Bioinformatic Analysis of Genes and MicroRNAs Associated With Atrioventricular Septal Defect in Down Syndrome Patients

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Summary

Down syndrome (DS), also known as trisomy 21, is a genetic disorder caused by the presence of part or all of a third copy of chromosome 21. It is the most common chromosome abnormality in humans, occurring in about 1 per 1000 new-born babies annually. DS is typically associated with physical growth delays, intellectual disability, and characteristic facial features. The mental abilities of adults with DS are generally similar to that of an 8- or 9-year-old child. In addition, adults with DS can also have various health problems, including congenital heart disease, leukemia, thyroid disorders, and mental illness. Approximately 45% of children with DS have congenital heart disease, of which 35-40% suffer from atrioventricular septal defect (AVSD).

AVSD, also known as endocardial cushion or atrioventricular canal defect, is a clinically significant congenital heart malformation, and it is generally combined with other diseases like pulmonary valve stenosis. AVSD is the most commonly occurring heart defect in DS. Recent studies have indicated that AVSD is regulated by a series of molecules and signaling pathways. Numerous genes, such as CRELD1 and COL6A1, have been found to participate in AVSD development in DS patients. The mutation of COL6A1 in exon 35 genes has been identified in DS patients with AVSD. Reportedly, Ciliome and VEGF-A pathways are involved in the genetic underpinnings of AVSD in humans. Ripoll, et al identified several GO (gene ontology) functions and pathways associated with AVSD in DS patients by using gene expression profiling. However, no in-depth studies have identified the protein-protein interactions (PPIs) between candidate genes of AVSD. In addition, there is evidence that microRNAs (miRNAs) play crucial roles in heart development and diseases, such as miR-155, miR-652, microRNA-340-5p, and miR-210. Circulating miRNAs can also serve as prognostic or diagnostic biomarkers for various cardiovascular diseases. However, there have been few studies on miRNAs involved in AVSD in DS patients. Thus, ongoing efforts are needed to further identify candidate genes and miRNAs related to this disease.

In this study, we first identified the differentially ex-
pressed genes (DEGs) between DS patients without congenital heart disease and DS patients with AVSD. Next, the functions and pathways enriched by DEGs were investigated. Furthermore, PPIs between DEGs and miRNA-target pairs were predicted, and miRNA-DEG regulatory networks were constructed. Consequently, a variety of genes regulated by miRNAs were predicted to participate in AVSD in DS patients.

**METHODS**

**Microarray data:** Gene expression profiling of GSE34457 was downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), based on the platform of GPL6102 (Illumina human-6 v2.0 expression beadchip). A total of 22 samples from DS patients without congenital heart disease and 7 samples from DS patients with AVSD were utilized in this study. As only the samples of AVSD and control groups were utilized, the characteristics of DS patients were re-analyzed, and patients in these groups had similar age distribution ((19.3 ± 2.4) for DS; (17.1 ± 3.7) for AVSD + DS), similar gender ratios (Male/Female = 1.0 for DS; Male/Female = 0.75 for AVSD + DS), and the same ethnicity (Caucasian). In addition, samples were peripheral blood lymphocyte-derived lymphoblastoid cells immortalized with the same Epstein-Barr virus batch, and it was confirmed that immortalization did not cause other chromosomal rearrangements based on the detection of trisomic/euploid status.

**Data preprocessing and DEGs screening:** In data preprocessing, probe-level data were first converted into gene expression values. For each sample, the expression values of probes mapped to a given gene were averaged. Then, missing data were imputed, and median normalization was performed by using preprocessCore package in R. Finally, limma package in R, the most widely used software in DEGs screening, was used to identify significant DEGs between DS patients without congenital heart disease and DS patients with AVSD. For each DEG, both P < 0.05 and |log2 FC| > 0.5 need to be met.

**Pathway and functional enrichment analyses:** The Database for Annotation, Visualization, and Integrated Discovery (DAVID) provides a comprehensive set of functional annotation tools for investigators to understand the biological meaning of genes. In this study, DAVID was applied to investigate the GO functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involving DEGs. The significant functions and pathways might relate to AVSD in DS patients. A P-value < 0.05 was chosen as the cut-off criterion.

**Construction of PPI networks:** The online server Search Tool for the Retrieval of Interacting Genes (STRING) stores PPI information obtained from functional experiments and prediction based on bioinformatics technologies. In the present study, WebGestalt was used to predict the potential miRNAs targeting the DEGs mined above. For miRNA-DEG pairs, the criterion was set as P < 0.05, while for each miRNA, the number of target DEGs should ≥ 2. By integrating protein-protein pairs and miRNA-DEG pairs, miRNA-DEG regulatory networks were constructed and visualized by Cytoscape software.

**RESULTS**

**Data preprocessing and DEGs screening:** Microarray data before and after normalization are presented in Figure 1. After normalization of microarray data, median expression values of all samples were centered on a straight line, with the exception of GSM849488. GSM849488 was a sample from a DS patient with AVSD, and it was then deleted in this study. After DEGs screening, 179 significant DEGs (P < 0.05 and |log2 FC| > 0.5) were identified, including 59 up-regulated and 120 down-regulated DEGs.

