

Original Article

Screening key genes associated with congenital heart defects in Down syndrome based on differential expression network

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Abstract: Background: Down syndrome (DS) is the most common viable chromosomal disorder with intellectual impairment and several other developmental abnormalities. Forty to fifty percent of newborns with DS have some form of congenital heart defects (CHD). The genome of CHD in DS has already been obtained, but the underlying genomic or gene expression variation that contributes to the manifestation of a CHD in DS is still unknown. Objective: This study was aimed to analyze key genes of patients with CHD in DS. Methods: Differential expression network (DEN) approach was employed to analyze the dysregulated genes and pathways in this study. First, the differentially expressed genes (DEGs) between CHD in DS and normal subjects were screened based on the microarray expression data. Next, the differential interactions were identified using spearman correlation coefficients of edges in different conditions. The DEN was then constructed combining both DEGs and differential interactions, and HUB genes were gained by degree centrality analysis of DEN. Meanwhile, disease genes included in the DEN were also ascertained. Results: When analyzing gene expression values in different conditions, no DEGs were identified. While, a total of 984 gene pairs with significant differential expression were identified. Finally, the DEN was constructed only using differential edges in our study. In this network, eight HUB genes were identified, and thereinto four genes (UBC, APP, HUWE1 and SRC) were both HUB genes and disease genes. Conclusions: DEN approach should be taken as a useful complement to traditional differential genes methods. We provide several potential underlying biomarkers for CHD in DS.

Keywords: Congenital heart defects, Down syndrome, differential expression network, centrality analysis

Introduction

Down syndrome (DS) is a genetic disorder caused by trisomy of chromosome 21 [1]. It is the most commonly occurring chromosomal abnormality in live-born infants [2] and affects 1 to 2 per 1000 live births [3-5]. Despite increasing in antenatal detection, the prevalence of babies born with DS has risen by 25% during the past 30 years and parallels the increase in advanced maternal age pregnancies [6]. Some of its phenotypes (e.g., cognitive impairment) are consistently present in all DS individuals, while others show incomplete penetrance [7-9]. The most notable phenotypes with reduced penetrance are the congenital heart defects (CHD), forty to fifty percent of newborns with DS have some form of CHD [3, 10-12]. Of those with CHD, about 80% have an atrioventricular septal defect or ventricular sep-

tal defect [13]. The relatively greater infant mortality rate in the DS population has been largely attributed to their having a higher incidence of CHD [11, 14, 15]. Given the relative prevalence of patients with both DS and CHD, additional information with regard to expect cardiac surgical outcomes for this particular population might provide parents and medical providers with important guidance. The fact that the frequency of CHD in DS is much higher than normal euploid individuals indicates that dosage-sensitive genes on chromosome 21 greatly increase the risk for CHD [16]. However, the main genomic or gene expression variation that contributes to the manifestation of a CHD in DS is still unknown.

Genes are tightly regulated to execute the proper biological functions in a cell for responding internal or external perturbations [17], and thus

their expression variations during disease deterioration process are causally associated with the phenotype changes. Recent rapid advance on high-throughput technologies provides unprecedented opportunities to measure dynamical behaviors of various tissues at a genome-wide level [18, 19]. To date, gene expression profiles have been widely used for complex diseases research and a variety of methods have been proposed to identify molecular biomarkers or select features out of the gene expression profiles [20]. Considering the large volume of the data obtained through high-throughput techniques and the negative influence from noise, a natural strategy is to look for those genes that are dramatically different in disease state and normal state.

Network-based systems biology offers a quantifiable description of the molecular networks, which characterize the complex interactions and the intricate interwoven relationships. These relationships govern cellular functions among those tissues and disease related genes to explain the molecular processes during disease development and progression [21-24]. Network-based approaches have been developed to extract informative genes relying on biomolecular networks (e.g., protein-protein interaction (PPI) network and gene regulatory network), rather than individual genes. Differential expression network (DEN), developed by Sun et al. [25], is a new form of molecular network, which is based on both molecular network (e.g. PPI network) and gene expression profiles to characterize biological process, in contrast to traditional 'differential genes' (DG) or 'differential network' (DN). DEN is composed of 'non-differential interactions' and 'differential interactions'. From the network viewpoint, the DEN actually not only covers DG and DN, but also includes disease-related 'non-differential interactions' which are missed in DN. Meanwhile, the DEN scheme provides a novel way to predict disease genes and further disease interactions, it can cover 3-4 folds more known disease genes than traditional differentially expressed genes (DEGs) [25]. Also, DEN fully explores all disease-related interactions including non-differential interactions among the proteins, and therefore is able to identify disease genes and disease interactions in an accurate manner [25].

