Co-expression network analysis of Down's syndrome based on microarray data

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Abstract. Down's syndrome (DS) is a type of chromosome disease. The present study aimed to explore the underlying molecular mechanisms of DS. GSE5390 microarray data downloaded from the gene expression omnibus database was used to identify differentially expressed genes (DEGs) in DS. Pathway enrichment analysis of the DEGs was performed, followed by co-expression network construction. Significant differential modules were mined by mutual information, followed by functional analysis. The accuracy of sample classification for the significant differential modules of DEGs was evaluated by leave-one-out cross-validation. A total of 997 DEGs, including 638 upregulated and 359 downregulated genes, were identified. Upregulated DEGs were enriched in 15 pathways, such as cell adhesion molecules, whereas downregulated DEGs were enriched in maturity onset diabetes of the young. Three significant differential modules with the highest discriminative scores (mutual information>0.35) were selected from a co-expression network. The classification accuracy of GSE16677 expression profile samples was 54.55% and 72.73% when characterized by 12 DEGs and 3 significant differential modules, respectively. Genes in significant differential modules were significantly enriched in 5 functions, including the endoplasmic reticulum (P=0.018) and regulation of apoptosis (P=0.061). The identified DEGs, in particular the 12 DEGs in the significant differential modules, such as B-cell lymphoma 2-associated transcription factor 1, heat shock protein 90 kDa beta member 1, UBX domain-containing protein 2 and transmembrane protein 50B, may serve important roles in the pathogenesis of DS.

Introduction

Down's syndrome (DS), also known as trisomy 21, is a genetic disorder caused by trisomy of part of, or all of, chromosome 21, and is associated with significant intellectual disability, physical growth delays and slanted eyes (1,2). DS may cause other complications, such as autism, coeliac disease, hypothyroidism and leukemia (3-5). Over the last several decades, numerous genes on trisomy 21 have been suggested to be associated with DS (6).

Modern genetic pathology states the hypothesis that phenotypes of DS are determined by an extra copy of one or several genes, such as oligodendrocyte transcription factor 1 present on human chromosome 21 (Hsa21) (7). A previous study demonstrated that sorting nexin family member 27, which is suppressed by a bioactive substance encoded on Hsa21, is upregulated in the brains of DS mice (8). The DS critical region (DSCR) is composed of 6 genes, including v-ets erythroblastosis virus E26 oncogene homolog 2 (*ETS2*) and ETS-related gene (*ERG*), which encode transcription factors (9). *ERG* and *ETS2* belong to the E26 transformation-specific family, which contains key regulators of embryonic development (10). Numerous sensitive genes such as ubiquitin specific peptidase 16, which are associated with the reduced ubiquitination of cyclin-dependent kinase inhibitor 2A and expedited senescence in Ts65Dn fibroblasts, have been confirmed to be associated with DS (11,12).

The identification of sensitive genes on Hsa21 has become the focus of investigations with the aim of elaborating suitable therapeutic strategies for DS, and global gene expression profiling techniques have been widely used to analyze the molecular mechanisms underlying trisomy 21, as the expression levels of trisomic genes and those in the wider genome can be assessed (13). The majority of DS studies have been performed

in animal models, such as the Ts65Dn mouse model of DS, which can be used to analyze the association between hippocampal pathways and cognitive dysfunction (14-16). Various samples, such as fibroblasts, whole blood, T cells and placenta, have been used to investigate the pathogenesis of DS (17-20). The samples used in the present study are derived from the brain tissue samples of patients with DS. Lockstone *et al* (21) screened differentially expressed genes (DEGs) in DS and performed functional analysis, and investigated significant differential modules from a co-expressed network (21).

In the present study, GSE5390 and GSE16677 were downloaded from the Gene Expression Omnibus (GEO) database, DEGs were screened for pathway enrichment analysis, then a co-expressed network was constructed and screened for significant differential modules. In addition, the classification accuracy of GSE16677 expression profile samples were evaluated, characterized by the value of average expressed DEGs and all DEGs in significant differential modules. Finally, functional enrichment analysis of DEGs in significant differential modules was performed.

Materials and methods

Microarray data. The transcription profiles of GSE5390 and GSE16677 were acquired from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) (22,23). The platform names were GPL96 Affymetrix Human Genome U133A Array and GPL570 Affymetrix Human Genome U133 plus 2.0 Array, respectively. A total of 15 samples from GSE5390 were studied, including 8 normal samples and 7 DS samples of human adult brain tissue (dorsolateral prefrontal cortex) from healthy controls and individuals with DS (23). A total of 11 samples from GSE16677 were studied, including 5 normal samples and 6 DS samples of megakaryocytic leukemia blasts from patients with DS-acute megakaryoblastic leukemia (AMKL) or French-American-British M7 leukemia, and patients with non-DS-AMKL. The raw data were downloaded for further analysis. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) and performed in accordance with ethical standards (22,23).

