

Bioinformatics Analysis of Differentially Expressed Genes Involved in Irritable Bowel Syndrome With Diarrhea

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Research article

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Abstract

Purpose: Irritable bowel syndrome with diarrhea (IBS-D) is a common functional gastrointestinal disorder around the world. However, the molecular mechanisms of IBS-D are still not well understood. This study was designed to identify key biomarkers and immune infiltration in the rectal mucosa of IBS-D by bioinformatics analysis.

Methods: The gene expression profiles of GSE36701 were downloaded from the GEO database. The differentially expressed genes (DEGs) were identified and functional enrichment and pathway analyses were performed. Using STRING and Cytoscape, protein-protein interaction (PPI) networks were constructed and core genes were identified. Subsequently, 22 immune cell types of IBS-D tissues were explored by the Cell type Identification by Estimating Relative Subsets of RNA Transcripts. Finally, the co-expression network of DEGs was estimated by the weigh gene co-expression network analysis method to identify IBS-D-related modules and deeply hub genes.

Results: 224 up-regulated and 171 down-regulated genes in IBS-D patients: Our analysis indicated that several DEGs might play crucial roles in IBS-D, such as CDC20, UBE2C, AURKA, CDC26, CKS1B and PSMB3. Later, we found that immune infiltrating cells such as T cells CD4 memory resting, M2 macrophages are crucial in IBS-D progression. In the end, a total of 9 co-expression gene modules were calculated and the black module was found to have the highest correlation. 15 hub genes were identified both in DEGs and the black module.

Conclusions: This study identified molecular mechanisms and a series of candidate genes as well as significant pathways from the bioinformatics network, which may provide a diagnostic method and therapeutic targets for IBS-D.

Introduction

Irritable bowel syndrome (IBS) is a frequent functional bowel disorder which is the characteristics of abdominal discomfort and change in bowel evacuation habit [1]. IBS is one of the most common gastrointestinal diseases causing the decline of quality of life and the reason for patients to seek medical advice. Compared with the general population, the physical functioning, sense of well-being, social functioning of IBS patients are significantly limited, accompanied by obvious discomfort, pain and fatigue. It exerts an enormous economic burden and takes responsibility for a considerable high incidence rate worldwide [2–4]. The ultimate cause of IBS is not yet completely understood. It seems to be multifactorial and many pathogenic factors can play a significant role (1). IBS can be classified into three predominant subtypes: IBS with constipation; IBS with diarrhea (IBS-D); mixed IBS. It was reported that 31%-48% of IBS was IBS-D, whose prevalence was higher than the other two subtypes [5].

Studies have shown that the richness of the intestinal microbiota in the luminal niche has been found to reduce in IBS-D patients [6, 7]. Altered gastrointestinal motility, visceral hypersensitivity, post inflectional reactivity, brain-gut interactions, alteration in fecal microflora, bacterial overgrowth, food sensitivity,

carbohydrate malabsorption, and intestinal inflammation all have been implicated in the pathogenesis of IBS [8]. Also, IBS-D may be associated with increased platelet depleted plasma 5-HT concentrations [9, 10]. However, the mechanisms of gene and protein expression in IBS-D are still unclear.

Microarray analyses have been increasingly used to explore the pathogenic processes of several diseases and identify disease-associated genes and pathways, which presents as an important technology for the prevention, diagnosis and treatment of diseases [11]. Therefore, the IBS-D-related genes downloaded from the GEO database provide genetic support for future research and recognition of IBS-D.

Material And Methods

Microarray data screening

Gene expression profile dataset GSE36701 [12] was downloaded from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). The data was produced using the GPL570 [HG-U133 Plus 2] Affymetrix Human Genome U133 Plus 2.0 Array. The GSE36701 dataset contained data from 93 volunteers, including 40 healthy control (HC) and 53 IBS-D patients.

Data processing and DEG screening

The downloaded platform and series of matrix files were preprocessed using the Affymetrix package [13] in R version 4.0.3 language software (<cran.at.r-project.org>). The limma package in the Bioconductor package [14] (<http://www.bioconductor.org/>) is used for Gene differential expression analysis [15]. The P-value of each gene symbol was normalized by R software using the Limma package and then saved as a TXT file. Only the difference between DEGs with adjusted- $P < 0.05$ and $|\log FC(\text{fold change})| > 1$ was statistically significant.

GO and KEGG pathway analysis of DEGs

Gene Ontology (GO) [16] (www.geneontology.org) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [17] (www.genome.ad.jp/kegg) are essential tools to systematically extract the pathway information from molecular interaction networks and identify the differentially expressed genes (DEGs). R was widely used to perform GO and KEGG pathway analysis to determine the biological significance of DEGs. Adjusted- $P < 0.05$ was considered a statistically significant difference.

PPI network and functional modules screening

PPI networks are mathematical analysis of interactions between proven protein and predicted protein using The Search Tool for the Retrieval of Interaction Genes (STRING) database [18] (<http://string-db.org/>), which provide information to construct the PPI networks by selecting protein interaction with a combined score > 0.4 . Besides, the PPI networks were visualized using Cytoscape software [19] (<http://www.cytoscape.org>) and the cytohubba in Cytoscape software was used to screening the hub genes. Ranked by MCC, the top 10 genes were colored marked in the pictures.

Estimation of CIBESORT

To study the proportions of immune cells in the IBS-D samples, the standard annotated gene expression data were analyzed by the CIBESORT [20] (<http://cibeersort.stanford.edu/>). CIBESORT uses Monte Carlo sampling to derive a P-value for the deconvolution of each sample. The CIBESORT is at a threshold of adjusted- $P < 0.05$, indicating that the result of the inferred fraction of immune cell populations are accurate and the samples are considered available for subsequent analysis [21]. No further analysis is made for the cells with a matrix expression of 0.

Co-expression network construction by WGCNA

The co-expression network analysis was performed to identify IBS-D-related modules using weigh gene co-expression network analysis (WGCNA) [22]. First of all, select the soft threshold for network construction. The soft threshold makes the adjacency matrix a continuous value between 0 and 1. Based on the soft threshold, generate a scale-free topology plot and then identify gene co-expression modules by the module partition analysis. The module partition analysis can group genes that have similar expression patterns. Dynamic tree cut method algorithm was used to define the selected modules and color to indicate modules [23]. Module eigengenes (ME) is used to analyze the major component of genes with the same expression profile in the module. The relationship between ME and the clinical traits in each module was analyzed. Then, the gene significance (GS) in the module was further calculated to manifest the correlation between gene and sample.

Results

The microarray data information and identification of DEGs in IBS-D

The IBS-D expression microarray datasets GSE36701 were downloaded from GEO and normalized by the limma package, and the results were shown in figures 1a and 1b. 395 DEGs were obtained using the limma package (adjusted- $P < 0.05$, $|\log FC| > 1$), including 224 up-regulated genes and 171 down-regulated genes. The up-regulated genes and down-regulated genes in the top 50 were shown in Table 1. The volcano of total DEGs between the HC and IBS-D sample data in GSE36701 is shown in figure 2. The cluster heatmap of the top 100 DEGs was shown in figure 3. The top 10 up-regulated and down-regulated DEGs identified between HC and IBS-D tissue data were shown in Table 2.

Gene Ontology (GO) enrichment

To identify the biological function of the DEGs, the R software was used to integrate microarray data with GO term enrichment and divided into up-regulated and down-regulated genes with an adjusted- P -value of < 0.05 . In general, Go enrichment includes three functional groups: biological processes (BP), molecular function (MF) and cellular component (CC). The enriched biological process was shown in figure 4a and figure 4b. The significant DEGs analysis results were demonstrated in Table 3. In the biological process group, the up-regulated genes are mainly concerned with RNA and mRNA splicing, rRNA and ncRNA

processing, protein-containing complex localization, regulation of cellular amide metabolic process RNA localization. The down-regulated genes were mainly played roles in the protein catabolic process, regulation of chromatic segregation, protein-DNA complex assembly, regulation of mitotic nuclear division and positive regulation of ubiquitin protein ligase activity. In the cellular component group, the up-regulated genes mainly concerned with nuclear speck, Cajal body, spliceosomal complex. The down-regulated genes were mainly enriched in ubiquitin ligase complex, transfer complex, protein-DNA complex and RNA polymerase complex. In the molecular function group, the up-regulated genes are mainly concerned with threonine-type activity, general transcription initiation factor activity and TBP-class protein binding. The down-regulated genes mainly played roles in small nucleolar RNA (snoRNA) binding, transferase activity, protein serine/threonine kinase activity and glycosyltransferase activity. These results indicated that DEGs significantly enriched in cell division, gene expression, transcription and translation activity as well as protein catabolism.

