Identification of key gene biomarkers and pathways related to Dent disease in CLCN5 knockout mice by bioinformatics analysis

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Abstract

Background Dent disease is an X-linked inherited renal disease that occurs almost exclusively in males. Abnormal CLC-5 function might play a role in the development of Dent disease, but the genetic interaction changes and biomarkers in Dent disease are not fully understood. The aim of this study was to explore the potential key gene biomarkers and pathways related to Dent disease in CLCN5 knockout mice model.

Methods The gene expression profile GSE10162 was analyzed differentially expressed genes (DEGs), between 3 samples of CLC-5 transporter gene knockout mouse model of Dent disease and 3 samples from wild type mouse. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied for the enriched pathway by the online tool DAVID. A protein-protein interaction (PPI) network of DEGs was constructed to find the hub genes by STRING, and visualized with Cytoscape software.

Results Three samples from were incorporated into this study. A total of 500 DEGs were filtered, consisting of 231 upregulated genes and 269 downregulated genes. GO analysis indicated that the up-regulated DEGs were significantly enriched in the regulation of transcription form RNA, regulation of cell proliferation, and ion transport, whereas down-regulated genes were mainly enriched in oxidation-reduction process, and metabolic process. KEGG analysis demonstrated that the DEGs were enriched in the metabolic pathways, neuroactive ligand-receptor interaction, nicotine addiction, morphine addiction, fatty acid elongation, TNF signaling pathway, calcium signaling pathway, and cAMP signaling pathway. PPI network analysis found 17 hub genes with greater than 10 degrees of connectivity. The hub genes might participate in TNF signaling pathway, fat digestion and absorption, and enrich in lipid metabolic process, regulation of blood pressure, cellular response to hypoxia, positive regulation of angiogenesis, positive regulation of developmental growth, and positive regulation of cytosolic calcium ion concentration.

Conclusions Our study suggests that Apob, Lep, C3, Cxcl1, Acly and Mmp9 may play key roles in the progression of Dent disease. Blood lipid profiles and calcium levels might be potential prognostic biomarkers for Dent disease.

Background

Dent disease, a rare an X-linked disorder of proximal renal tubular dysfunction\(^1\), was first described in
1964. It is characterized by low-molecular-weight proteinuria (LMWP), hypercalciuria, nephrocalcinosis, kidney stones, renal failure, and rickets. The exact prevalence of Dent disease is unknown; to date, >250 families have been described. Dent disease is inherited in an X-linked manner. The father of an affected male will not have the disease nor will he be hemizygous for the pathogenic variant. Molecular genetic testing is often used for Dent disease diagnosis; include single-gene testing, use of a multigene panel, and more comprehensive genomic testing. Patients with Dent disease often carry mutations in genes encoding the Cl-/H+ exchanger CLC-5 and/or inositol polyphosphate 5-phosphatase (OCRL1). About 60% of patients with Dent disease are found to have a CLCN5 mutation, 15% have an OCRL mutation, and in the remaining 25% a mutation cannot be identified. Gene mutations in Dent disease have been identified at least 20 sites in the CLC-5 channel sequence, which might lead to the formation of nonfunctional channels. The various types of loss-of-function were found since the mutation of the OCRL gene with nearly 200 different genetic alterations. But, the physiologic function of CLC-5 and OCRL1 is not well known, and also, the pathway and mechanism of these genes are not known. Moreover, the phenotype of Dent disease varies and the phenotype can overlap with other kidney disease, which can make diagnosis difficult. Thus, the disease is likely under-diagnosed or diagnosed in late stage. So far, the biochemical effects of these mutations and the pathogenic mechanism are not fully understood.

