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Identifying Key Genes and Therapeutic Targets in Down Syndrome: A Comprehensive Analysis Using RNA-Seq, PPI Networks, and Molecular Docking

S. Yogigiridhar, J. Jino Blessy*, Shalini Urumaiya and D. Jaswanthi

Department of Bioinformatics, Sri Ramachandra Faculty of Engineering and Technology, Sri Ramachandra Institute of Higher Education and Research, Chennai 600116, India

**Corresponding author*

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A B S T R A C T

Down syndrome (DS) is a widespread genetic disorder associated with a range of physical and intellectual disabilities, and it can also lead to several metabolic and health-related issues. Modernization and technological innovation have simplified next-generation sequencing through the use of open-source online software like Galaxy, which enables users to easily share their data and workflow mappings. Current study is to identify candidate genes for DS by performing differential expression of genes. RNA-Seq analysis was performed for different datasets retrieved from the GEO database. The 10 datasets from DS patients and 10 datasets from healthy control were analysed for differentially expressed genes (DEGs). DEGs analysis showed 10 upregulated and 10 downregulated genes with log2FC counts > 2.5 and p-values <0.05. To further investigate these differentially expressed genes (DEGs), WebGestalt was used for comprehensive in silico analysis, visualizing enrichment via volcano plots. Additionally, protein-protein interaction (PPI) networks were constructed using STRING, identifying three gene modules and ten hub genes through Cytoscape cluster analysis. Molecular docking studies were then conducted on these hub genes using PyRx software. This included the addition of polar hydrogen atoms, the assignment of partial charges, and the removal of water molecules to prepare for efficient molecular docking. This research enhances our understanding of gene interactions and protein-phytochemical binding mechanisms, thereby contributing to therapeutic advancements in the biopharmaceutical sector.

Introduction

DS is one of the most common genetic disorders worldwide, affecting approximately 1 in 700 live births. The condition results from an extra copy of chromosome 21, leading to a total of three copies instead of the usual two, which is why it is also known as trisomy 21 (Antonarakis *et al.,* 2020). This chromosomal anomaly leads to a range of physical and intellectual disabilities. Family history, maternal age, and genetic predisposition are known factors that increase the risk of having a child with down syndrome (Khoury and Erickson, 1992). The likelihood of having a baby with down syndrome increases with maternal age, particularly for women aged

35 and older. It was estimated that there were 6 million people with DS worldwide (Fortea *et al.,* 2024).

Many microarray-based efforts have been conducted to identify key genes associated with DS. The use of microarray technology has simplified the examination of genetic alterations underlying the onset and progression of DS cells with validation through real-time PCR, study of the DNA methylation and RNA-sequencing methods. Each of these techniques has its own strengths and limitations when it comes to finding key gene targets. Although many studies have detected a large number of DEGs, consistent changes in particular genes, gene sets, or pathways have not been identified to indicate a causative role. Genetic mutations, or changes, play a significant role in the development of DS. These genetic alterations are inherited and can vary from one individual to another (Park *et al.,* 2021; Chapman *et al.,* 2024).

The risk of DS is often associated with maternal age and genetic predisposition. While dietary and environmental factors have minimal evidence linking them to the risk of DS, the genetic basis remains a significant aspect. Early signs of DS can be detected through prenatal screening methods such as the nuchal translucency scan, cell-free fetal DNA testing, and amniocentesis. These methods help in early diagnosis, allowing for better planning and management (Wojcik *et al.,* 2020). Studies have shown that individuals with DS have distinct DEGs as determined by gene expression profiling (Yu *et al.,* 2020; Chapman *et al.,* 2024). However, recent techniques demonstrate that a vast number of genes may not be directly related to each other. The primary focus of research is to discover significant genes related to DS development to enhance detection, prognosis, and treatment. Understanding the molecular signature of DS compared to normal genetic profiles is crucial for improving patient outcomes.