KEGG pathway analysis

The pathways of these DEGs were identified by KEGG pathway analysis. The significantly enriched pathway concerned with IBS-D were 'Spliceosome'(22%), 'Human T-cell leukemia virus 1 infection'(26%), 'Progesterone-mediated oocyte maturation'(16%), 'Oocyte meiosis'(18%) and 'Ubiquitin mediated proteolysis'(18%), the results were shown in figure 5a and 5b and Table 4.

PPI network construction and analysis

Up- and down-regulated PPI networks of DEGs were built and visualized based on the STRING database. Among the 395 DEGs, 141 DEGs were filtered into the PPI network complex of DEGs, including 70 up-regulated genes and 71 down-regulated genes. In addition, there were 20 nodes and 166 edges of 20 up-regulated DEGs in the PPI network, the results were shown in figure 6a. For the 20 down-regulated genes, there were 20 nodes and 254 edges, the results were shown in figure 6b. The top 10 up-regulated hub genes showing significant correlation were SRSF1, SNRNP70, SRSF6, HNRNPA2B1, HNRNPR, SRRM2, FUS, HNRNPU, CCAR1, PRPF3, the results were shown in figure 6c. The top 10 down-regulated hub genes were CDC20, UBE2C, AURKA, CDC26, CKS1B, PSMB3, PTTG1, CCNB, PSMA2, PSMB9, the results were shown in Figure 6d.

The distribution of immune infiltration in IBS-D

Based on the CIBERSORT algorithm, the immune infiltration cells (IICs) in IBS-D tissue in 22 subpopulations of immune cells were identified. The proportions of immune cells in each IBS-D tissue were shown in figure 7a. Different color represents different immune cells and the length of the bars in the barplot represent the proportion of the immune cell population. This barplot indicates that the percentage of T cells CD4 memory resting and M2 Macrophages, B cell memory, plasma cells of IBS-D tissue is relatively high, accounting for approximately 60% of the 22 immune cells. On the contrary, the percentage of T cell gamma delta, mast cells resting and Eosinophils of IBS-D tissue is relatively low, only accounting for approximately 8%. The unsupervised hierarchical clustering was used to identify the level

of 22 immune cells, the results were shown in figure 7b. The heatmap showed that the level of T cells CD4 memory resting, M2 macrophages, Plasma cells, B cells memory and Mast cells resting is relatively high of the 22 immune cells. The results of IICs found that M0 macrophages were not expressed in DEGs. Therefore, only the remaining 21 types of immune infiltrating cells were analyzed for their correlation. The quantified contrast of the distribution of IICs subsets between HC and IBS-D tissues was shown in figure 8a. The results indicate that the proportion of T cells CD8 ($p=0.03$) and Mast cells activated ($P=0.04$) is relatively high in HC tissues compared to IBS-D tissues. The results are shown in figure 8b. Based on the above results, the anomalous immune infiltration in IBS-D tissues and its nonuniformity and heterogeneity indicates that it may have vital significance to guide clinical practice as a tightly regulatory process.

Construction and analysis of WGCNA associate with IBS-D and HC

To find the hub genes, the WGCNA was used to identify the co-expression set of genes and modules. WGCNA analyzed 395 DEGs to explore the co-expression network. Pearson's correlation coefficient was used to perform the cluster analysis and drew a clustering tree shown in figure 9a. The power equal to 3 when the scale free² reached 0.9 was chosen as the soft threshold for further analysis. Then, the cluster dendrograms of HC and IBS-D tissues were performed to detect gene modules. The results were shown in figures 9b and 9c. 9 distinct co-expression modules were detected, including brown, blue, red, black, pink, green, magenta, yellow, turquoise. After correlation analysis, the black module ($cor=0.35, p=7 \times 10^{-4}$) is highly related to the pathological process of IBS-D, the module-trait relationships were shown in figure 10a. The correlation between each gene in the black module was identified by the scatter plot, the results were shown in figure 10b. Then, the 395 DEGs and 172 genes in the black module were superimposed to identify the hub genes, the results were shown in figure 10c. Finally, 15 hub genes were identified both in DEGs and the black module, including DFNB59, FLJ45513, GOLGA8A, HIST1H2AE, HIST1H3C, LINC00893, LOC100506114, LOC101927391, LOC101928068, LOC101929988, LOC286367, PFDN2, RP11-395I6.3, RP11-676J12.4, SEC31B.

Discussion

IBS is a chronic condition and the largest gastrointestinal clinical subgroup affecting 9% and 23% of the general population. It has the most significant impact on patients' work, life, health-care and society. The proportion of 15%-43% of patients has to pay for the treatment expense for immediate cure [8, 24, 25]. So far, researches about IBS-D focused on the multiple types of immune cells that infiltrated the intestinal mucosa and released inflammatory mediators, disrupting the intestinal epithelial barrier and nervous system signaling [26]. Several studies showed that low-grade was highly associated with the pathophysiology of IBS-D [27, 28]. However, the micro-level of the pathogenic mechanism of IBS-D remains doubtful because IBS-D has no structure and metabolic abnormalities accounting for its syndrome [29, 30]. Therefore, it is of significant importance to explore the gene and molecular mechanisms and development of the IBS-D to identify the underlying cause of the disease. The differentially expression genes (DEGs) of IBS-D have become a hot spot since the establishment of gene

database of TCGA, SEER and GEO. GEO is a comprehensive genes database of both tumor and non-tumor when TCGA and SEER only contain genes of tumor diseases. There, this study aims to explore the DEGs, protein-protein interaction (PPI) network, immune infiltrating cells and gene co-expression network so as to do further statistical analysis about IBS-D using the GEO database. GO and KEGG effectively clusters the functional genes into different biological processes to systematically analyze the gene function in the biological pathway [31, 32]. PPI network plays a crucial part in predicting the function of interacting genes or proteins as well as providing evidence for evolutionary conservation of gene interacting [33].

395 DEGs were obtained using the robust multiarray averaging algorithm, including 224 up-regulated genes and 171 down-regulated genes. We constructed a DEGs-encoded protein network and explored 10 up-regulated hub genes: SRSF1 (serine and arginine rich splicing factor 1), SNRNP70 (small nuclear ribonucleoprotein U1 subunit 70), SRSF6 (serine and arginine rich splicing factor 6), HNRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2/B1), HNRNPR (heterogeneous nuclear ribonucleoprotein R), SRRM2 (serine/arginine repetitive matrix 2), CCAR1 (cell division cycle and apoptosis regulator 1), FUS (FUS RNA binding protein), HNRNPU (heterogeneous nuclear ribonucleoprotein U), PRPF3 (pre-mRNA processing factor 3). And 10 down-regulated hub genes: CDC20 (cell division cycle 20), UBE2C (ubiquitin conjugating enzyme E2 C), AURKA (aurora kinase A), CDC26 (cell division cycle 26), CKS1B (CDC28 protein kinase regulatory subunit 1B), PSMB3 (proteasome 20S subunit beta 3), PTTG1 (regulator of sister chromatid separation, securin), CCNB (cyclin B), PSMA2 (proteasome 20S subunit alpha 2), PSMB9 (proteasome 20S subunit beta 9). Researches showed that the HNRNP is a specific selection of auto-antigens stimulating T cells by activating antigen-presenting cells with Toll-like receptor to initiate inflammation [34, 35]. Kalva S et al. [36] showed that FUS had an impact on the occurrence of IBS among the thousands of DEGs from the PPI network. Our research indicates that the biological process of RNA splicing and spliceosomal complex and the pathway of spliceosome play major roles in the pathogenic mechanism of IBS-D. Wohlfarth C et al. [37] signified that the reduction of miR-16 AND miR-10 weaken the function of 5-HT₄. The h5-HT₄ splicing variants were expressed in the human intestinal tract to regulate the intestinal function indifferent way [38]. Therefore, it is possible that the hub genes of SRSF1, SRSF6, SRRM2 and the pathway of the spliceosome and up-regulated gene ontology enrichment of RNA splicing and spliceosomal complex contribute to the expression of 5-HT receptor and cause IBS-D. In addition, the biological process of proteasomal protein catabolic process has a deep impact on the occurrence and development of IBS-D.