Bioinformatics is a multidisciplinary field that can provide useful methods to identify and analyze the associations and molecular mechanisms among key genes and central signaling pathways. Gene expression profiling analysis is a useful method with broad clinical application for identifying gene changes, from molecular diagnosis to pathological classification. In this study, we downloaded an original microarray dataset containing CLCN5 Knockout samples and wild type mouse samples from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). Then, we screened the gene expression profiles of CLCN5 Knockout samples and wild type mouse samples in order to identify
the differentially expressed genes (DEGs) and hub genes through a DEG interaction network. We subsequently performed the functional enrichment analyses, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and protein-protein interaction (PPI) network, by online software. After analyzing the biological functions and pathways, we further explored the potential biomarkers for diagnosis and prognosis by survival analysis. This study may offer better insight into potential molecular mechanisms for Dent disease, allowing the exploration of novel therapeutic strategies.

Results

Identification of DEGs in Dent Disease

A GSE10162 data profile was downloaded from NCBI GEO with 21723 genes in the microarray data. GEO2R was used to identify the DEGs from the expression profile data P value <0.05. A total of 500 DEGs in the proximal tubule tissues of CLCN5 knockout mice compared with wild type mice, including 231 upregulated DEGs and 269 downregulated DEGs (Supplement Table 1). Volcano plot was generated to show the correlation between DEGs (Figure 1). Top 10 up-regulated DEGs were Slc10a2, Tmprss11g, March2, LOC102639518, BC018473, Ear12///Ear3///Ear2///Ear1, Ankrd1, Gdpd3, Pde4b, and Acly. Top 10 down-regulated DEGs were Cyp4a14, Lmod1, Gm8096///Phgdh, Rnase4, B3gnt3, Hmgcs2, 9130214F15Rik, Gm31210, Smarce1, and 4930579H20Rik, and the cluster heat map of the top 20 genes is shown in Figure 2.

GO analysis and signaling pathway enrichment of DEGs in Dent Disease

All DEGs were uploaded to the DAVID website to analyze the GO terms enrichment. The GO term analysis included the MF, BP and CC group respectively. The results were considered statistically significant if P <0.05 and gene counts ≥10. The GO enrichment analysis of the up-regulated and down-regulated differential genes is shown in Table 1 and Figure 3. The up-regulated genes were mainly enriched in negative regulation of transcription from RNA polymerase II promoter (ontology: Biological Process, BP), dendrite (ontology: Cellular Component, CC), and protein binding (ontology: Molecular Function, MF); the down-regulated genes were mainly enriched in oxidation-reduction
process (ontology: BP), extracellular region (ontology: CC) and oxidoreductase activity (ontology: MF).

**KEGG pathway analysis of DEGs**

A KEGG pathway analysis of the DEGs was also conducted with the online DAVID database, and the results of significantly enriched pathway (P value <0.05) are shown in Table 2. The results of the pathway enrichment analysis indicated that the pathways of the DEGs were mainly enriched in eight pathways: Metabolic pathways, Neuroactive ligand-receptor interaction, Nicotine addiction, Morphine addiction, Fatty acid elongation, TNF signaling pathway, Calcium signaling pathway, and cAMP signaling pathway.

**Integration of protein-protein interaction (PPI) network and hub gene Analysis**

The STRING online database was used to identify the interactions and key genes of the DEGs and to dissect the PPI networks. The results were downloaded and analyzed using Cytoscape software. There were 119 nodes and 692 edges in the PPI network, which represented proteins and interactions (Figure 4). The top 17 hub genes were screened according to their degree values and the identified hub genes in were Apob, Lep, C3, Cxcl1, Acly, Mmp9, Hmgcs2, Sox2, Edn1, Hmox1, Plk1, Fabp1, Ftcd, Ttr, Cyp4a14, Mchr1, and Spp2, as shown in Table 3. The hub genes were again analyzed by DAVID. GO analysis showed that the 17 hub genes enriched in lipid metabolic process, regulation of blood pressure, cellular response to hypoxia, positive regulation of angiogenesis, positive regulation of developmental growth, and positive regulation of cytosolic calcium ion concentration. KEGG pathway analysis showed that the 17 hub genes might participate in the TNF signaling pathway, fat digestion and absorption.