DEG analysis. Gene expression data from GSE5390 were processed through background correction, quantile normalization and probe summarization using the robust multi-array average algorithm in the oligo software package (version 1.8.3) in Bioconductor (https://bioconductor.org/packages/release/bioc/html/oligo.html). The probe in the matrix file corresponded to the gene symbol based on the downloaded platform annotation files. The average value of the gene symbol with multiple probes was calculated. Significant Analysis of Microarray approach in the R package samr (version 2.0) (24) was used to screen the DEGs in DS samples by assigning a score based on the change in gene expression relative to the standard deviation of the repeated measurements. Corrected P<0.05 was considered to indicate a statistically significant difference.

Pathway enrichment analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID) provides

analytical tools for extracting biological meaning from large lists of genes (25,26), and was used for pathway enrichment analysis of upregulated and downregulated DEGs in the current study.

Co-expressed network construction. Gene co-expression network analysis is a fusion product of microarray profile techniques and network theory, that offers an intuitive concept for illustrating the association between two genes (27). The gene expression data set GSE5390 was applied to build a co-expressed network. Cytoscape is an open software platform for visualizing complex networks and integrating these with any type of attributed data (28). The Pearson correlation coefficient is a popular coefficient for evaluating the dependence of two variables (29) and, in the present study, Pearson's coefficient analysis was performed using the Perl script program (version 5.22.1; https://www.perl.org/get.html). R>0.9 was chosen as the criterion for selecting co-expressed genes. The co-expression network was visualized using Cytoscape software (version 2.8; http://www.cytoscape.org/).

Analysis of differentially co-expressed gene modules. GraphWeb (http://biit.cs.ut.ee/graphweb/) is a public web server used to mine heterogeneous biological networks for significant function gene modules (30). The hidden Markov model (HMM) of GraphWeb can be used for modeling by defining and observing the joint probability of sequences and label sequences (31). In the present study, HMM was used for mining network modules. Mutual information (MI) between the average expression value of modules and sample labels was calculated according to the following formula (32):

$$MI(a',c) = \sum_{a' \in x}\sum_{c \in y} P(x,y) = \log\frac{p(x,y)}{p(x)p(y)}$$

In this formula, a' is the discretization average expression value; c is the corresponding vector of the sample label. To derive a' from a, activity levels are discretized into $[\log_2 (\text{number of samples}) + 1]$ values (33). Differentially co-expressed gene modules were screened with the threshold of discriminative score (defined as MI) >0.35. Then, functional enrichment analysis of differentially expressed genes in the differentially co-expressed gene modules was performed by DAVID. P<0.1 was used as the cut-off criterion.

Assessment of classification efficiency. Normal and DS samples were classified by the characteristics of differentially co-expressed gene modules and differentially expressed genes. The accuracy of classification was confirmed by leave-one-out cross-validation. The leave-one-out estimate was calculated as follows (34): Each specimen was excluded from the training set one at a time and then classified on the basis of the predictor built from the data for all of the other specimens.

Results

Differentially expressed gene screening. Based on the differential expression analysis of GSE5390, a total of 997 DGEs were obtained with corrected P<0.05, including 638 upregulated genes and 359 downregulated genes.

Table I. Pathway enrichment analysis for upregulated and downregulated DEGs.

DEGs	Term ID	Description	Gene no.	P-value
Upregulated	hsa04514	Cell adhesion molecules	23	1.68E-07
	hsa05416	Viral myocarditis	14	2.19E-05
	hsa04940	Type I diabetes mellitus	9	6.58E-04
	hsa05330	Allograft rejection	8	1.26E-03
	hsa04612	Antigen processing and presentation	12	1.68E-03
	hsa05332	Graft-versus-host disease	8	2.06E-03
	hsa04142	Lysosome	14	3.34E-03
	hsa05320	Autoimmune thyroid disease	8	9.57E-03
	hsa00380	Tryptophan metabolism	7	1.05E-02
	hsa05310	Asthma	6	1.07E-02
	hsa05322	Systemic lupus erythematosus	11	1.81E-02
	hsa00600	Sphingolipid metabolism	6	3.54E-02
	hsa00330	Arginine and proline metabolism	7	3.79E-02
	hsa00071	Fatty acid metabolism	6	3.90E-02
	hsa04640	Hematopoietic cell lineage	9	4.98E-02
Downregulated	hsa04950	Maturity onset diabetes of the young	4	1.56E-02
	hsa04260	Cardiac muscle contraction	6	2.53E-02

DEGs, differentially expressed genes; ID, identification.