Researches showed that IBS-D patients had increased faecal serine protease activity which took a significant part in visceral hypersensitivity and gave rise to increased colonic paracellular permeability causing allodynia and diarrhea [39, 40]. Thus, the down-regulated hub genes of PSMB3, PSMA2, PSMB9 and gene ontology enrichment of proteasomal protein catabolic may inhibit the degradation of serine protease and may be a promising candidate in IBS-D pathophysiology. In addition, the down-regulated genes of CDC20, CDC26, CKS1B, PTTG1 and its biological process such as regulation of sister chromatid segregation, regulation of mitotic sister chromatid segregation, gene pathway of progesterone-mediated

oocyte maturation and Oocyte meiosis indicate that the reduction in cell mitosis and meiosis is the cause of IBS-D. El-Salhy M et al. [41] showed that the decrease of cells expressing Musashi 1 (Msi-1), neurogenin 3 (NEUROG3) were found in patients with IBS. Human T-cell leukemia type 1 (HTLV-1) is the first demonstrable retrovirus found to cause T-cell leukemia/lymphoma, or other lymphocyte-mediated disorders [42, 43]. In addition, CD4 T cells are susceptible to HTLV-1 infection, which deregulates their differentiation, function and homeostasis. It indicates the pathogenic mechanisms of HTLV-1, including the induction of CD4 T cells transformation and chronic inflammatory disease [44]. Kirsch R et al. [45] Showed that chronic, low-level, subclinical inflammation was involved in the progression of IBS-D and was the cause of the persistence of IBS-D symptoms.

CIBERSORT provides a new method to explore immune biomarkers for diagnosis and prognosis, which can accurately determine the diversity and proportion of immune infiltrating cells of disease [46]. Our research showed that the T cells CD4 memory resting, M2 macrophages, B cells, plasma cells and mast cells resting played an important role in IBS-D disease progression. Many researches showed that the immune infiltrating cells such as T cells and mast cells existing in the intestinal mucosa of patients with IBS-D [47]. Cremon C et al. [48] showed that the proportion of CD4 T cells and mast cells in IBS-D patients is relatively high compared with healthy controls. The elevated T cell activation corresponds with the low-level immune activation hypothesis of IBS-D and may be associated with the development of symptoms of IBS-D [49]. Visceral hypersensitivity refers to abnormal pain in the gut caused by stimuli below the pain threshold and increased response to painful stimuli [50]. Most IBS-D patients saw increased visceral sensitivity but the biological process associated with visceral hypersensitivity was unclear [51]. Mujagic Z et al. [52] showed that inflammatory factors activate COX-2 to prompt abnormal synthesis of PEG2 by mast cells and cause visceral hypersensitivity in patients with IBS-D. Boyer J et al. [53] denoted that the number of macrophages increased in IBS-D than HC. Vicario M et al. [54] indicated the unique feature of increased number and activation of mucosal B cells and plasma cells contributing to the partial inflammation and clinical symptoms of IBS-D patients. The immune cells with a significant positive correlation included Neutrophils and T cells CD4 naive (0.99), Neutrophils and NK cells resting (0.57), NK cells resting and T cells CD4 naive (0.56), Mast cells resting and NK cells activated (0.46), Mast cells resting and Plasma cells (0.44), NK cells activated and Plasma cells (0.42), M1 macrophages and T cells CD4 memory activated (0.42) B cells memory and T cells CD4 memory activated (0.44) and mast cells activated and T cells CD4 memory activated (0.46). The immune cells with a significant negative correlation included plasma cells and B cells memory (-0.77), Plasma cells and T cells CD4 memory activated (-0.56), M2 macrophages and T CD4 memory resting (-0.55), mast cells resting and mast cells activated (-0.47), T cells gamma delta and T cells CD4 memory resting (-0.42) and Mast cells resting and B cells memory (-0.45).

WGCNA is an approach to cluster genes based on expression patterns, giving a sight into the relationship between gene modules and traits and is a method of identifying candidate biomarkers or therapeutic genes [55, 56]. A total of 9 co-expression gene modules were calculated by WGCNA and the black module was found to have the highest correlation. The 15 hub genes both in DEGs and the black module were screened, including DFNB59 (deafness, autosomal recessive 59), FLJ45513, GOLGA8A (golgin A8 family

member A), HIST1H2AE (histone cluster 1, H2ae), HIST1H3C (H3 clustered histone 3), LINC00893 (long intergenic non-protein coding RNA 893), LOC100506114, LOC101927391, LOC101928068, LOC101929988, LOC286367, PFDN2 (prefoldin subunit 2), RP11-395I6.3, RP11-676J12.4, SEC31B (SEC31 homolog B, COP β coat complex component). DFNB59 is a member of the gasdermin family, which has pore-forming activity and mediates homeostasis as well as inflammation in the gastrointestinal tract and various immune cells, leading to the inflammation death related to damaged cell membrane integrity and increased inflammatory mediators [57, 58]. Heyd F et al. [59] showed that the reduced expression of HIST1H2AE resulted in increased T cell apoptosis and decreased cell number. Li S et al. [60] showed that the over-expression of LINC00893 increased the expression of PTEN. At the same time, our research showed that PTEN was a crucial gene of the Human T-cell leukemia virus 1 infection pathway causing IBS-D. PFDN2 was discovered to regulate the cytoskeleton organization [61, 46]. Kuznetsova IM [62] hypothesized that proteins in natural states were characterized by instability, but the kinetic stable state of the proteins was expressed by the intracellular folding mechanism of PFEN2. In the meanwhile, the protein instability was associated with the colonic transit in IBS-D [63]. The relationship between IBS-D and other hub genes has not been reported yet, which needs to be explored.

Conclusion

In this study, we found the differentially expressed genes of IBS-D through the GEO database. As a result, we analyzed these DEGs and identified the biological function, pathways, IICs and WGCNA hub genes to indicate the function of interacting genes or proteins, which might be potential targets and biomarkers for IBS-D treatment.

Declarations

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Competing interest

The authors declare that they have no competing interests.

Availability of data and material

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

Code available

Not applicable.

Authors' contributions

Yuan-Mei Lou and Yan-Zhi Ge designed and supervised the study; Yan-Zhi Ge and Wen Chen performed the data processing; Lin Su, Jia-Qi Zhang, Gui-Yue Wang and Ya-Qin Qi contributed to the data analysis; Jin-Ying Yang, Zu-Xiang Chen performed data mining and downloading; Hong Song organized and revised the paper. All authors reviewed the final manuscript.

Ethics approval and consent to participate

The data of this research was downloaded from the GEO database, a public website. All institutional and national guidelines for the care and use of participants were followed.

Patient consent for publication

Not applicable.

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Tables

Table 1 Screening DEGs by integrated microarray	
DEGs	Gene names
Up-regulated	SETD4, MDN1, ZNF783, ABCC5, ITPA, PHF14, WDR4, ANAPC5, STAMBOPLI, AP4B1, THUMPD3-AS1, ZNF589, ATR, CHT0P, SNHG17, TTC27, N0P58, CDC16, NSUN5P1, CAPN8, FBRSL1, TNFSF15, THADA, GGCX, RP11-97C16.1, RP-676J12.4, IRAK1, N0L8, SCARNA15, N0M1, VRK2, METAP1D, IFR1D, IFRD1, RP11-846E15.4, DNM1L, ZNF606, SRSF6, RP11-350F4.2, EME2, RP11-1007024.2, ERMARD, PDAS, UBE3D, LOC102606465, DLG5, NPIPA1, SFPQ, SRRM2, RP12-39P12.3, LOC102724356, L0100506114, FANCL, CCDC84, NR2C1, KLHDC4, GPCPD1, ZNF767P, ENTPD1-AS1, LUC7L, HSP90AA1, SEPT9, KIAA0368, L0H12CR1, CEP83, TACC2, LENG8, LINC00342, M0N1B, MFSD8, CHERP, RBM19, SF1, TUBGCP5, MAT2A, SUGP2, CSTF2, MARK3, ZNF839, MST01, GOLGA8A, ZC3H4, LOC10927391, EIF4G3, TCF7L2, ZNF513, EIF5B, BC039686, LOC101928068, METTL3, SCRIB, IKBKB, PARP4, USP48, ANKRD10, E0GT, CDK5RAP1, CRTC2, TAF1D, PNN, EFCAB4B, AUTS2, HEATR6, ANAPC7, LIG3, CCAR1, TUFT1, CLK2, MNT, PABPC1L, NFX1, MTMR4, OSBPL3, PUS7, CEP170B, MUC4, PRRC2C, EIF3B, INTS10
Down-regulated	PRSC1, GPR160, PET100, NDUFA4, ATP5L, SELK, IFNGR2, PLGRKT, SEC61G, H2AFZ, ZBED5-AS1, CHPT1, HMGB2, WRB, TCEAL8, HIST1H3C, NEUPL1B, LSM1, TMEM50A, GABARAPL2, EIF1B, UQCRB, CRK, SET2D1, MPC2, GTF2B, HIST1H2AE, PSMA2, NR1D2, RNASE4, NOP16, TMEM60, PTGDR, ETFA, PTTG1, EIF1, SS18L2, PSMB3, COMMD6, POLR2J, SNX7, LEPROTL1, PRDX3, LXN, RPA3, MRPL13, ASF1A, DPY30, AURKA, LOC1007459, BEX4, SDHC, PFDN2, C2orf47, CKS1B, STMN1, WBP5, SF3B6, RPA30S, GOLGA7, AS3MT, C7orf55, HES1, FAM200B, LSM2, FAM220A, SMIM3, THAP1, FUNDC1, ACAT1, LAMTOR1, SGCE, SDHB, LINC01420, IMMMP1L, QPCT, GTF2A2, CDC26, CDC26, NSMCE2, CBY1, TBPL1, PDE6D, PEX2, C16orf80, FAM172A, C11orf74, PTEN, PSMB9, TRIQK, CASP6, RCHY1, MSRB1, CDKN3, CCNDBP1, IFT57, MIS18A, UBE2C, BECN1, C18orf21, SPSB1, ISCA2
Abbreviation: DEGs, differentially expressed genes.	