**Pathway and functional enrichment analyses:** To investigate the specific functions and pathways associated with DEGs, pathway and functional enrichment analyses were performed. The results demonstrated that 5 GO functions (P < 0.05) (Table I) and 1 KEGG pathway (P < 0.05) (Table II) were significantly enriched by up-regulated DEGs, while 4 functions (P < 0.05) were significantly enriched by down-regulated DEGs (Table I). Moreover, genes like IL12RB2 and IL1B were enriched in more functions than other DEGs.

**PPI networks:** PPIs between DEGs were investigated using the online server STRING, and a total of 22 protein-protein pairs (combined score > 0.4) were identified. Based on these protein-protein pairs, PPI networks were constructed (Figure 1).
2), involving 16 down-regulated DEGs, 17 up-regulated DEGs, and 22 PPIs. In this network, *IL1B* had the highest node degree, and it was defined as the hub-gene of PPI networks.

**miRNA-DEG regulatory networks:** Based on Webgestalt (*P* < 0.05 and target DEGs ≥ 2), miR-518a, miR-518e, miR-518f, miR-528a, and miR-96 were predicted to regulate the identified DEGs. After integrating PPI networks with the 18 miRNA-DEG regulatory pairs, miRNA-DEG regulatory networks were constructed (Figure 3), involving 24 down-regulated DEGs, 19 up-regulated DEGs, and 5 miRNAs. Among these DEGs, *AUTS2* was predicted to be co-regulated by miR-518e, miR-518f, and miR-528a, whereas *KIAA2022* was predicted to

### Table I. GO Functional Enrichment Analysis of DEGs

<table>
<thead>
<tr>
<th>Category</th>
<th>GO ID</th>
<th>Term</th>
<th>Gene counts</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>GO:0032496</td>
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<tr>
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<td>Integral to plasma membrane</td>
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<td>Intrinsic to plasma membrane</td>
<td>9</td>
<td>0.012561</td>
</tr>
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<td>Up</td>
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<td>Sugar binding</td>
<td>4</td>
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<tr>
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<td>4</td>
<td>0.016134</td>
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<tr>
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<td>0.006144</td>
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<tr>
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<td>3</td>
<td>0.012029</td>
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<tr>
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</tr>
<tr>
<td>Down</td>
<td>GO:0000790</td>
<td>Nuclear chromatin</td>
<td>3</td>
<td>0.021011</td>
</tr>
</tbody>
</table>

GO indicates gene ontology; DEGs, differentially expressed genes; Up, up-regulated genes; Down, down-regulated genes; and ID, identifier.

### Table II. KEGG Pathway Enrichment Analysis of Up-Regulated DEGs

<table>
<thead>
<tr>
<th>KEGG pathway</th>
<th>Gene Counts</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa04640-Hematopoietic cell lineage</td>
<td>4</td>
<td>0.003721382</td>
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</tbody>
</table>

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

**Figure 2.** Protein-protein interaction networks of DEGs. Dark nodes: up-regulated DEGs; light nodes: down-regulated DEGs; lines: interactions between DEGs. DEGs indicates differentially expressed genes.

**Figure 3.** miRNA-DEG regulatory networks. Dark nodes: up-regulated DEGs; light nodes: down-regulated DEGs; normal lines: interactions between DEGs; diamond nodes: miRNAs; arrowed lines: regulatory relationships between miRNAs and DEGs. miRNAs indicates microRNAs and DEGs; differentially expressed genes.
be co-regulated by miR-518a, miR-518e, miR-518f, and miR-96.

**DISCUSSION**

To gain insight into the molecular mechanism of AVSD in DS patients, gene expression profiling was systematically analyzed in this study. Consequently, a total of 179 DEGs were screened out, which were significantly enriched in 9 functions and 1 pathway. Furthermore, PPI and miRNA-target analyses indicated that IL1B was the hub-gene of PPI networks, and AUTS2 and KIAA2022 might be co-regulated by miR-518a, miR-518e, miR-518f, miR-528a, and miR-96 in miRNA-DEG regulatory networks.

In comparison with a previous study based on the same microarray profiling, a different DEG screening method and criteria were utilized in our study. The limma package used in the present study is the most widely used software in DEG screening, and the criteria of \( P < 0.05 \) and \( \log_2 FC > 0.5 \) are strict enough to ensure the significance of DEGs. Therefore, the accuracy of the 179 DEGs identified in our study might be higher than that of the 889 DEGs found in the study by Ripoll, *et al.* As proteins/genes generally function via interacting with each other, we investigated the PPIs between DEGs and find the hub-gene IL1B, which was not identified by Ripoll, *et al.* study. Furthermore, miRNAs that might target DEGs were discovered, including miR-518a, miR-518e, miR-518f, miR-528a, and miR-96, shedding light on the post-transcriptional regulation of DEGs.