**Discussion**

Dent disease is a chronic kidney disorder that occurs almost exclusively in males. Genetics plays a strong role in urinary stone pathogenesis. Patients with Dent's disease often carry mutations in genes of CLCN5 or OCRL1 that are located on chromosome. A gene mutation can affect different genes the cell that influence physiological, morphological and pathological variation. Previous
studies indicate that Dent's disease may be associated with aminoaciduria, phosphaturia, glycosuria, uricosuria, kaliuresis, and impaired urinary acidification, and is often complicated by rickets or osteomalacia. The clinical diagnosis of Dent's disease is based on urinary LMWP, hypercalciuria, and at least one of the following: nephrocalcinosis, kidney stones, hematuria, hypophosphataemia, or renal insufficiency. The clinical diagnosis is confirmed by genetic tests of the identification of mutation in either CLCN5 or OCRL. The CLC-5 chloride channel knock-out mouse was established as an animal model for Dent's disease. This mouse model had elevated serum 1alpha,25-dihydroxyvitamin D3, alkaline phosphatase, osteocalcin, and urinary deoxypyridinoline that similar with the serum parameters of Dent disease patients.

In this study, the original microarray analysis showed that hundreds of genes are expressed differentially in the proximal tubule samples of the Dent related CLCN5 knockout mice compared with the wild-type mice, 8-week-old. A total of 500 DEGs were identified from dataset of GSE10162 by GEO2R analysis. These DEGs were then subjected to BP, CC and MF enrichment analysis. The up-regulated genes were mainly enriched in negative regulation of transcription from RNA polymerase II promoter (ontology: BP), dendrite (ontology: CC), and protein binding (ontology: MF), and the down-regulated genes were mainly enriched in oxidation-reduction process (ontology: BP), extracellular region (ontology: CC) and oxidoreductase activity (ontology: MF). KEGG pathway analysis showed the down-regulated genes were mainly enriched in metabolic pathways and fatty acid elongation, and the up-regulated genes were mainly enriched in neuroactive ligand-receptor interaction, nicotine addiction, and morphine addiction, TNF signaling pathway, calcium signaling pathway, and cAMP signaling pathway and oxidation-reduction process.

In addition to the pathway discussed above, the candidate hub genes were identified through PPI network construction and analysis, and ranking the hub genes by degree of connectivity. Our results indicated that 17 hub genes had significant correlations with Dent disease. The 17 hub genes enriched in lipid metabolic process, regulation of blood pressure, cellular response to hypoxia, positive regulation of angiogenesis, positive regulation of developmental growth, and positive
regulation of cytosolic calcium ion concentration. KEGG pathway analysis showed that the 17 hub genes might participate in the TNF signaling pathway, fat digestion and absorption. The first hub genes are Apob and Lep, which serve as critical function in lipid metabolism. Apob gene encodes the main apolipoprotein of chylomicrons and low density lipoproteins. Lep gene encodes a protein that is secreted by white adipocytes into the circulation and plays a major role in the regulation of energy homeostasis, such as the regulation of energy balance and body weight control. The gene C3, a Protein Coding gene, plays a central role in the activation of the complement system. There were some research indicated that C3 had relationship with acylation stimulating protein, which can stimulate triglyceride (TG) synthesis and glucose transport in adipocytes. The Cxcl1 gene encoded protein is a secreted growth factor that signals through the G-protein coupled receptor and CXC receptor 2. The Acly gene encodes the primary enzyme (ATP citrate lyase), which responsible for the synthesis of cytosolic acetyl-CoA in many tissues. The Mmp9 gene is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Based on gene function description of the top five ranking hub genes, we could find them playing important roles in lipid metabolism.

These results suggested that these DEGs are involved in the lipid metabolism, calcium signaling pathway and. Dent disease has multiple defects attributed to proximal tubule malfunction including LMWP, hypercalciuria, aminoaciduria, phosphaturia, glycosuria, and nephrolithiasis, but symptoms of Dent’s disease vary by patient. Some patients have typical symptoms such as kidney stones, nephrocalcinosis, hypercalciuria; and some patients exhibit other typical symptoms of nephrotic syndrome, including hypoproteinemia, hyperlipemia, or edema. Scheinman SJ’s study indicated that inactivation of CLCN5 can be found in the setting of hypercalciuria. However, Dent's disease is often underdiagnoses since the mild clinical and biochemical signs, and unobvious of the X-linked inheritance.