Table 2 Top 10 up-regulated and down-regulated DEGs identified between HC and IBS-D tissue calculated by limma package					
Up-regulated			Down-regulated		
DEG	LogFC	Adjusted-P value	DEG	LogFC	Adjusted-P value
GNE	238.918971	0.00084078	PRAC1	-117.8097143	5.53E-05
LENG8	156.632009	8.11E-05	GPR160	-91.80168052	0.000679182
HSP90AA1	138.1588401	6.85E-05	PET100	-86.18600778	0.000213857
HNRNPA2B1	111.0993318	0.000350182	NDUFA4	-81.83637453	0.000686655
TUFT1	101.3611916	0.000200998	IFNGR2	-68.19248005	0.000193654
OPHN1	94.47427679	0.000845769	PLGRKT	-67.88231684	1.54E-05
DNMBP	93.70029476	0.000326541	SEC61G	-66.89338208	0.000851522
CAPN8	88.30087551	4.63E-06	H2AFZ	-64.0459408	0.000201415
MAT2A	86.0075925	0.000101693	ZBED5-AS1	-58.91459849	2.33E-05
ZMPSTE24	83.92053014	0.000418344	CHPT1	-55.22476906	0.000504226
Abbreviation: DEGs, differentially expressed genes; HC: healthy control; IBS-D, Irritable bowel syndrome with diarrhea; LogFC, log fold change.					

Table 3 Go analysis of DEGs between HC and IBS-D tissue

Category	Term	Description	Adjusted-P value	Count
Upregulated				
BP	GO:0008380	RNA splicing	3.16E-09	20
	GO:0006397	mRNA processing	4.85E-09	21
	GO:0000377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	1.53E-08	17
	GO:0000398	mRNA splicing, via spliceosome	1.53E-08	17
	GO:0000375	RNA splicing, via transesterification reactions	1.81E-08	17
CC	GO:0016607	nuclear speck	5.92E-08	17
	GO:0015030	Cajal body	5.28E-05	6
	GO:0005681	spliceosomal complex	8.95E-05	8
MF	GO:0030515	snoRNA binding	0.000879666	3
Downregulated				
BP	GO:0031145	anaphase-promoting complex-dependent catabolic process	1.07E-07	8
	GO:0010498	proteasomal protein catabolic process	3.26E-06	13
	GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	3.80E-06	12
	GO:0033045	regulation of sister chromatid segregation	1.02E-05	6
	GO:0033047	regulation of mitotic sister chromatid segregation	5.16E-05	5
CC	GO:0031461	cullin-RING ubiquitin ligase complex	3.45E-05	7
	GO:0016591	RNA polymerase II, holoenzyme	0.000168354	5
	GO:0030992	intraciliary transport particle B	0.000197272	3
MF	GO:0070003	threonine-type peptidase activity	0.000482122	3

Abbreviation: GO, gene ontology; DEGs, differentially expressed genes; HC, healthy volunteer; IBS-D, irritable bowel syndrome with diarrhea; BP, biological process; CC, cellular component; MF, molecular function.

Table 4 KEGG pathway analysis of DEGs concerned with IBS-D

Pathways	ID	Gene counts	Adjusted-P value	Genes
Spliceosome	hsa03040	11	0.019262642	ISY1, LSM2, PRPF18, SF3B6, SRSF6, CHERF, SNRNP70, FUS, PRPF3, RBM25
Human T-cell leukemia virus 1 infection	hsa05166	13	0.019262642	CDC16, IKBKB, CRTC2, ANAPC6, MAP2K1, CDC26, PTTG1, CCNB2, TBPL1, PTEN
Progesterone-mediated oocyte maturation	hsa04914	8	0.031371796	CDC26, CCNB2, AURKA, ANAPC5, CDC16, HSP90AA1, ANAPC7, MAP2K1
Oocyte meiosis	hsa04114	9	0.031371796	ANAPC5, CDC16, ANAPC7, MAP2K1, CDC20, CDC26, PTTG1, CCNB2, AURKA
Ubiquitin mediated proteolysis	hsa04120	9	0.045291051	UBE2Q1, CDC20, CDC26, RCHY1, UBE2C, ANAPC5, CDC16, FANCL, ANAPC7

Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; IBS-D, irritable bowel syndrome with diarrhea.

Figures

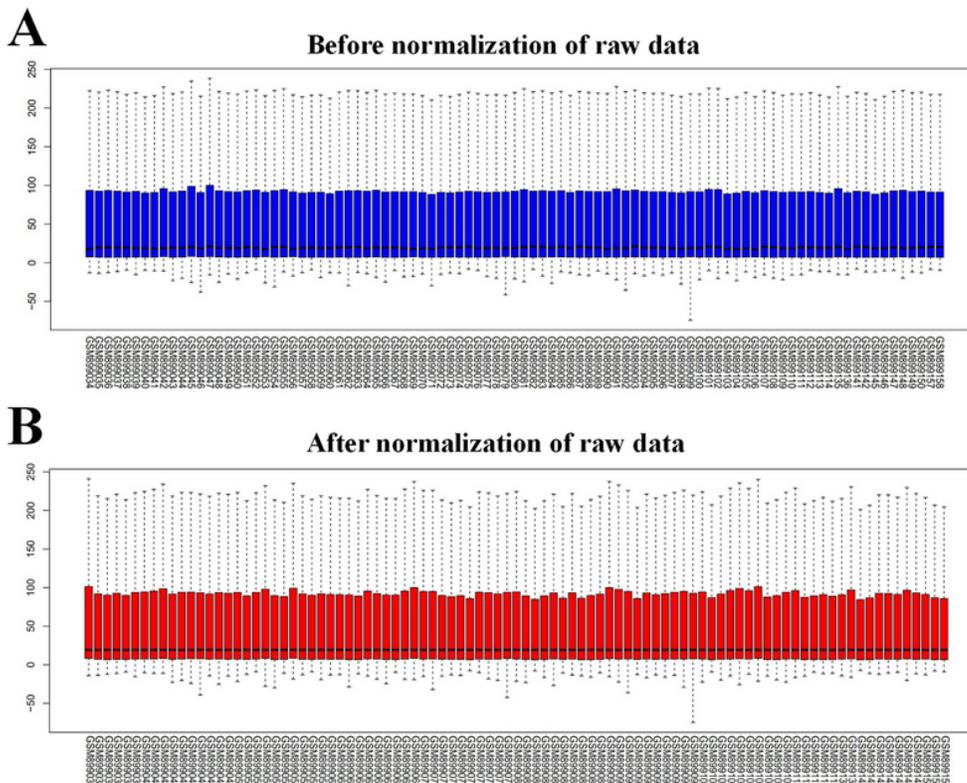


Figure 1

Normalization of IBS-D expression microarray datasets GSE36701 a GSE36701 datasets before normalization; b GSE36701 datasets of normalization. Abbreviation: IBS-D, irritable bowel syndrome with diarrhea.

