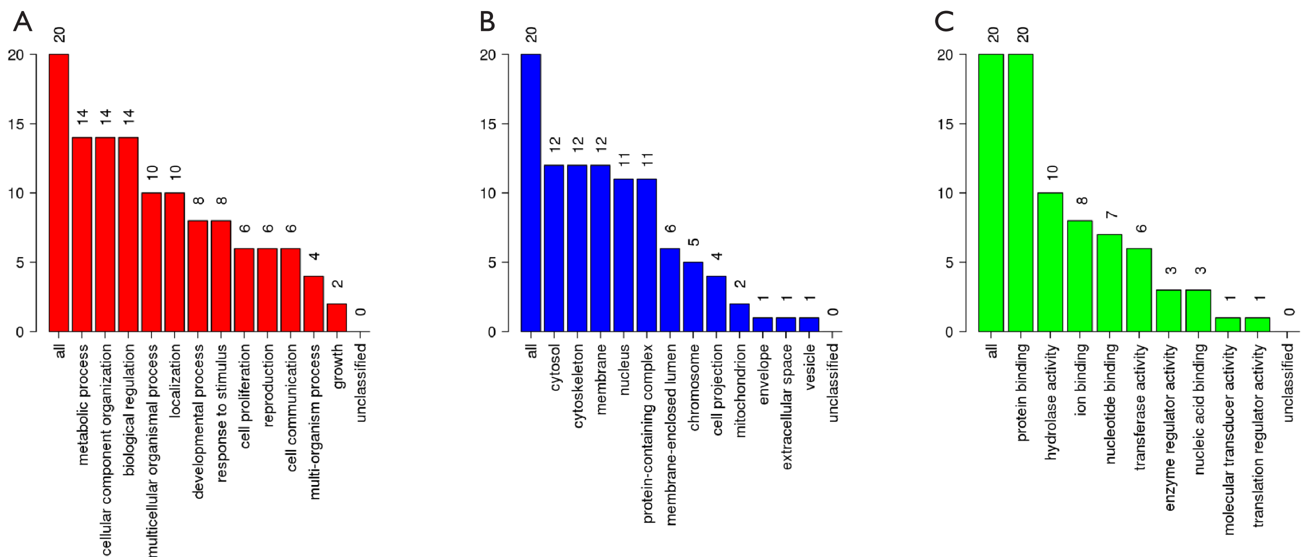


**Figure 5** GO map of 20 hub genes. (A) Biological process categories. (B) Cellular component categories. (C) Molecular function categories. GO, Gene ontology.

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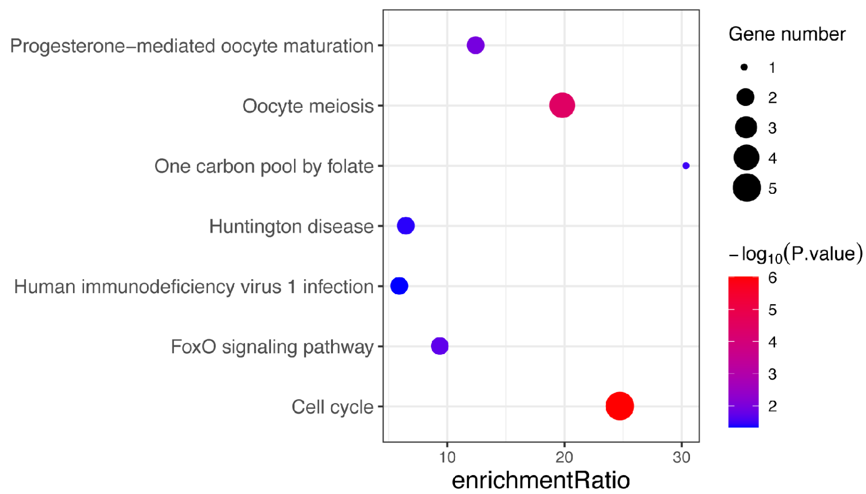