Pathway enrichment analysis. Pathway enrichment analysis of upregulated and downregulated DEGs was performed by DAVID. A total of 17 pathways were enriched for the DEGs (Table I). The upregulated DEGs were enriched in 15 pathways, specifically in cell adhesion molecules ($P=1.68x10^{-7}$) which involved 23 genes, such as golgi glycoprotein 1 (*GLG1*), human leukocyte antigen class II histocompatibility antigen (*HLA-DRB1*) and claudin-10. Type I diabetes mellitus ($P=6.58x10^{-4}$), a complication of DS, was enriched by 9 genes, including carboxypeptidase E and *HLA-DRB1*. Downregulated DEGs were primarily enriched in two pathways: Maturity onset diabetes of the young ($P=1.56x10^{-2}$) and cardiac muscle contraction ($P=2.53x10^{-2}$). These 17 pathways may serve important roles in the pathogenesis of DS.

Significant differential module analysis. The constructed co-expression network included 997 nodes and 19,049 edges (Fig. 1). The co-expressed network was processed by HMM, and 36 modules were obtained. A total of 3 significant differential modules with MI>0.35 were identified (Fig. 2). Module 13 contained 5 upregulated DEGs [heterogeneous nuclear ribonucleoprotein R, B-cell lymphoma 2-associated transcription factor 1 (*BCLAF1*), *GLG1*, UBX domain-containing protein 2 (*UBXD2*) and transmembrane protein (*TMEM*) 106B] and 1 downregulated DEG (cullin 7). A total of 2 upregulated DEGs [*TMEM50B* and heat shock protein 90 kDa beta member 1 (*HSP90B1*)] combined with the downregulated DEG (receptor for advanced glycation end-products) combined to form module 31. Module 35 consisted of 3 downregulated DEGs, including D-beta-hydroxybutyrate dehydrogenase, solute carrier family 36 member 1 (*SLC36A1*) and keratin 85, type II.

Classification efficiency of significant differential modules. The accuracy of the sample classification for GSE1667 was 72.73%, based on the average expression values of the 3 significant differential modules. The accuracy of the classification was 54.55% according to the expression values of all 12 DEGs in the 3 significant differential modules.

Functional enrichment analysis of significant differential modules. The functional enrichment analysis of the 12 DEGs in the 3 significant differential modules, according to the DAVID database, demonstrated that 4 genes, including *SLC36A1*, *UBXD2*, *HSP90B1* and *TMEM50B*, were significantly enriched in the endoplasmic reticulum (P=0.018; Table II). Similarly, *BCLAF1*, *HSP90B1* and dopamine β-hydroxylase were associated with 3 functions, including the biological process of apoptosis (P=0.061), regulation of programmed cell death (P=0.062) and regulation of cell death (P=0.02) (Table II). Genes *GLG1*, *UBXD2* and *HSP90B1* were enriched in the endomembrane system (Table II).

Discussion

DS is a common chromosomal disorder which, to date, cannot be treated. In the present study, GSE5390 and GSE16677 were downloaded from the GEO database in order to investigate the molecular mechanisms underlying DS. A total of 997 DEGs, including 638 upregulated and 359 downregulated DEGs, were screened based on GSE5390. The DEGs in the 3 significant differential modules screened from a co-expressed network were significantly involved with the endoplasmic reticulum, endomembrane system, regulation of apoptosis, programmed cell death and cell death. Based on the pathway enrichment

Table II. Functional enrichment analysis for the twelve DEGs in the three identified significant differential modules.