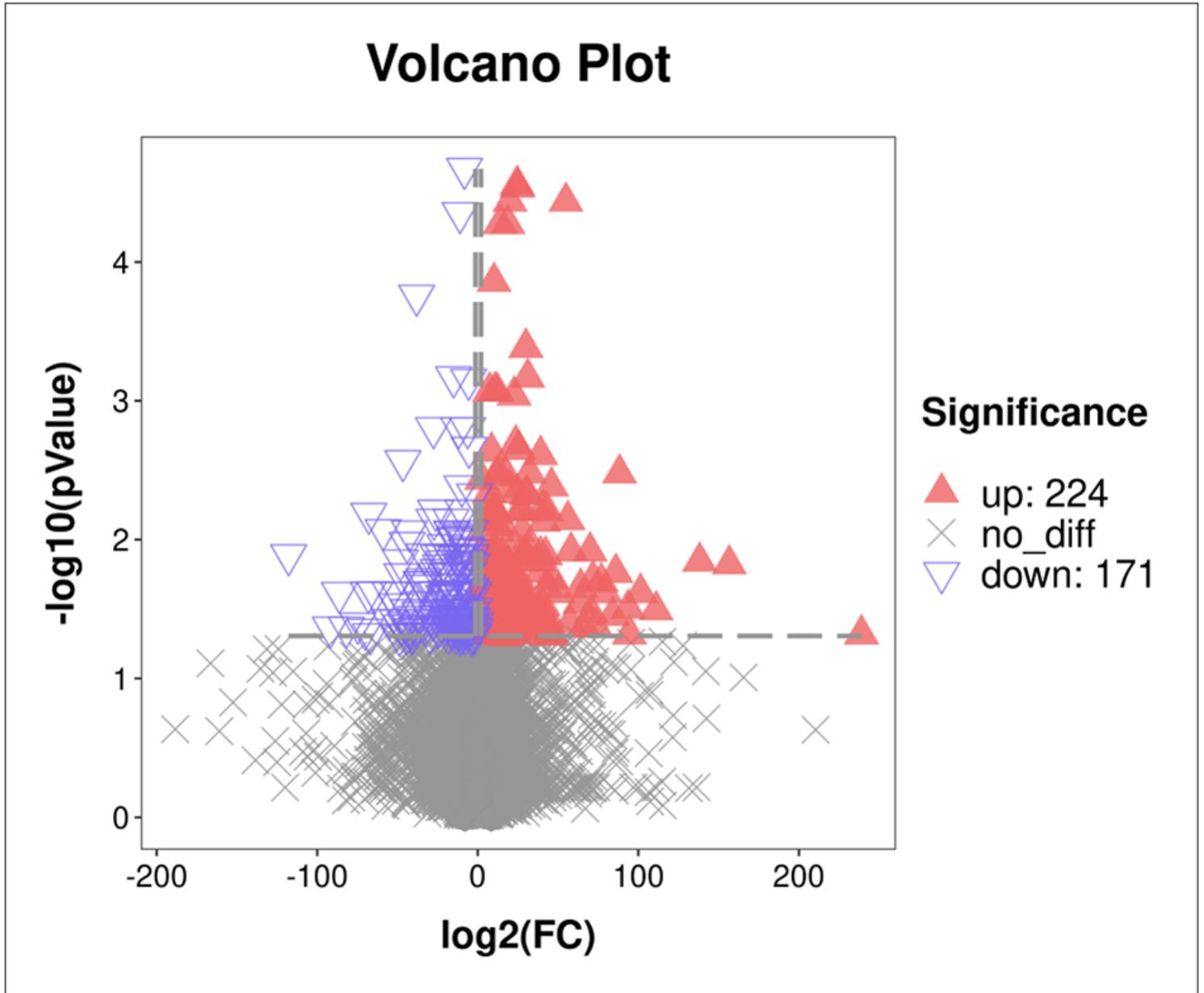


Figure 2

Volcano plot of DEGs Notes: Red spots represent up-regulated genes. Green spots represent down-regulated genes and the black spots represent genes with no significant difference. All the data is on the basis of adjusted- $P < 0.05$ and $|\text{LogFC}| > 1$. Abbreviation: DEGs, differentially expressed genes; LogFC, log fold change.

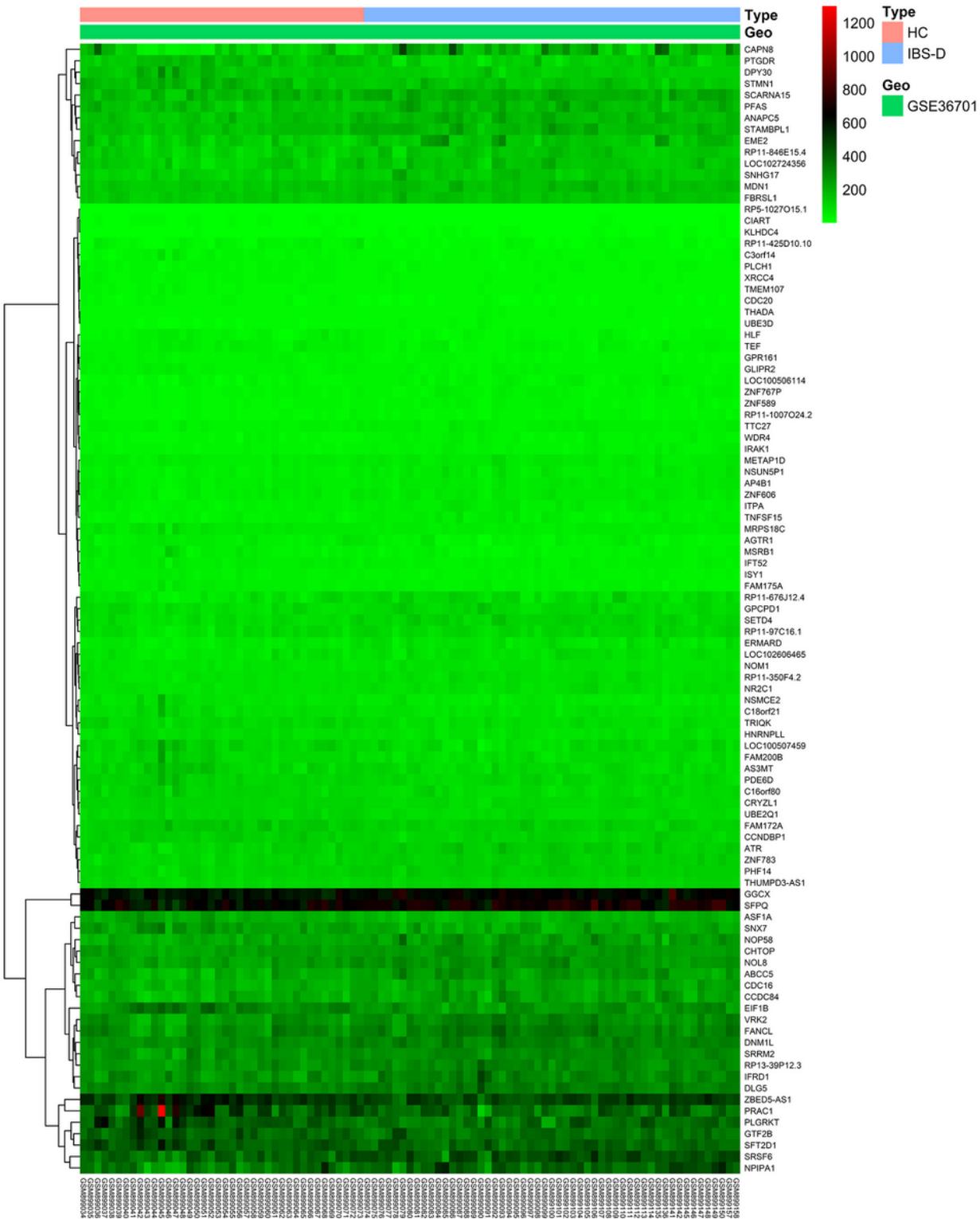


Figure 3

Cluster heatmaps of top 100 DEGs ($P < 0.05$, $|\text{LogFC}| > 1$) Notes: Red color indicates upregulations. Green color indicates downregulations and black color indicates genes with no significant difference. All the data is on the basis of $P < 0.05$ and $|\text{LogFC}| > 1$. Abbreviation: DEGs, differentially expressed genes; LogFC, log fold change.