Some of the significant DEGs identified in this study were discovered in the study of Ripoll, *et al.*, such as AUTS2 (autism susceptibility candidate 2), showing the validity of our bioinformatics methods. Reportedly, AUTS2 contributes to the differentiation of human embryonic stem cells into cardiomyocytes, as well as the following differentiation of cardiomyocytes into beating embryoid bodies. AUTS2 is involved in the Notch signaling pathway, which takes part in mammalian cardiac development and is related to human congenital heart defects like bicuspid aortic valve disease, ventricular septal defects, and calcification of the heart valves. Moreover, AUTS2 is a transcriptome signature of heart malformations, and it is associated with supravalvular aortic stenosis.

In the present study, AUTS2 was remarkably upregulated in DS patients with AVSD, and this result coincided with the previous study, in which AUTS2 mRNA was increased about 80% in DS patients with AVSD in comparison with DS patients without congenital heart disease, based on both microarray analysis and quantitative polymerase chain reaction (qPCR). These results implied the validity of our analysis based on limma package, and the involvement of AUTS2 in AVSD development in DS patients.

Novel candidate AVSD genes were identified in this study, such as IL1B (interleukin-1b), IL12RB2 (IL-12 receptor \( \beta 2 \)), and KIAA2022. Among these genes, IL1B and IL12RB2 were found to interact with each other, and they were jointly enriched in various GO functions and KEGG pathways. Reportedly, IL1B is an important mediator of an inflammatory response, and it participates in the MAPK signaling pathway, which is implicated in cardiac regulation and heart diseases like hypertrophic cardiomyopathy, dilated cardiomyopathy, ischemic/reperfusion injury, and cardiac fibrosis. Further-more, IL12RB2 is a subunit of the interleukin 12 (IL12) receptor complex of type 1 transmembrane protein. IL12RB2 and IL12 are associated with the JAK/STAT signaling pathway, which plays a role in the recovery of cardiac functions. By activating Enhancer-of-split complex genes, JAK/STAT signals regulate the expression of T-box transcription factor H15, directing heart precursor diversification. These findings suggest that the dysregulation of IL1B and IL12RB2 may contribute to AVSD in DS patients by affecting MAPK and JAK/STAT signaling pathways.

KIAA2022 is also known as an X-linked mental retardation protein related to neurite extension (XPN), and it plays a role in regulating cell-cell and cell-matrix adhesion and migration. It has been shown that the dysregulated cell-cell and cell-matrix adhesion signaling pathways contribute to heart birth defects during heart organogenesis. In this study, KIAA2022 was significantly down-regulated in DS patients with AVSD. Therefore, KIAA2022 was expected to play a role in AVSD in DS patients through regulating cell-cell and cell-matrix adhesion and migration.

Moreover, miR-518a, miR-518e, miR-518f, miR-528a, and miR-96 were predicted to target genes dysregulated in DS patients with AVSD. Specifically, miR-518a, miR-518f, and miR-528a were predicted to jointly target AUTS2, while miR-518a, miR-518e, miR-518f, and miR-96 were predicted to co-regulate KIAA2022 in miRNA-DEG networks. Additionally, miR-96 mediates cell apoptosis by targeting the forkhead box protein O1 (FOXO1) gene, which induces mice death via impairing cardiomyocyte proliferation, reducing heart size and myocardium thickness, and causing heart failure. Therefore, it was predicted that miR-518a, miR-518e, miR-518f, miR-528a, and miR-96 might be associated with AVSD progression in DS patients via targeting AUTS2 and KIAA2022.

Interestingly, genes were differently expressed among DS patients, namely, some genes were up-regulated or down-regulated in some individuals with trisomy 21 in comparison with others with trisomy 21. One of the potential reasons is that DS is characterized by the presence of part or all of a third copy of chromosome 21. The genes on chromosome 21 may directly change their expression levels in a dosage compensation manner in DS patients, and these changes may further influence the expression of genes on other chromosomes via for example transcription factor-target regulation, miRNA-target modulation, and chromatin remodeling proteins. The appearance of different additional parts of chromosome 21 may cause different expression profiling in DS patients.

In conclusion, based on gene expression profiling, we identified DEGs between DS patients without congenital heart disease and DS patients with AVSD. Furthermore, bioinformatics analyses were conducted, including functional and pathway enrichment, PPI investigation, as well as miRNA prediction. The results indicated that IL1B, IL12RB2, AUTS2, and KIAA2022 might participate in AVSD in DS patients, and miR-518a, miR-518e, miR-518f, miR-528a, and miR-96 might target AUTS2 and KIAA2022.

However, several limitations exist in the present study. The sample size is small, and no time-series experiment or validation experiment was performed. In a future study, we plan to perform experiments like qPCR and Western blot to confirm the expression changes of DEGs, as well as miR-518a, miR-518e, miR-518f, miR-528a, and miR-96 that might target
**REFERENCES**