In this study, we aimed to analyze key genes related to patients with CHD in DS basing on

the DEN approach. First, we obtained the microarray data of healthy human beings and DS with CHD from the ArrayExpress database. Next, DEGs between DS patients with CHD and normal controls were screened. Then differential interactions were identified using spearman correlation coefficient based on the gene expression values under different conditions. Finally, the DEN was constructed and HUB genes were discovered by centrality analysis. This study may provide information to explore the potential key genes and the underlying molecular mechanisms of CHD in DS.

Methods

Data recruitment and preprocessing

The gene expression profile of E-GEOD-1789 which was contributed by Conti et al. [26] was obtained from ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress/>). The data was gained from cardiac tissue which was obtained from fetuses at 18-22 weeks of gestation after therapeutic abortion. Ten samples from fetuses trisomic for Hsa21 and 5 from euploid control fetuses were studied [26]. The microarray data and annotation files of healthy human beings and CHD in DS were downloaded for further analysis.

In order to eliminate the influence of nonspecific hybridization, we carried out background correction and normalization by robust multichip average (RMA) method [27] and quantile based algorithm [28]. And we used Micro Array Suite 5.0 (MAS 5.0) algorithm to revise perfect match and mismatch value [29], the value of which was selected via the median method. The gene expression value was transformed to a comparable level. Then we used feature filter method of genefilter package screened the data. Each probe was mapped to one gene, where the probe was discarded if it couldn't match any genes. The expression value averaged over probes was used as the gene expression value if the gene had multiple probes.

Identifying DEGs

After data preprocessing, we used empirical Bayes method (F test) that was implemented in the linear models for microarray data (LIMMA) package [30] to identify DEGs between CHD in DS and normal controls. Values of $|\log \text{Fold Change (FC)}| > 2.0$ and $P\text{-value} < 0.05$ were selected as the cut-off criteria.

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Identifying dysfunctional interactions

In this study, the original PPI network was integrated from Biological General Repository for Interaction Datasets (BioGrid, <http://thebiogrid.org/>). In the BioGrid, a total of 15,750 genes and 248,584 interactions of human beings were included. Based on the transcript data of E-GEOD-1789, the PPI network including 10,630 genes and 184,940 interactions was then constructed.

Calculating spearman correlation coefficients of gene pairs

Spearman correlation coefficient we used in this study is a popular methods to describe the interaction strength between genes [31]. In the network, the spearman correlation coefficient of each edge was computed separately based on the gene expression values under different conditions (controls and CHD in DS), which denoted as A1 and A2, respectively.

Determining the threshold of P value

In order to determine how to choose the gene relationships for further research, we built two models (one for the control group, the other for case group) randomly, each model contains 200,000 gene relationships. The spearman correlation coefficients of edges in two models (A1, A2) were calculated respectively, and the absolute value of the correlation coefficients of them ($|A1-A2|$) were obtained. To get a more powerful and less bias subject, multiple testing was employed in this work. The absolute value of the correlation coefficients was set in descending order. We found that when the threshold of *P*-value was set to 0.05, the absolute value of the correlation coefficient was 1.397. Therefore, we conducted the spearman value of the 184,940 relationships that we obtained from BioGrid in descending order. And then we chose out these gene relationships that whose absolute value of the correlation coefficient was greater than 1.397, as well as at least one of the spearman correlation coefficients was greater than 0.7.

Constructing DEN

After both DEGs and differential interactions were identified, the DEN was then constructed using differential interactions and non-differential interactions whose two endpoints were

both DEGs. The network was constructed using Cytoscape 2.1 software.

Centrality analysis

In any network structure, the role of a node depends, not only on the features of the node itself, but also on the topological structure of the network and on the other nodes features [32]. In the present study, centrality analysis, which was particularly useful to identify key players in biological processes was implemented to study the DEN. Formally, a centrality is a function which assigns every vertex of a graph a numeric value. Centrality measures mainly contain degree centrality, closeness centrality and shortest path between centrality, in which degree is the simplest topological index. Nodes with high degree (highly connected) are called “hubs”, which interact with several other genes, suggesting a central role in the interaction network. Genome-wide studies show that deletion of a hub protein is more likely to be lethal than deletion of a non-hub protein, a phenomenon known as the centrality-lethality rule [33]. In this work, the degree of the genes which were equal or greater than 9 were considered as hub genes. An obvious order of the vertices of a graph can be established by sorting them according to their degree [32]. Networks displaying a degree distribution approximating a power-law are called scale-free networks [34], which are intrinsically robust to random attacks. The degree distribution of the DEN was also analyzed in this work.