Category	Term	Genes	P-value
GOTERM_CC	Endoplasmic reticulum	SLC36A1, UBXD2, HSP90B1, TMEM50B	0.018
GOTERM_BP	Regulation of apoptosis	BCLAF1, HSP90B1, DBH	0.061
GOTERM_BP	Regulation of programmed cell death	BCLAF1, HSP90B1, DBH	0.062
GOTERM_BP	Regulation of cell death	BCLAF1, HSP90B1, DBH	0.062
GOTERM_CC	Endomembrane system	GLG1, UBXD2, HSP90B1	0.082

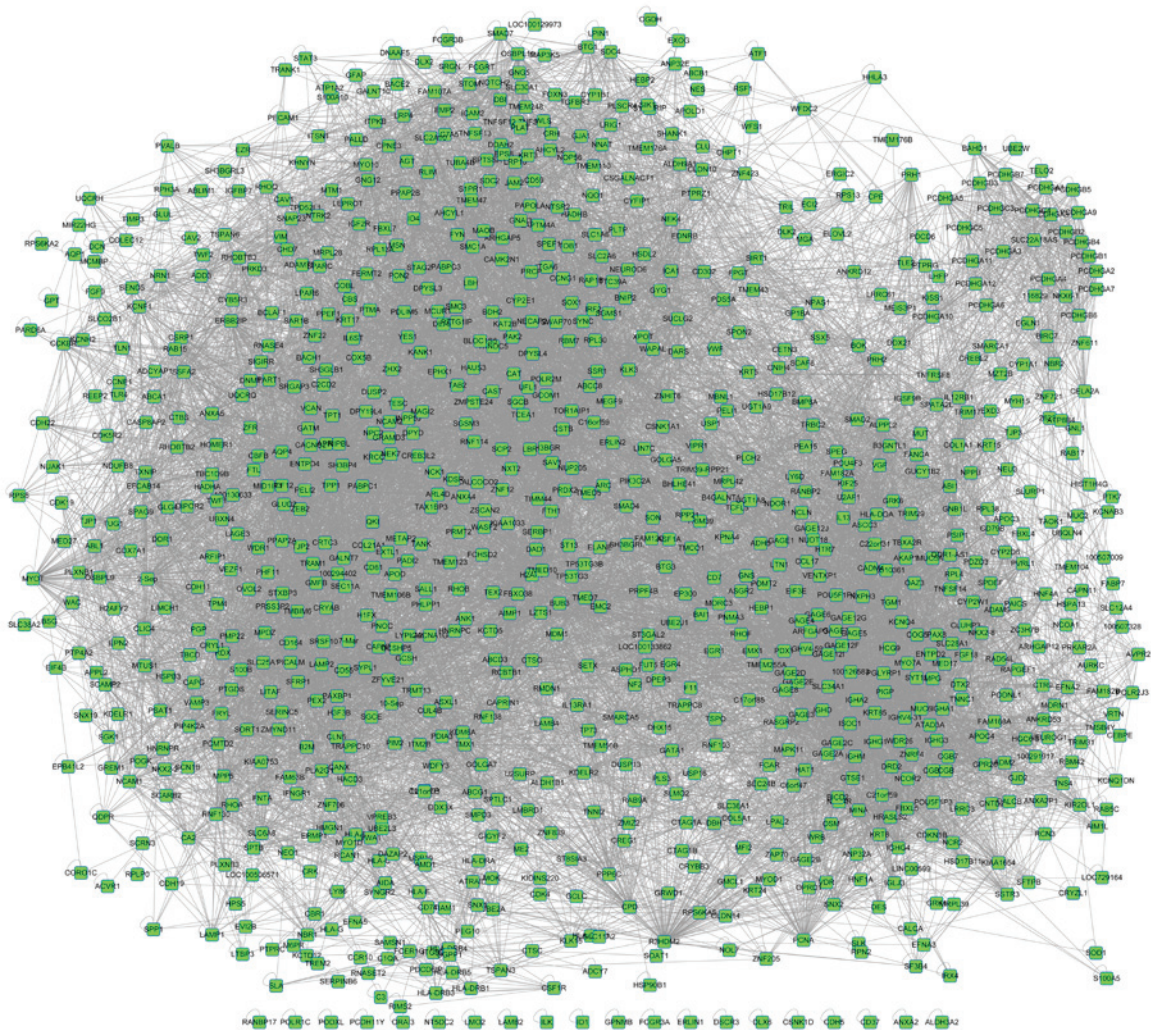DEGs, differentially expressed genes.