**Figure 6** KEGG pathway analysis of 20 hub genes. KEGG, Kyoto Encyclopedia of Genes and Genomes.

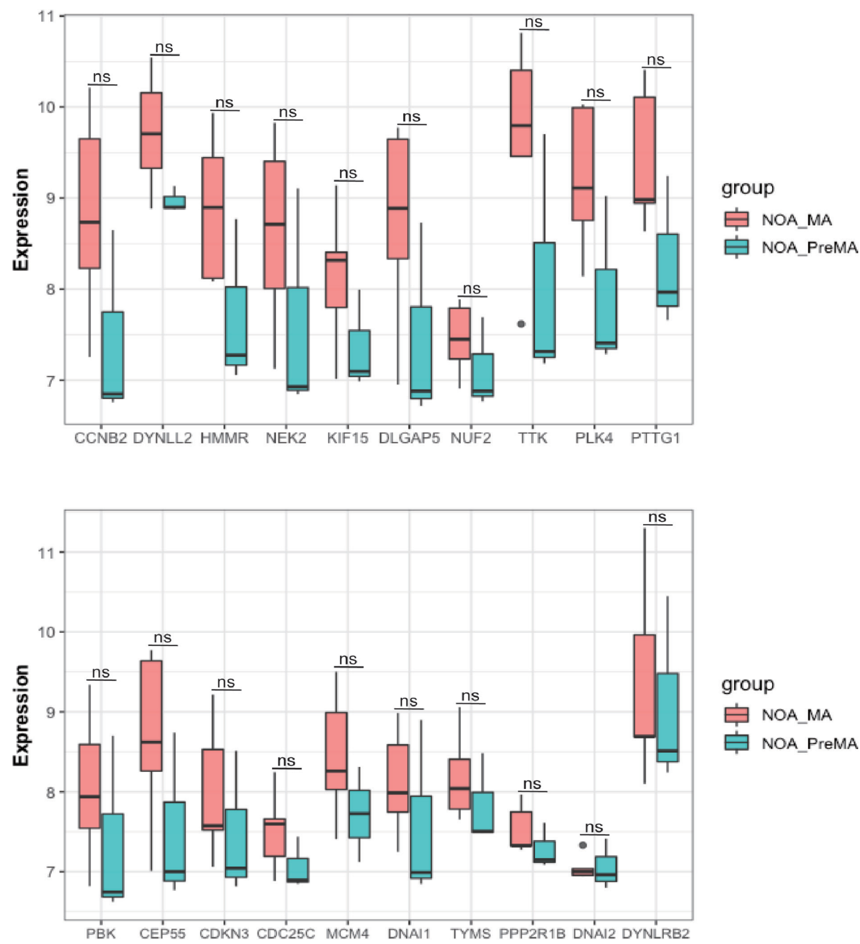


**Figure 7** The differential expression level of 20 hub genes between NOA-MA and NOA-PreMA subgroups of NOA in GSE108886 dataset (*, P<0.05). NOA-MA, non-obstructive azoospermia with meiotic arrest; NOA-PreMA, non-obstructive azoospermia with pre-meiotic arrest.

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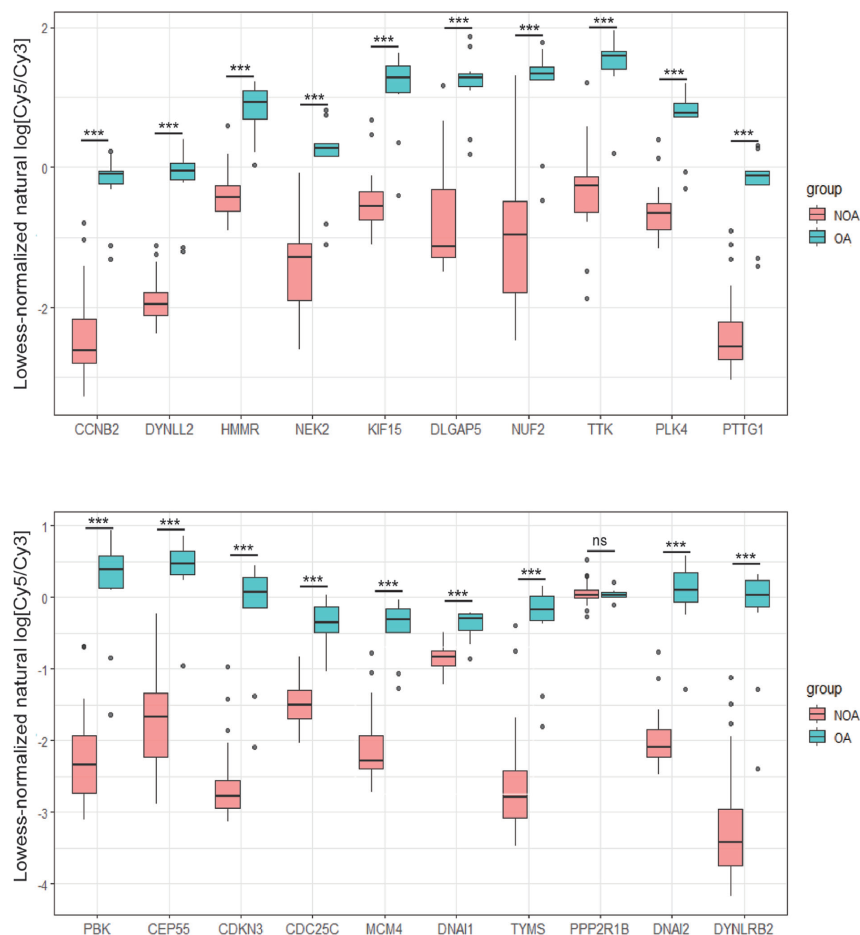