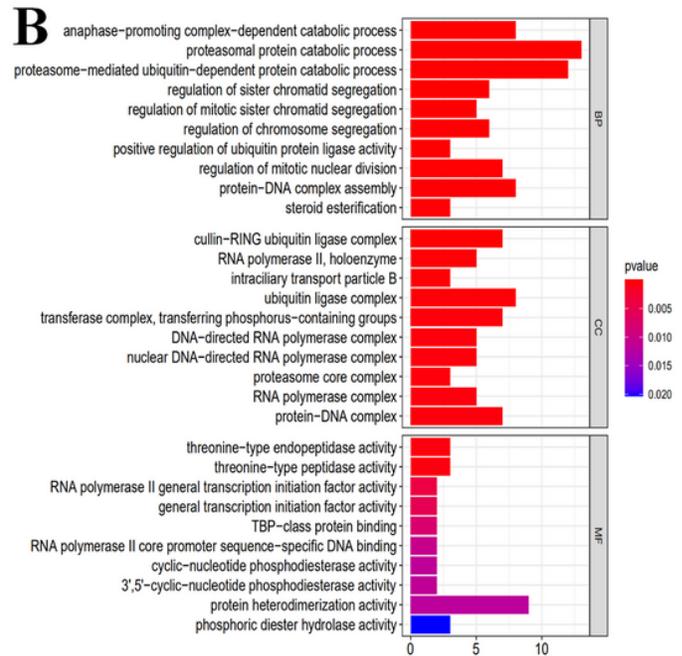
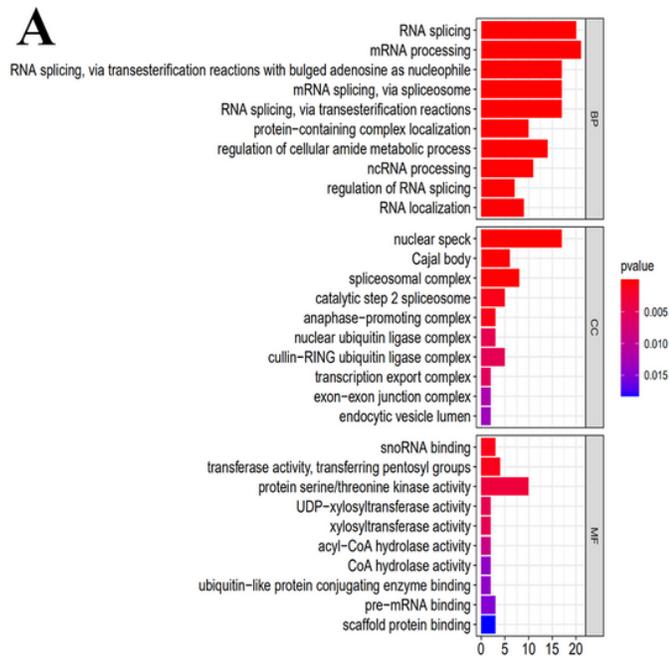


Figure 4

Go enrichment analysis a Go enrichment analysis of up-regulated DEGs in IBS-D tissue; b Go enrichment analysis of down-regulated DEGs in IBS-D tissue. Notes: Go enrichment includes three functional groups: BP, MF and CC. GO enrichment significance items of up-regulated DEGs in different functional groups. Abbreviation: IBS-D, irritable bowel syndrome with diarrhea; DEGs, differentially expressed genes; BP, biological processes; MF, molecular function; CC, cellular component; GO, gene ontology.

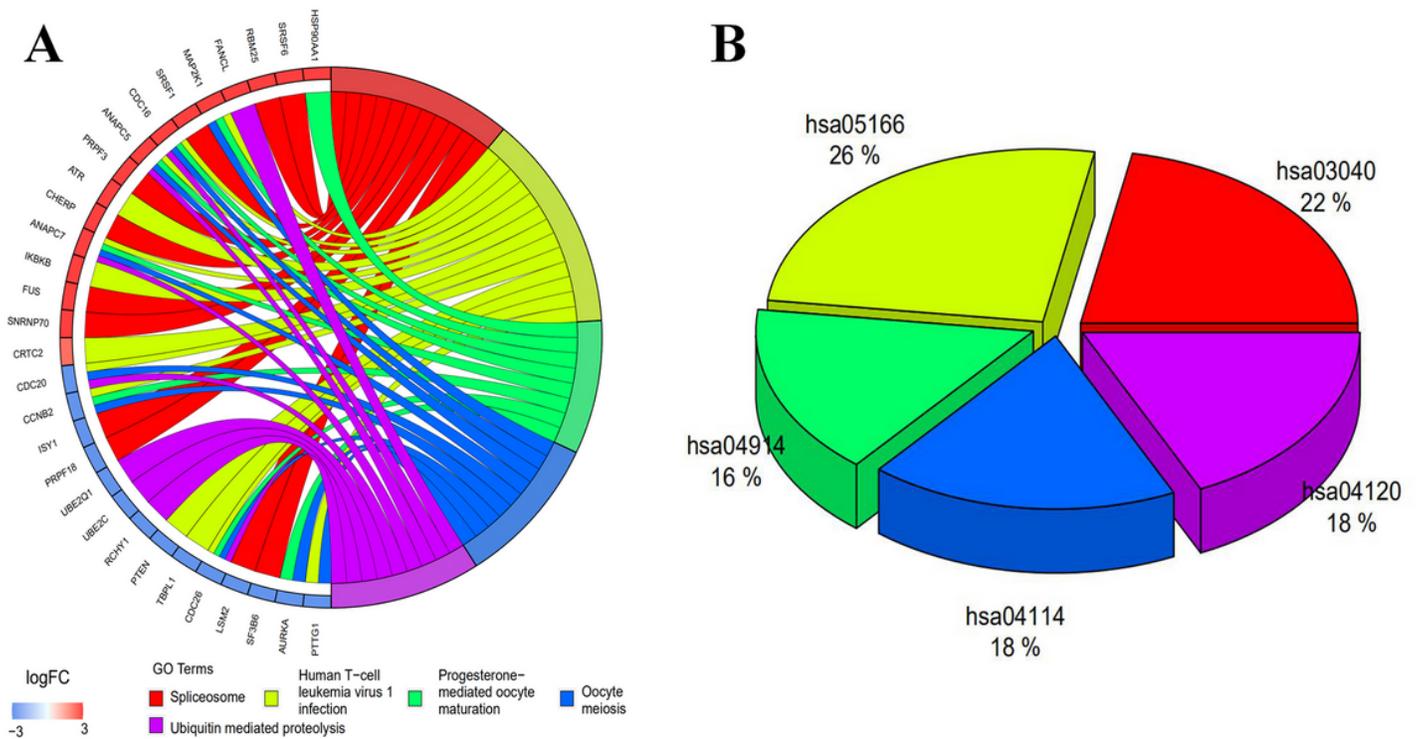


Figure 5

KEGG pathway analysis of DEGs concerned with IBS-D a Circos graph analysis of the KEGG pathway; b Pie chart analysis of the correlation between the KEGG pathway and IBS-D. Abbreviation: KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes, IBS-D, irritable bowel syndrome with diarrhea.