Some limitations exiting in our studies should be mentioned. There is only one available dataset from
CLCN5 knockout mouse model in online GEO database. In this study, we conducted bioinformatics analysis with GO and KEGG pathway enrichment, and PPI network construction. All these analysis was based on the gene expression data from the comparison between 3 CLCN5 knockout mouse samples and 3 wild type mouse samples. In DEG identification, we identified 500DEGs with P value <0.01 and a \(|\log_2\text{fold change (FC)}| \geq 1\) as the thresholds, and 63 DEGs with adjust P value <0.05 and a \(|\log_2\text{fold change (FC)}| \geq 1\) as the thresholds. Comparing to these two methods, the 63 DEGs were also ranked in the top 63 list DEGs screened by p value. It is acceptable to use the p value instead adjust p value to identify the DEGs, since the small number of sample size. Multi-central and large sample studies on Dent disease expression profiles were needed for validation of our findings in the future. Integrating more omics data such as epigenetic or epidemiological data would help illustrate the genetic, epigenetic, and environmental factors for Dent disease. Moreover, the lack of experimental validation is another limitation of our research. And further experimental research is needed to verify these findings with a larger sample size.

Conclusions
The present study, we presumed these key genes and pathways identified by a series of bioinformatics analyses on DEGs between CLCN5 knockout mouse samples and wild type mouse samples. The results showed that several pathways are altered and numerous hub genes. The hub genes and the enriched pathways indicated lipid and calcium might be importantly associated with the pathogenesis of Dent disease. According to the study, down-regulation of Apob and Mmp9, up-regulation of Lep, C3, Cxcl1 and Acly might be considered as biomarkers or therapeutic targets for Dent disease. The blood lipid profiles and urinary calcium may be valuable as prognostic markers for Dent disease patients. This study provided new insight for the understanding of molecular mechanisms in Dent disease. However, further experiments are required to confirm and validate these predicted results.

Methods
**Microarray data information**
The aim of this study was to explore the potential key gene biomarkers and pathways related to Dent
disease in CLCN5 knockout mice model from public database. Raw gene expression data of GSE10162 used in the present study was downloaded from the National Center of Biotechnology Information Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10162). The original gene expression profile was contributed by Wright JM et al which included 6 mouse kidney proximal tubule samples from 3 wild type (GSM256956, GSM256957,) and 3 CLCN5 knockout mice (GSM256958 GSM256959, GSM256960, GSM256961) 23. This dataset comprised from and was generated from renal proximal tubule samples using the platform [Mouse430_2] Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). The microarray of GeneChip initial expression analysis was performed using the GCOS 1.2 software (Affymetrix, Santa Clara, CA). The 3′-to-5′ ratio for GAPDHMur was confirmed to be <2 for all six chips used in the analysis23.

**Data preprocessing and identification of DEGs**

GEO2R is an interactive online analysis tool based on R language that allows users to compare two or more groups of samples in a GEO series in order to identify genes that are differentially expressed across experimental conditions 31. The 6 mouse kidney proximal tubules samples were classified into two groups that had similar expression patterns. We analyzed the raw data by GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r/). Statistically significant DEGs were defined with $P$ value $<0.01$ and a $|\log_2$ fold change (FC)| $\geq1$ as the thresholds described in the references$^{32-34}$.

**Gene Function Enrichment Analysis of DEGs**

Gene Ontology (GO) enrichment analysis of DEGs was implemented via a common functional annotation tool DAVID (https://david.ncifcrf.gov/). All genes in the genome were used as the enrichment background. $P$ values were calculated based on accumulative hypergeometric distribution, and q-values were calculated using the Benjamini-Hochberg procedure to account for multiple testing. GO enrichment analysis was performed in order to distinguish the biological
attributes, such as molecular function (MF), biological process (BP), and cellular component (CC) of important DEGs. The results were considered statistically significant if P values < 0.05 and gene counts ≥10. For each given gene list, pathway and process enrichment analyses were performed using the following ontology sources: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. The results were considered statistically significant if P values < 0.05.