**Figure 8** The differential expression level of 20 hub genes between NOA and OA groups in GSE9210 dataset. (*, P<0.05, **, P<0.01, ***, P<0.001). NOA, non-obstructive azoospermia; OA, obstructive azoospermia.

an essential role in spermatogenesis. Hyaluronan-mediated motility receptor (HMMR), also known as receptor for hyaluronan-mediated motility (RHAMM), is a hyaluronic acid-mediated motor receptor (31). One study showed that the downregulation of HMMR is related to a decrease in sperm count, motility, and number of sperm with normal morphology (20). Meanwhile, NIMA-related kinase 2 (Nek2) is a serine/threonine kinase associated with G2/M phase transition of the cell cycle (21). In the testes of *Oreochromis niloticus*, Nek2 was generally found to be expressed in primary and secondary spermatocytes (21). Another study reported that Nek2 plays an important role in chromatin condensation during meiosis in male mice (32). TTK (or Mps1) is a dual specificity protein kinase with the ability to phosphorylate tyrosine, serine, and threonine. In reproductive tissues of male zebrafish, mps1$^{zp1}$ mutation was found to reduce mitotic checkpoint activity, resulting in abnormal chromosomes in male germ cells, and severe developmental defects in aneuploid progeny (22). Polo-like kinases (Plks) are a conserved family of mitotic serine-threonine protein kinases that play a key role in centrosome function (33). PLK4 is a member of the Plks family and necessary for centriole duplication. In a study of PLK4 mutation, the majority of spermatids in *Drosophila* could not form flagella due to a lack of centrioles, while the depletion of PLK4 in human cells was also seen to induce apoptosis due to mitotic abnormalities (23). PLK4 mutations might also be associated with human Sertoli - cell - only syndrome (SCOS) (34,35). PDZ binding kinase (PBK) is a serine/threonine protein kinase, which was found in the outer cell layer of spermatogenic tubules by *in situ* hybridization, indicating that it plays an important role in the process of spermatogenesis (24). CEP55 (Centrosomal protein 55), located in the centrosomes of interphase