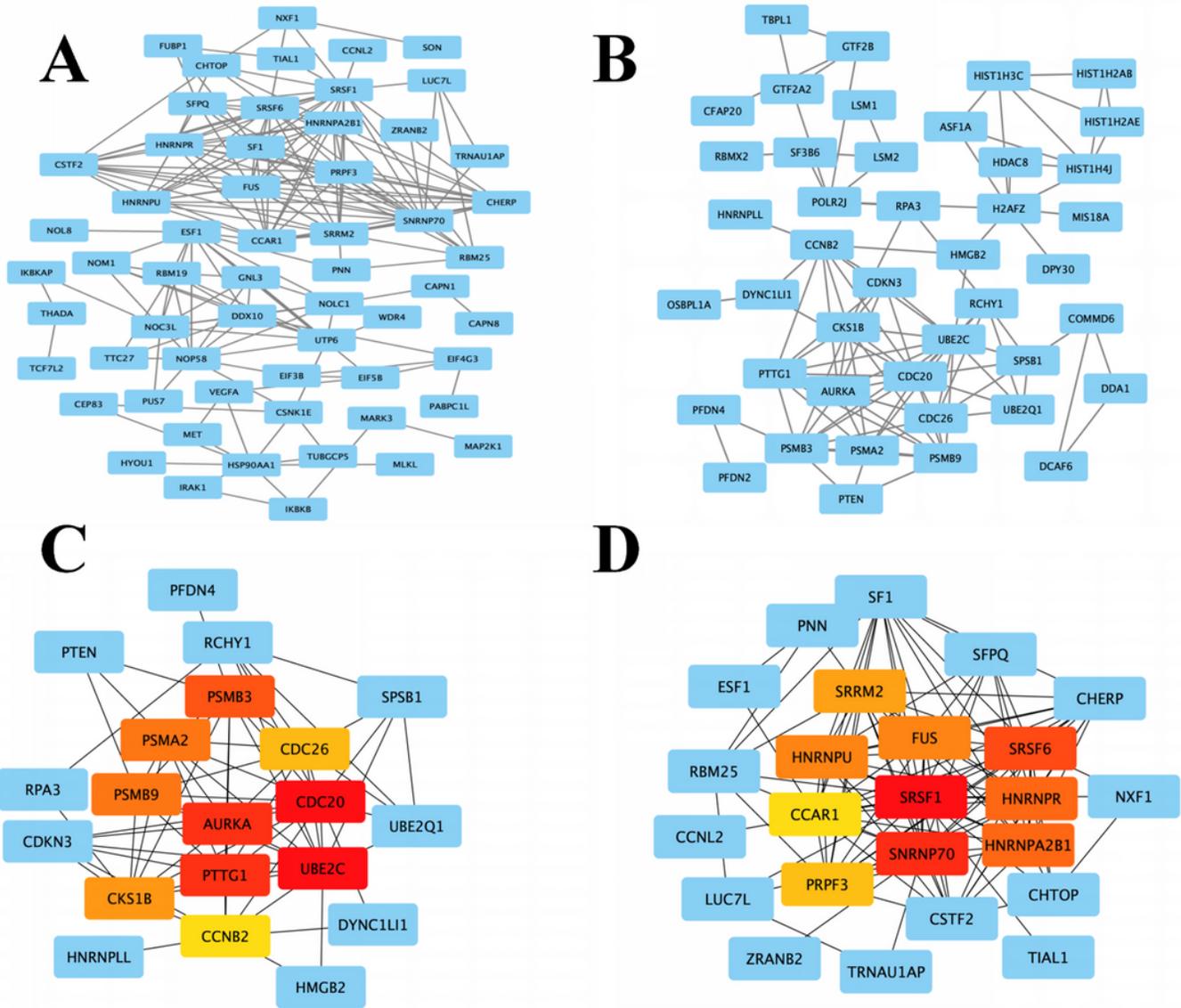


Figure 6

Visualization of PPI network and hub genes of the identified DEGs by Cytoscape a PPI network of up-regulated DEGs; b 10 hub genes of the up-regulated DEGs; c PPI network of down-regulated DEGs; d 10 hub genes of the down-regulated DEGs. Notes: Blue nodes represent the DEGs. Edges represent the protein-protein associations. Other color nodes represent the top ten hub genes. Abbreviation: PPI, protein-protein interaction; DEGs, differentially expressed genes.

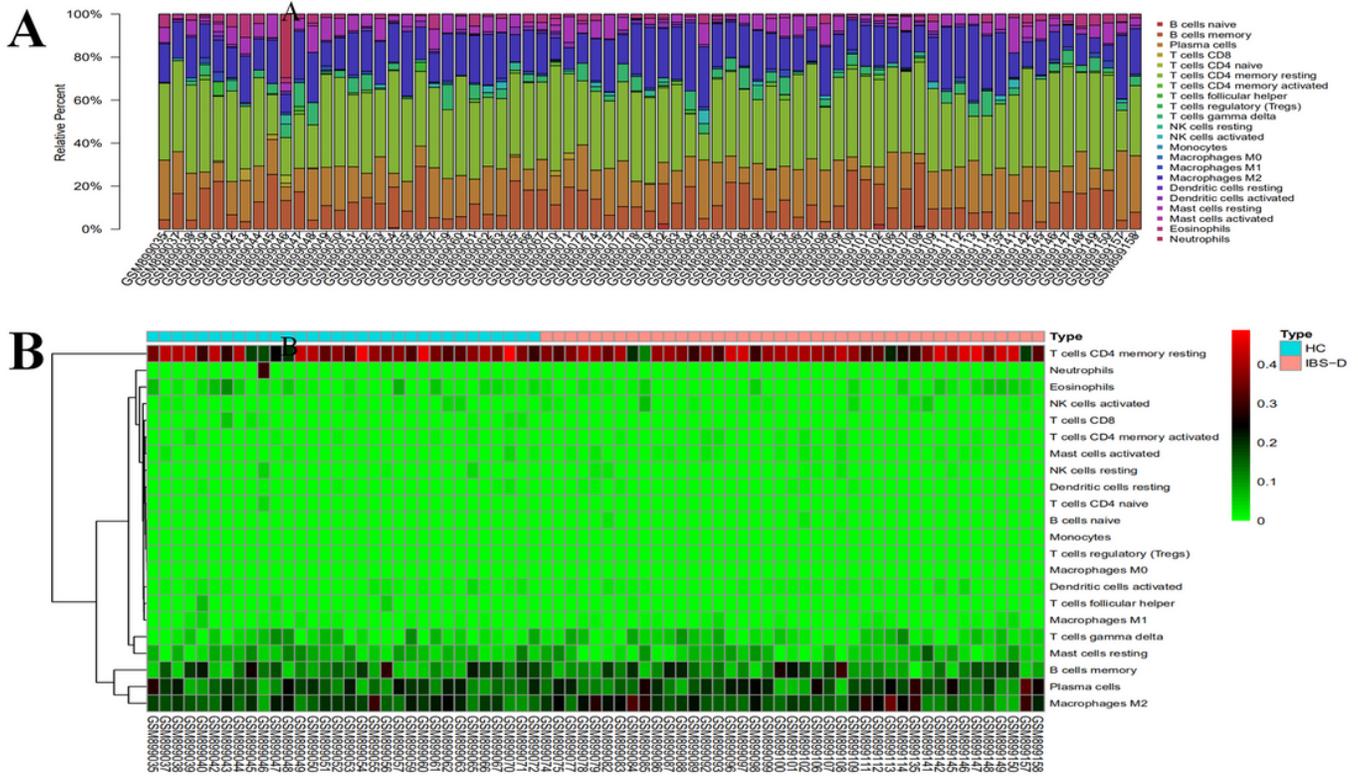


Figure 7

The distribution of immune infiltration in IBS-D a Immune infiltration in IBS-D tissue in 22 subpopulations of immune cells. Notes: Different color represents different immune cells and the length of the bars in the barplot represents the proportion of the immune cell population. b Heatmap identifying the level of 22 immune cell proportion. Note: Each column represents a tissue and each row represents one of 22 immune cells. Different color represents different immune cells populations, and the proportions gradually increased from green to red. Abbreviation: IBS-D, irritable bowel syndrome with diarrhea.

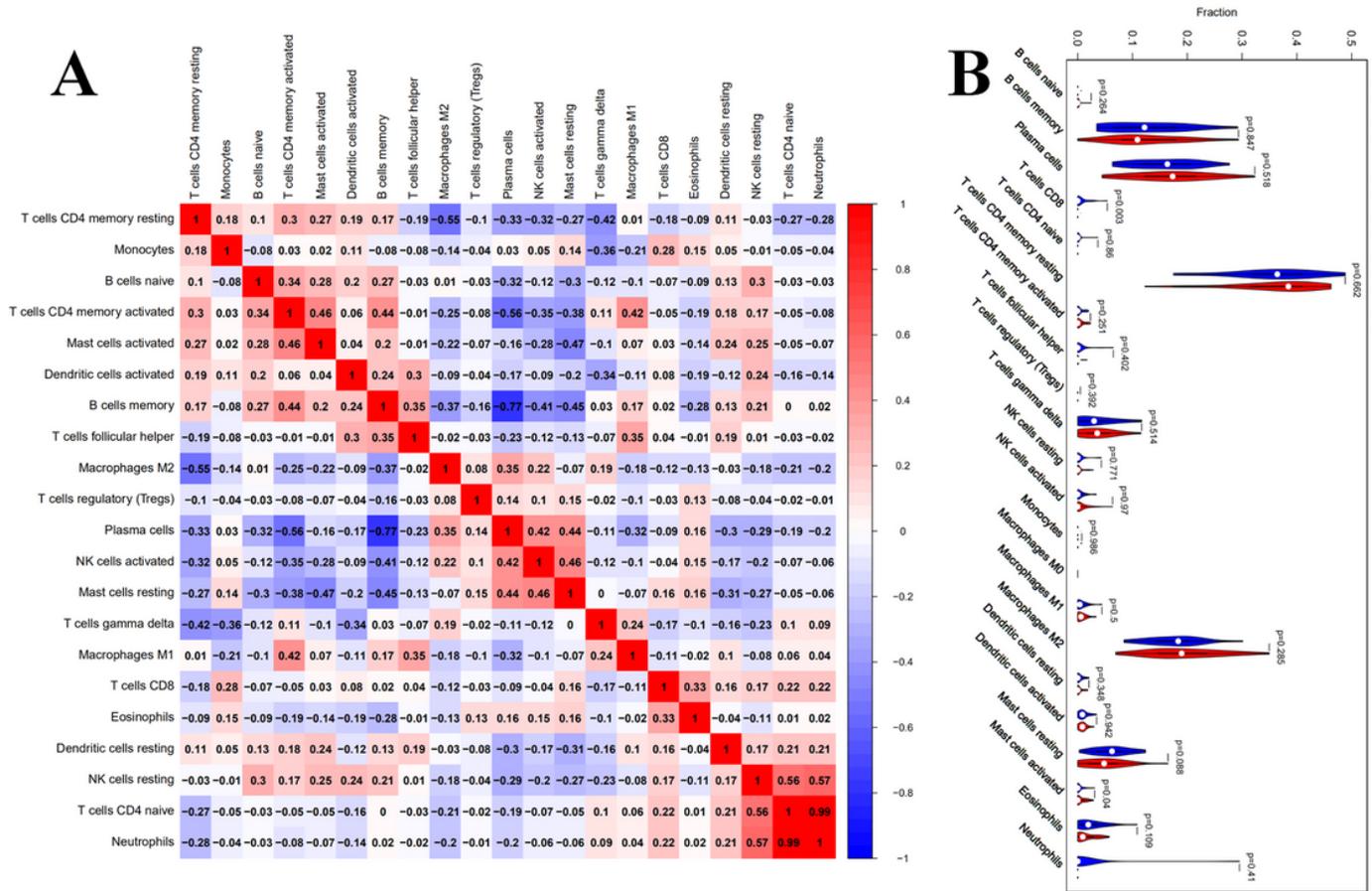


Figure 8

The distribution of immune infiltration in IBS-D a Corheatmap of the correlation matrix for filtered 21 immune cell proportions. Note: The red color represents the positive relationship between two immune cells and the blue color represents the negative relationship between two immune cells. The darker the color, the higher the correlation was ($P < 0.05$). b The violin plot of immune cells. The blue bar represents HC tissues and the red bar represents the IBS-D tissues. Abbreviation: IBS-D, irritable bowel syndrome with diarrhea; HC, healthy control.