Ascertaining disease genes included in the DEN

Genecards is a database of human genes that provides genomic, proteomic, transcriptomic, genetic and functional information on all known and predicted human genes [35]. In the present study, disease genes that related to CHD in DS were obtained from Genecards database (<http://www.genecards.org/>). There are 1614 disease genes of CHD in DS in all in the database. We downloaded all the data for further research. The disease genes including in the DEN was ascertained by comprehensive statistical analysis.

Functional enrichment analysis of the nodes

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database is a recognized and

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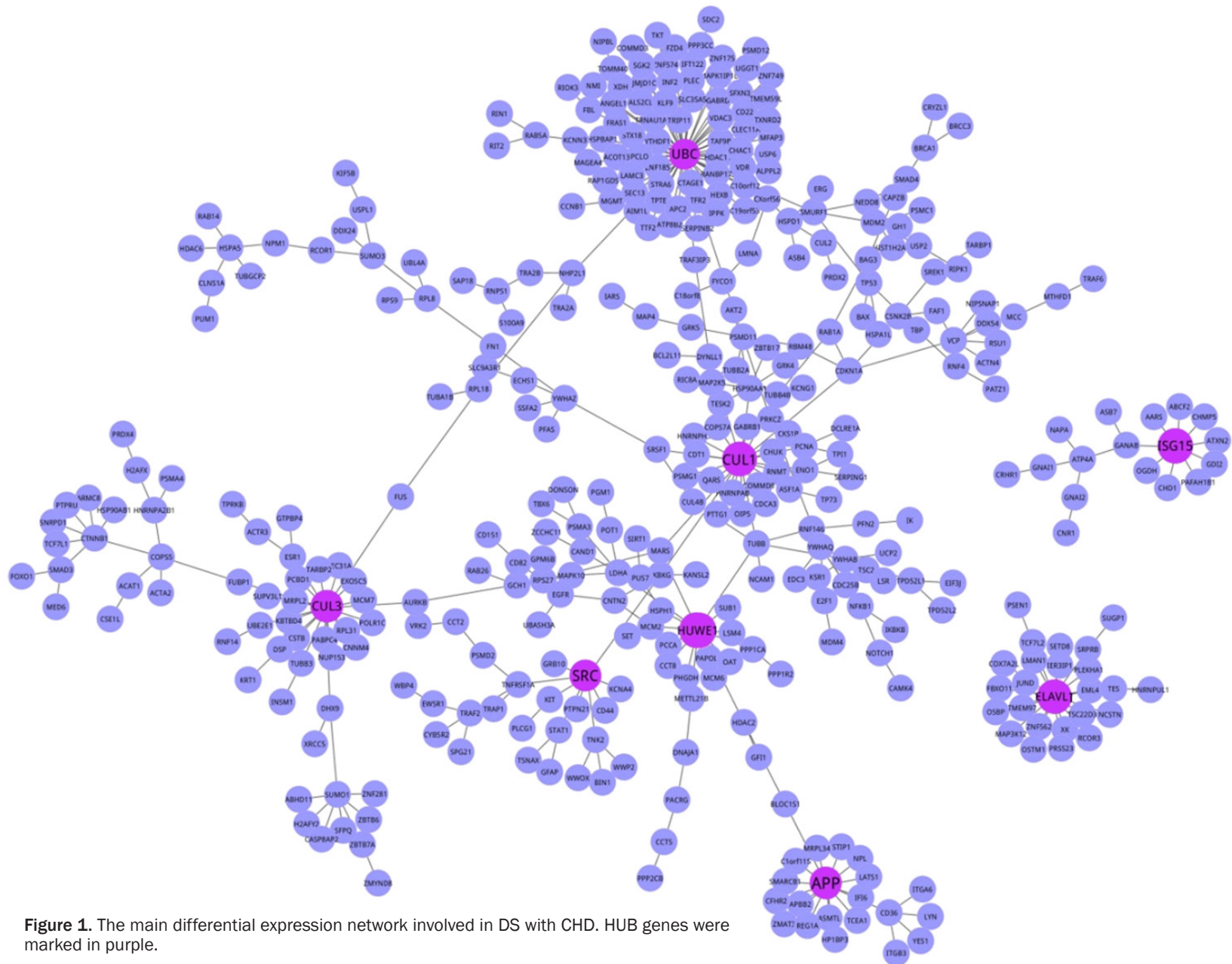


Figure 1. The main differential expression network involved in DS with CHD. HUB genes were marked in purple.