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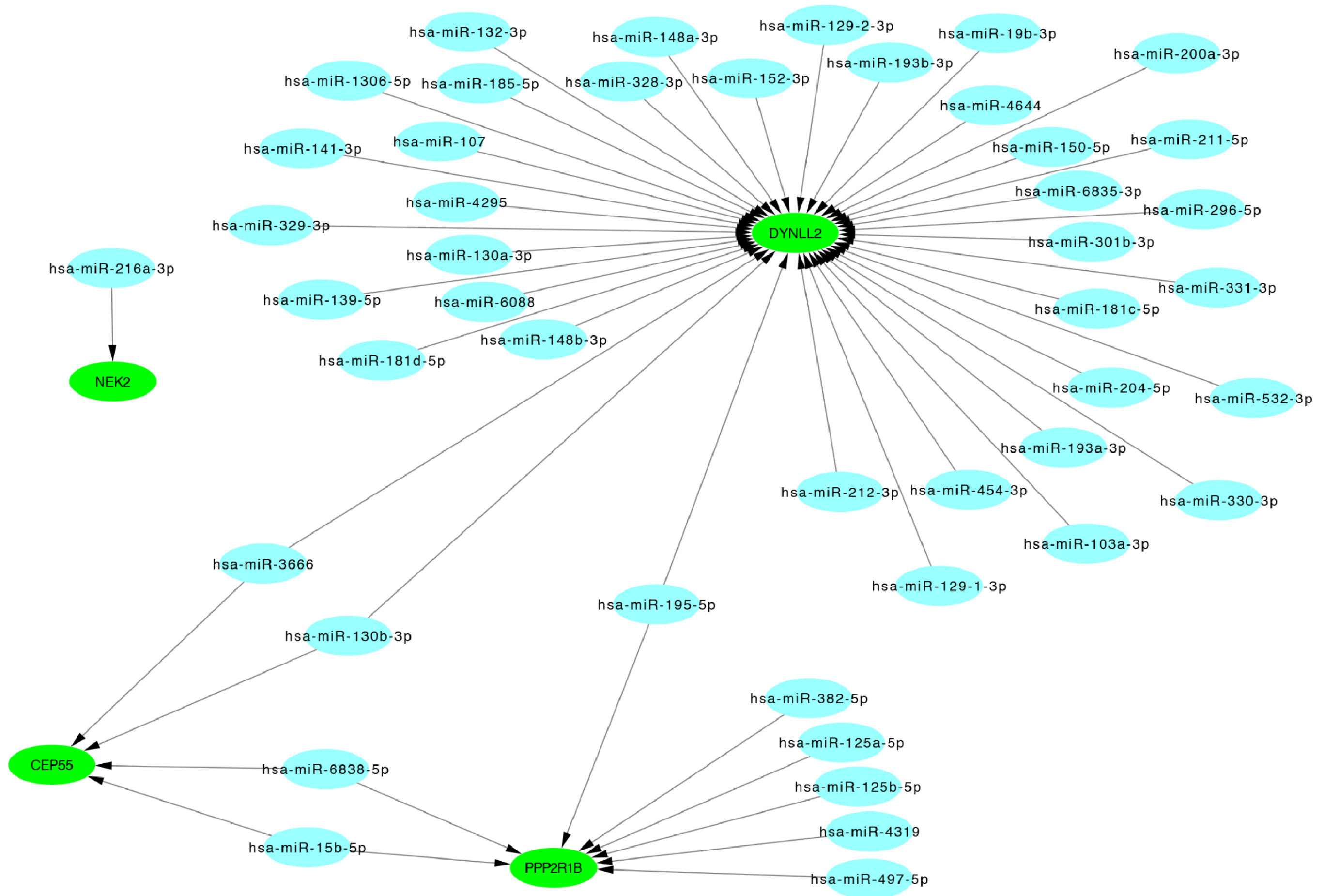


**Figure 9** The miRNA-gene regulated network, green color: down- regulated hub genes, blue color: miRNAs.

**Table 4** Hub genes and corresponded target miRNAs

| Gene | miRNA |
|---|---|
| *NEK2* | hsa-miR-216a-3p |
| *CEP55* | hsa-miR-130b-3p hsa-miR-6838-5p hsa-miR-3666 hsa-miR-15b-5p |
| *PPP2R1B* | hsa-miR-382-5p hsa-miR-497-5p hsa-miR-125a-5p hsa-miR-6838-5p hsa-miR-15b-5p hsa-miR-195-5p hsa-miR-125b-5p hsa-miR-4319 |
| *DYNLL2* | hsa-miR-193a-3p hsa-miR-148a-3p hsa-miR-139-5p hsa-miR-329-3p hsa-miR-330-3p hsa-miR-296-5p hsa-miR-19b-3p hsa-miR-129-2-3p hsa-miR-204-5p hsa-miR-4644 hsa-miR-1306-5p hsa-miR-130a-3p hsa-miR-130b-3p hsa-miR-150-5p hsa-miR-6088 hsa-miR-181c-5p hsa-miR-185-5p hsa-miR-148b-3p hsa-miR-193b-3p hsa-miR-195-5p hsa-miR-200a-3p hsa-miR-211 -5p hsa-miR-454-3p hsa-miR-331-3p hsa-miR-141-3p hsa-miR-103a-3p hsa-miR-129-1-3p hsa-miR-212-3p hsa-miR-328-3p hsa-miR-301b-3p hsa-miR-3666 hsa-miR-4295 hsa-miR-532-3p hsa-miR-152-3p hsa-miR-181d-5p hsa-miR-107 hsa-miR-132-3p hsa-miR-6835-3p |

cells, plays an important role in maintaining stable germ cell intercellular bridges during spermatogenesis and spermiogenesis in mice (25). In one study, the expression level of CEP55 in patients with maturation arrest was significantly lower than in patients with normal spermatogenesis (36). However, CEP55 overexpression