**Protein-Protein Interaction (PPI) Network Construction and hub gene Analysis**

The PPI network was constructed using the Search Tool for the Retrieval of Interacting Genes online database (STRING Version: 11.0, https://string-db.org/)\(^{35}\), a biological predictive web resource including numerous proteins and known interactive functions. A combined score of >0.4 was designated as the cutoff standard. Genes with degrees >10 were selected as hub genes and sorted according to the ranking order of connectivity degree. The PPI network was visualized by the Cytoscape software (Version 3.7.1) (The Cytoscape Consortium, New York, NY), and module of PPI network was screened by the cytoHubba in Cytoscape.

**Abbreviations**

DEGs: Differentially Expressed Genes

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

DAVID: Database for Annotation, Visualization and Integrated Discovery software

PPI: Protein-Protein Interaction

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

LMWP: Low-Molecular-Weight Proteinuria

BP: Biological Process

MF: Molecular Function

CC: Cellular Component.

**Declarations**

**Ethical approval and consent to participate**
Not applicable. This article does not contain any studies with human participants or animals performed by any of the authors. We performed all bioinformatic procedures based on online public database.

**Consent for publication**

Not applicable.

**Availability of data and material**

The gene expression profile GSE10162, used and/or analyzed during the current study, was downloaded from the largest fully public online free database (The GEO database - NCBI - NIH, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10162).

**Competing interests**

The authors declare that they have no competing interests.

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**Contributions**

ZJ LIN performed the data analysis and wrote the manuscript and paper submission. Y WANG performed double check of the data analysis and revised the manuscript draft. FF GUO and B ZHANG
revised the manuscript. All authors have read and approved the manuscript.

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**Tables**

Table 1: Gene ontology analysis of DEGs in Dent disease
| Expression     | Category   | Term                                                                 |
|---------------|------------|----------------------------------------------------------------------|
| Up-regulation | BP_DIRECT  | GO:0000122—negative regulation of transcription from RNA polymerase II promoter |
|               | BP_DIRECT  | GO:0007275—multicellular organism development                        |
|               | BP_DIRECT  | GO:0008284—positive regulation of cell proliferation                 |
|               | BP_DIRECT  | GO:0006811—ion transport                                            |
|               | CC_DIRECT  | GO:0030425—dendrite                                                |
|               | CC_DIRECT  | GO:0005737—cytoplasm                                               |
|               | CC_DIRECT  | GO:0005783—endoplasm reticulum                                     |
|               | CC_DIRECT  | GO:0030424—axon                                                    |
|               | CC_DIRECT  | GO:0005887—integral component of plasma membrane                    |
|               | CC_DIRECT  | GO:0005576—extracellular region                                     |
|               | CC_DIRECT  | GO:0016020—membrane                                               |
|               | CC_DIRECT  | GO:0042995—cell projection                                         |
|               | MF_DIRECT  | GO:0005515—protein binding                                         |
|               | MF_DIRECT  | GO:0042803—protein homodimerization activity                       |
|               | MF_DIRECT  | GO:0043565—sequence-specific DNA binding                            |
|               | MF_DIRECT  | GO:0003700—transcription factor activity, sequence-specific DNA binding |
| Down-regulation| BP_DIRECT  | GO:0055114—oxidation-reduction process                             |
|               | BP_DIRECT  | GO:0008152—metabolic process                                        |
|               | CC_DIRECT  | GO:0005576—extracellular region                                     |
|               | CC_DIRECT  | GO:0005615—extracellular space                                      |
|               | CC_DIRECT  | GO:0005829—cytosol                                                 |
|               | MF_DIRECT  | GO:0016491—oxidoreductase activity                                 |
|               | MF_DIRECT  | GO:0046982—protein heterodimerization activity                     |

Notes: BP, biological process. CC, cellular component. MF, molecular function.