Figure 1. Co-expression network of differential expression genes. Square nodes represent significant differential genes, and lines represent the association between the genes.
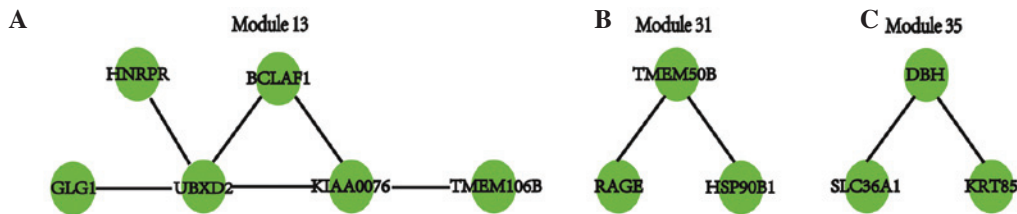

Figure 2. Significant differential modules of Down's syndrome screened from co-expressed networks using the method of mutual information. The nodes are differentially expressed genes. A, B and C indicate the three modules extracted from the network.

analysis of DEGs, 17 significant signaling pathways involving 158 DEGs were obtained in the current study. The upregulated DEGs were significantly enriched in 15 signaling pathways, such as cell adhesion molecules, type I diabetes mellitus, autoimmune thyroid disease and lysosome. The pathway of cell adhesion molecules has previously been confirmed to be upregulated in infants with DS (35). Previous epidemiological surveys have demonstrated that the prevalence of type I diabetes mellitus in patients with DS was higher than that in the general population (36).

The present study demonstrated that 8 upregulated DEGs were enriched in the signaling pathway of autoimmune thyroid disease, of which regulator of calcineurin 1 (*RCAN1*) has been confirmed to contribute to the DS phenotype and autoimmune disorders (37,38). *RCAN1*, also known as *DSCR1*, is located in the minimal candidate region and is expressed as four protein isoforms using four alternative first exons (39,40). *RCAN1* encodes a novel protein which may affect the process of transcriptional regulation or signal transduction (39,40). Fuentes *et al* (41) demonstrated that the expression of the calcineurin-dependent gene was downregulated following the inhibition of nuclear factor of activated T-cell gene translocation to the nucleus by the overexpressed *DSCR1*. In addition, the lysosome was an important pathway enriched by 14 upregulated DEGs. A study by Kalanj-Bognar *et al* (42) showed that the activities of some lysosomal enzymes participated in the metabolism of sulfatides and gangliosides, and that there was a significant increase of β-galactosidase activity in DS leukocytes compared with controls.

In the current study, significant differential modules were identified from the co-expressed network and used as characteristics for classification of DS samples in GSE16677. Current studies on genomics focus on characterization of DEGs and co-expression networks, although this is a time-consuming and costly process (43-45). In order to solve these problems, Pavesi *et al* applied support vector machines (SVMs) with non-linear kernels, and the average accuracy of non-linear SVMs reached 66.5% (46). However, in the present study, the classification accuracy was 54.55 and 72.73% when characterized by 12 DEGs and 3 significant differential modules, respectively. More reliable results were obtained using this method by combining significant differential modules with the assessment of classification efficiency.

A total of 7 DEGs in significant differential modules were identified as being enriched in 5 pathways by functional enrichment analysis. The pathway of apoptosis regulation was enriched by *BCLAF1* (module 13), *HSP90B1* (module 31) and *DBH* (module 35). A previous study demonstrated that DS neurons have a defect, which may lead to mental retardation early in life and promote the metabolism of reactive oxygen species that cause neuronal apoptosis (47). In addition, *HSP90B1* can help stabilize protein-folding intermediates when the endoplasmic reticulum is involved in protein folding (48). *TMEM50B* was found to be involved with the function of endoplasmic reticulum in the current study. The Allen brain atlas investigated by Lein *et al* (49) showed that *TMEM50B* may be a candidate for DS brain phenotypes (49), in addition, experiments on a DS mouse model demonstrated that *TMEM50B* was overexpressed in the DS cerebellum, compared with a normal mouse (50). The present study identified that *UBXD2*

was enriched in two functions: The endoplasmic reticulum and the endomembrane system. A previous study demonstrated that *UBXD2* was a molecular chaperone involved in various cellular processes, such as endoplasmic-reticulum-associated protein degradation, and that the overexpression of *UBXD2* resulted in additional immunoprecipitation, which was associated with Alzheimer's disease (51). Therefore, *UBXD2* may be involved in the complications of DS. *BCLAF1*, which intrinsically inhibits tumor cells, was demonstrated to be enriched in three functions in the present study, including the regulation of apoptosis, programmed cell death and cell death. It has been reported that *BCLAF1* participates in the γH2AX-mediated regulation of apoptosis, DNA repair, and interacts with γH2AX as a tumor suppressor (52). An extra unpaired chromosome in DS is modified by phosphorylation of H2AX (53). Therefore, *BCLAF1* may be involved indirectly in the pathogenesis of DS. In conclusion, the DEGs in the selected differentially co-expressed gene modules may participate in the pathogenesis of DS.

Acknowledgements

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