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**Table 5** Several hub genes play a functional role in spermatogenesis

| Gene | Function | Reference |
|---|---|---|
| *CCNB2* | G2/M transition in both mitosis and meiosis | Baker *et al.* (19) |
| *HMMR* | Sperm count, motility and number of sperm with normal morphology | Abu-Halima *et al.* (20) |
| *Nek2* | G2/M phase transition of the cell cycle | Matsuoka *et al.* (21) |
| *TTK* | Mitotic checkpoint activity | Poss *et al.* (22) |
| *PLK4* | Necessary for centriole duplication | Bettencourt-Dias *et al.* (23) |
| *PBK* | In the outer cell layer of spermatogenic tubules | Zhao *et al.* (24) |
| *CEP55* | Maintaining stable germ cell intercellular bridges | Chang *et al.* (25) |
| *CDC25C* | G2/M phase transition of the cell cycle | Nilsson *et al.* (26) |
| *DNAI1* | Ciliary function and ultrastructure | Escudier *et al.* (27) |

causes change in the proportion of germ cells in mice, and it manifests as a Sertoli-cell-only–tubule phenotype, resulting in mouse infertility (37). Thymidylate synthase (TYMS) converts deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP) using a 5,10-methylenetetrahydrofolate cofactor (38). The TYMS mutation has been associated with failed post-implantation development of the embryo in mice (38). DNAI1 and DNAI2 are members of the dynein intermediate chain family, and their mutations can lead to abnormalities in respiratory ciliary function and ultrastructure, which are important mechanisms of primary ciliary dyskinesia (PCD) (27). DNAI1 is also highly expressed in the testes (39). In male patients with PCD, infertility can be caused by sperm tail dysmotility (40). The DNAI1 mutation in male *Drosophila* has been associated with infertility caused by motile sperm (41). However, DNAI2 has not been reported in the literature with regard to spermatogenesis. Nine other hub genes, namely *DYNLL2*, *KIF15*, *DLGAP5*, *NUF2*, *PTTG1*, *CDKN3*, *MCM4*, *PPP2R1B*, and *DYNLRB2*, have also not been reported.

We compared the expression levels of 20 hub genes in the two subgroups (MA and PreMA) of NOA in the GSE108886 dataset and found no statistical difference. This suggests that these hub genes are not affected by NOA classification. This might have been due to the small sample size in the subgroup. A larger number of samples may be needed to confirm this.

In our study, we used WebGestalt for KEGG analysis of 20 hub genes and identified other pathways, including FoxO signaling pathway and Huntington disease. Studies have shown that these pathways are also related to spermatogenesis (42,43).

*DYNLL2*, *CEP55*, and *PPP2R1B* are three important target genes in the miRNA-gene regulatory network. CEP55 is described above. DYNLL2 was not involved in any KEGG pathways but could be regulated by most miRNAs, while PPP2R1B was involved in the oocyte meiosis pathway. Among these miRNAs, hsa-miR-3666, hsa-miR-130b-3p, hsa-miR-15b-5p, hsa-miR-6838-5p, and hsa-miR-195-5p have garnered the most research attention. However, these five miRNAs have not been studied in NOA, and further research is required.

A limitation in our study is that the potential miRNAs and hub genes in NOA need to be elucidated through experiments. Further research is required to verify and explore this in-depth.

## Conclusions

In summary, this study involved a comprehensive bioinformatics analysis of DEGs between NOA and OA tissues, and successfully screened 20 hub genes, namely *CCNB2*, *DYNLL2*, *HMMR*, *NEK2*, *KIF15*, *DLGAP5*, *NUF2*, *TTK*, *PLK4*, *PTTG1*, *PBK*, *CEP55*, *CDKN3*, *CDC25C*, *MCM4*, *DNAI1*, *TYMS*, *PPP2R1B*, *DNAI2*, and *DYNLRB2*. We also predicted some potential miRNAs of hub genes including hsa-miR-3666, hsa-miR-130b-3p, hsa-miR-15b-5p, hsa-miR-6838-5p, and hsa-miR-195-5p. Some important pathways, including focal adhesion, PI3K-Akt signaling pathway, cell cycle, oocyte meiosis, AMPK signaling pathway, FoxO signaling pathway, and Huntington disease, are involved in spermatogenesis. Our results may provide a more detailed understanding of the molecular

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mechanism of NOA and offer potential therapeutic targets for its treatment.

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## Footnote

*Reporting Checklist:* The authors have completed the STROBE and MDAR reporting checklists. Available at http://dx.doi.org/10.21037/tau-20-1029

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tau-20-1029). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. As all the data in this study were from the GEO public database, the approval of the local ethics committee was not required.

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