**Table 2. KEGG pathway enrichment analysis of the DEGs**
| Pathway                          | ID        | Gene Count | P Value   | Genes                                                                 |
|---------------------------------|-----------|------------|-----------|------------------------------------------------------------------------|
| * Metabolic pathways            | mmu01100  | 23         | 0.0014    | ETNPPL, CYP24A1, COX11, FUT7, PIK3C2A, ASNS, GCH1, ACOT3, FDFT1, MTHFD2, GA, UGT2B34, AGPAT9, PKLR, CYP4A31, PHGE, CYP4A14, MTMR7 |
| *Fatty acid elongation          | mmu00062  | 3          | 0.0231    | ELOVL1, ACOT1, ACOT3                                                  |
| # Neuroactive ligand-receptor interaction | mmu04080  | 10         | 0.0021    | LEP, MCHR1, CRHR2, PTGER1, ADRB2, GA, GDKRB2, GABRQ                  |
| # Nicotine addiction           | mmu05033  | 4          | 0.0073    | SLC32A1, GABRA6, CACNA1A, GABRQ                                      |
| # Morphine addiction           | mmu05032  | 5          | 0.0139    | SLC32A1, GABRA6, PDE4B, CACNA1A, GA                                   |
| # TNF signaling pathway        | mmu04668  | 5          | 0.0234    | CXCL1, VCAM1, SOCS3, MMP9, EDN1                                       |
| # Calcium signaling pathway    | mmu04020  | 6          | 0.0339    | PTGER1, ADRB2, HTR4, RYR2, BDKRB2, C.                                 |
| # cAMP signaling pathway       | mmu04024  | 6          | 0.0470    | ADRB2, ROCK1, PDE4B, HTR4, RYR2, ARA                                   |

Note: * represents the down-regulated gene pathway; # represents the up-regulated gene pathway

| Table 3. Top 17 hub genes with higher degree of connectivity |
|-------------------------------------------------------------|
| Rank | Gene Name | Description | Degree |
|------|-----------|-------------|--------|
| 1    | Apob      | apolipoprotein B | 24     |
| 1    | Lep       | leptin      | 24     |
| 3    | C3        | complement 3 | 19     |
| 4    | Cxcl1     | chemokine (C-X-C motif) ligand 1 | 17     |
| 5    | Acly      | ATP citrate lyase | 16     |
| 5    | Mmp9      | matrix metalloproteinase 9 | 16     |
| 7    | Hmgs2     | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 | 15     |
| 7    | Sox2      | SRY (sex determining region Y)-box 2 | 15     |
| 9    | Edn1      | endothelin 1 | 14     |
| 9    | Hmox1     | heme oxygenase 1 | 14     |
| 11   | Plk1      | polo like kinase 1 | 13     |
| 12   | Fabp1     | fatty acid binding protein 1 | 12     |
| 12   | Ftdc      | formiminotransferase cyclodeaminase | 12     |
| 12   | Ttr       | transthyretin | 12     |
| 15   | Cyp4a14   | cytochrome P450, family 4, subfamily a, polypeptide 14 | 11     |
| 15   | Mchr1     | melanin-concentrating hormone receptor 1 | 11     |
| 15   | Spp2      | secreted phosphoprotein 2 | 11     |
Volcano plot of differentially expressed genes. Red, green and black denote up-regulated, down-regulated and nonsignificant expressed genes, respectively.
Figure 2

The heat maps of the top 20 DEGs. Each row represents a gene, and each column represents a sham control or a CLCN5 knockout mouse. Each colored square shows the relative change of a single gene in a single mouse. The red color (score=100) indicates highest and green color (score=0) indicates lowest expression levels.
Figure 3

Gene ontology (GO) annotation and enrichment analysis of 500 DEGs. The horizontal axis shows the negative log 10 of the P-Value, while the vertical axis represents biological process, molecular function, and cellular component, respectively.
Figure 4
Protein-protein interaction network of DEGs and hub genes. (A): The PPI network for Dent disease based on overall DEGs constructed by Cytoscape. (B): the PPI network of top 17 hub genes with high connectivity degree constructed by STRING. Notes: Red indicates the upregulated DEGs. Green indicates the downregulated DEGs. Abbreviations: DEGs, differentially expressed genes; PPI, protein–protein interaction.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
Final Supplementary material Tables 0824.docx