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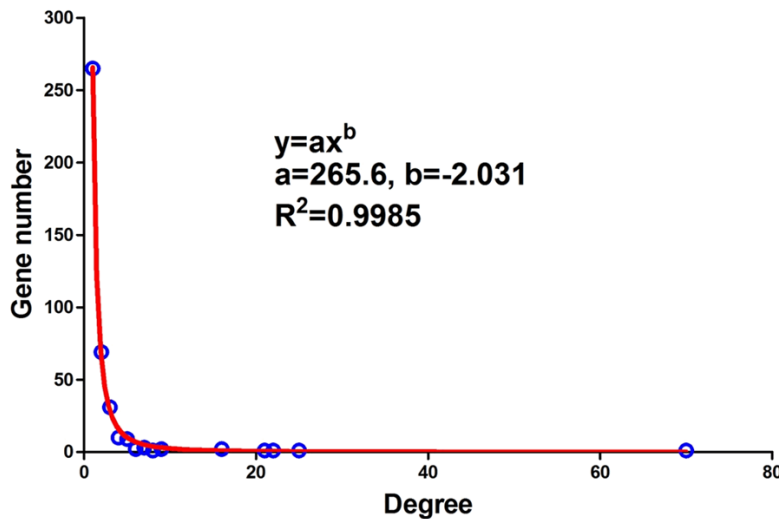


Figure 2. The scattergram of gene degree distribution in the main differential expression network. The degree distribution presents a power law, indicating the character of scale-free network.

comprehensive data base including all kinds of biochemistry pathways [36]. In this study, the KEGG database was applied to investigate the enrichment analysis of the nodes to find the biochemistry pathways which might be involved in the occurrence and development of CHD in DS. The Database for Annotation, Visualization and Integrated Discovery (DAVID) [37] was used to perform the KEGG pathway enrichment analysis with the p value < 0.05 and gene count > 5 .

Results

Constructing DEN

As we designed, the DEN should be constructed by complementarily considering both DEGs and dysfunctional gene pairs. Unfortunately, when identifying DEGs using the criteria of $|\log FC| > 2.0$ and $P < 0.05$ by LIMMA package, none DEGs were selected. So the DEN was built just basing on the differential interactions. We conducted the spearman value of the 184,940 gene pairs, and then selected gene pairs whose absolute value of the spearman correlation coefficients in two conditions were greater than 1.397, as well as at least one of the spearman correlation coefficients was greater than 0.7 to construct the DEN. Finally, the DEN was constructed using 984 gene pairs. However, there were some gene pairs not containing in the main DEN. By conducting statistical analysis on the major network, as shown in **Figure 1**, there

were 398 genes, 405 edges containing in it.

Disease genes including in the DEN

Through comprehensive statistical analysis, we found that among the 1614 disease genes that we obtained from the Genecards database, 293 disease genes were included in the DEN that we built above.

Centrality analysis to gain HUB genes

It had been introduced in the method, we mainly analyzed the degree centrality of the network in this study. As we

conducted analysis on the nodes degree of the main DEN that we built above, as shown in **Figure 2**, the degree distribution displayed approximating a power-law, suggesting that the DEN was a scale-free network. Meanwhile, we set the degree of the genes containing in the main DEN in descending order, we obtained eight HUB genes as following: UBC, CUL1, CUL3, ELAVL1, APP, HUWE1, SRC, ISG15. Furthermore, UBC, APP, HUWE1 and SRC were also disease genes. Parameters of these disease genes were shown in **Table 1**.

KEGG pathways analysis

In order to gain further insights into the function of nodes, DAVID was applied to identify the significant dysregulated KEGG pathways. By setting the threshold P -value < 0.05 and gene count > 5 , we obtained 39 pathways. There were 9 pathways containing at least one HUB gene (**Table 2**). It could be obviously observed from the table that these genes mainly enriched in cell cycle, adherens junction, wnt signaling pathway, ubiquitin mediated proteolysis, RIG-I-like receptor signaling pathway, gap junction, oocyte meiosis, tight junction and endocytosis. And there were two HUB disease genes, HUWE1 and SRC contained in the pathways.