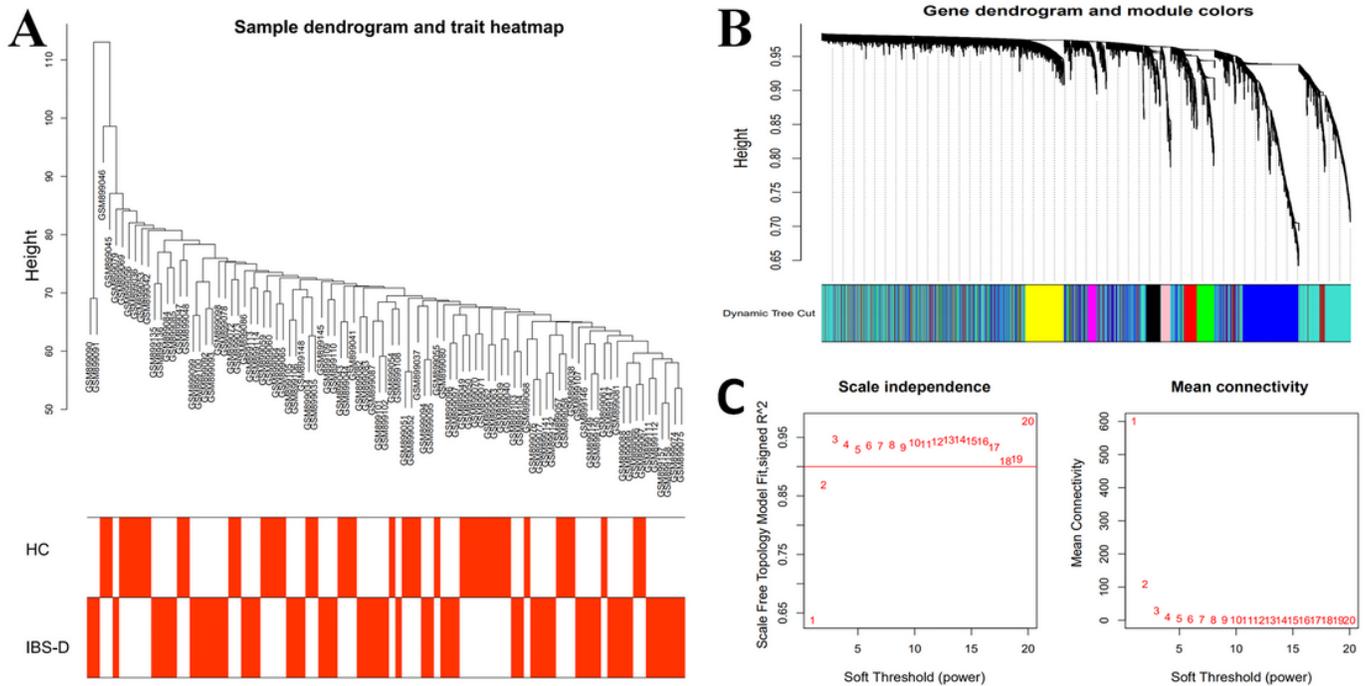


Figure 9

Construction and analysis of WGCNA associate with IBS-D and HC a Clustering tree detects outliers Note: All samples were in the clusters and passed the cutoff thresholds. b Analysis of scale-free topology for a set of soft threshold powers. Note: The X-axis indicates the function of the soft threshold power, and the Y-axis indicates the scale-free fit index of network topology in the left graph. The X-axis indicates the function of the soft threshold power, the Y-axis indicates the mean connectivity in the right graph. c Cluster dendrograms of HC and IBS-D tissues detecting gene modules. Note: Each color represents a gene module. Abbreviation: WGCNA, weigh gene co-expression network analysis; IBS-D, irritable bowel syndrome with diarrhea; HC, healthy volunteer.

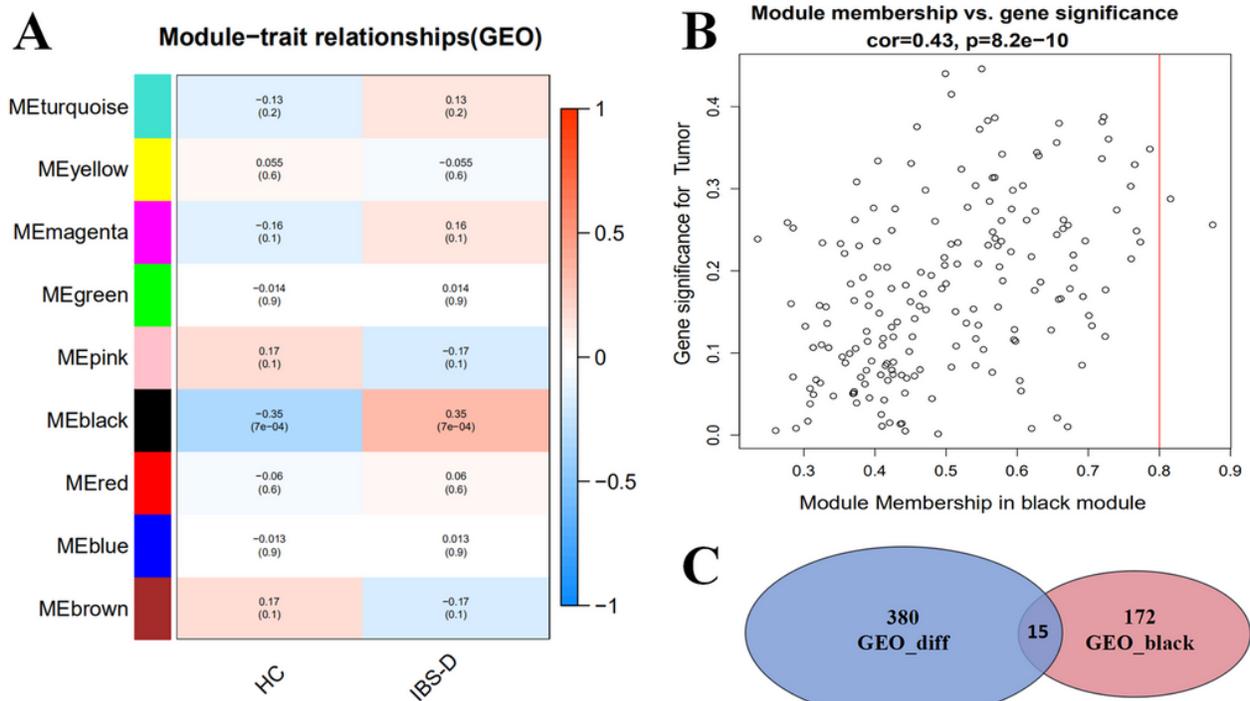


Figure 10

Construction and analysis of WGCNA associate with IBS-D and HC a Heatmap of module-trait relationships Note: Numbers in the table at the top of each row represent the corresponding correlation, and the number below represents the adjusted P-value. Scatter plot of the correlation between module membership in the black module and gene significance. Intramodular of genes identified in the black module, which comprises genes highly associated with IBS-D (correlation=0.43, $P < 8.2 \times 10^{-10}$). b The scatter plot of the correlation between each gene in the black module Notes: The X-axis indicates the gene significant for the tumor, and the Y-axis indicates the module membership in the black module. c Identification of black module genes in WGCNA and DEGs. Note: The blue represents the DEGs, the red represents the genes in black modules and the intersecting part represents the hub genes in both DEGs and the black module. Abbreviation: WGCNA, weigh gene co-expression network analysis; IBS-D, irritable bowel syndrome with diarrhea; HC, healthy control; DEGs, differentially expressed genes.