Discussion

A major challenge in the field of CHD in DS research is to recapitulate the disease features

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Table 1. The parameters the HUB disease genes

Gene symbol	Degree	Description	Category	GC id
UBC	70	Ubiquitin C	protein-coding	GC12M125396
APP	16	Amyloid beta (A4) precursor protein	protein-coding	GC21M027252
HUWE1	16	HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase	protein-coding	GC0XM053559
SRC	9	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog	protein-coding	GC20P035973

Table 2. The KEGG pathways contained HUB genes

ID	Term	Count	P-value
hsa04110	Cell cycle	19	9.04E-08
hsa04520	Adherens junction	10	8.48E-04
hsa04310	Wnt signaling pathway	14	1.26E-03
hsa04622	RIG-I-like receptor signaling pathway	9	2.09E-03
hsa04540	Gap junction	9	8.42E-03
hsa04120	Ubiquitin mediated proteolysis	11	0.0141
hsa04114	Oocyte meiosis	9	0.0274
hsa04530	Tight junction	10	0.0313
hsa04144	Endocytosis	12	0.0387

screened. Traditional methods used statistical techniques, such as *t*-test or fold change, to find DEGs showing significantly differential expressed patterns between case samples and a control group [43-45]. In this case, such traditional methods were not suitable, and DEN approach as a useful complement to traditional analysis analyzes gene interactions from a new vision.

and to understand the molecular mechanisms by which the extra copy of genes leads to the abnormalities observed in CHD in DS patients [38]. Extensive efforts over the past years to gain a better understanding of the genetic basis of CHD in DS using rare cases of partial trisomy 21 have led to identification of genomic regions on chromosome 21 that, when triplicated, are consistently associated with CHD. An initial study of rare partial trisomy 21 cases suggested a minimal CHD candidate region on 21q22.3 of ~5.27 Mb between markers D21S3 and PFKL [39]; and this region was later narrowed down to 1.77 Mb (DSCAM-ZBTB21) [40]. But the exact disease genes that give rise to the occurrence and development of the CHD in DS remain unclear.

The propensity of many diseases can be reflected in a difference of gene expression levels in particular cell types had been well confirmed [41]. For this reason, gene relationships showing a different expression levels in control crowds and case strains are likely related to the disease. However, in this study, when we used the LIMMA package to screen DEGs under the criteria of $|\log_{2}FC| > 2.0$ and $P < 0.05$, there was no DEG. To avoid artificial interference, significance analysis of microarrays (SAM) [42], another method to identify DEGs, and repeated trials were implemented, and no DEGs were

Since genes usually do not carry out their functions in isolation, in order to select out key genes individually, we further detected candidate genes and interactions from the network perspective. Genes tend to share common functional features that associated with the same disorder, reflected in the fact that their protein products have a tendency to interact with each other. Although there was no DEGs in the gene expression profiles, but the co-expression of some nodes were significantly changed. Take the gene pairs UBC and ALPPL2 as example, the correlation coefficient was -1 under normal state while it increased to 0.827 under the condition of CHD in DS, which meant that the co-participated function between the corresponding two proteins dysfunctions, while the interaction between them was regarded as differential interaction. In this paper, we chose a new form of molecular network named DEN, which was composed of 'non-differential interactions' and 'differential interactions', to obtain key genes of CHD in DS. This was a bran-new method for researching CHD in DS.

After conducting the gene expression profiles of healthy human beings and CHD in DS into the DEN, we could easily find that there were eight genes including four disease genes with higher degree than the others. Thereinto, the disease gene UBC had the highest degree of

70. In eukaryotes, UBC encode protein product that is processed to yield free ubiquitin [46]. In mammals, UBC gene codes for a polyubiquitin precursor with exact head to tail repeats [47]. The UBC mRNA is typically increased more than other ubiquitin mRNAs when the ubiquitin-proteasome system is activated in muscle of catabolic patients [48]. It was reported that RCAN1 was closely implicated with the neuropathological characteristics in Down syndrome [49], meanwhile, ubiquitin was found to be colocalized with RCAN1 aggregates [50]. In other words, there was a close relationship between ubiquitin and Down syndrome. Gardiner et al. [51] compared the gene content of human chromosome 21 with mouse modules of DS, and indicated that the orthologous genes encoded components of several cell pathways including the ubiquitin pathway. In our study, there were 9 enriched pathways containing at least one HUB gene, and the ubiquitin mediated proteolysis pathway was included. It further proved the close relationship between ubiquitin and CHD in DS.

However, there are some deficiencies of our study. First, the microarray data is from Array Express database but not generated by ourselves. Second, the data on CHD in DS was so limited that only allowed us to take advantage of a set of data for our study, and the outcome of gene expression profiles may have a high false positive rate. Therefore, further experimental studies should be carried out based on a larger sample size in order to confirm our results.

Conclusion

DEN approach which analyzes gene interactions from a new vision could be considered as a useful complement to traditional differential gene approaches. Based on the analysis of DEN method, several hub disease genes (UBC, APP, HUWE1 and SRC) might be target genes for diagnosing the CHD in DS.

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Disclosure of conflict of interest

None.

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