DS is increasing at an alarming rate leading to various life-threatening conditions. The identification of candidate genes for DS is highly important for treating this global epidemic crisis (Plaiasu, 2017). Modernization and technological innovation created novel sequencing technologies in genome sequencing whereby large DNA fragments were detected using NGS (Meldrum *et al.,* 2011). In recent years, RNA sequencing has been widely exploited to continuously monitor the changes in cellular transcriptome. The objective of RNA-Seq is to create profiling of gene expressions by identification of genes or their corresponding molecular pathways and understanding the differentially expressed genes among two or more biological conditions using a galaxy platform. The dataset for DS is imported from public databases to identify differentially expressed genes (DEG) involved in DS. The objective of this research is to identify significant genes associated with the development of DS. This may aid in improving the detection, prognosis, and treatment of the disease.

Materials and Methods

DESEQ2

Next-generation RNA sequencing samples were retrieved from the NCBI GEO Database from 10 different studies, namely GSE142221, GSE224687, GSE248895, GSE223758, GSE223405, and GSE223249 (Table 1) (Barrett *et al.,* 2012). A total of 60 samples were used, with 35 samples from Down syndrome (DS) patients and 25 samples from normal patients. The datasets were imported into the Galaxy Server (https://usegalaxy.org.au/) using the tool Faster Download and Extract Reads in FASTQ format from NCBI SRA (Galaxy Version 2.11.0 + galaxy0) (Jalili *et al.,* 2020). The read quality check was performed using the tool FastQC Read Quality Reports (Galaxy Version 0.73 + galaxy0). Sequence Mapping and Alignment were performed using HISAT2, a fast and sensitive alignment program (Galaxy Version 2.2.1 + galaxy1). The RNA sequence reads were mapped to the reference human genome version hg38. Feature counts were used to measure gene expression in RNA-Seq experiments from BAM files (Galaxy Version 2.0.1 + galaxy2). Annotations for gene regions were provided in the GTF format. Differential expression gene analysis was performed on two factors, namely, DS VS Non-DS, using Deseq2 (Galaxy Version 3.50.1 + galaxy0) (Anders & Huber, 2012). WebGestalt, a well-liked resource for interpreting gene lists obtained from extensive omics research, was used for further analysis. WebGestalt supports 12 organisms, 342 gene identities, 155,175 functioning classifications, user-uploaded functioning databases, and more with the 2019 edition (Liao *et al.,* 2019). For each category in the search database, the volcano plot displays the log of the FDR against the enrichment ratio. Major categories are located at the top sides. The dot's size and colour correspond to the category's dimensions. A dot's details can be viewed by hovering over it, and clicking on it updates the section with more specific information. A switch button that the user presses allows them to pan and zoom the plot. The

enriched subcategories have labels attached to them, and you can manually move the labels around with your mouse.

Protein-protein interaction network

Using PPI and eighty-seven gene ontology-ranked genes in order of preference, a network with PPI has been constructed using string (12.0). The web-based database STRING has around 24.6 million peptides and over 3.1 billion associations, which correspond to 5,090 different organisms (Szklarczyk *et al.,* 2021). The relationship between genotypes, biological systems, and gene expression is visualised via the Cytoscape software (Kohl *et al.,* 2011). In the present research, the scale-free property of the network led to the elevated score of central nodes.

String was used to construct a PPI network for the 24 genes with a high confidence score >0.900. Three gene modules were obtained from Cytoscape software. First gene module consists of 55 nodes and 1327 edges in the network. Second gene module consists of 15 nodes and 105 edges. Third gene module consists of 21 nodes and 71 edges. Using a cluster analysis of filtering nodes, 10 hub node genes were identified. The hub genes were FN1, ITGA1, ITGA2, ITGA2B, COL1A1, ESAM, ITGA6, ITGB7, CLDN20, CLDN23.

The biopharmaceutical sector of today is characterised by complexity due to the market's increasing expectations for new treatment classes, enhanced specificity and safety, and more complex disease mechanisms. Maintaining this level of intricacy calls for a more thorough comprehension of therapeutic conduct. An exceptional way to investigate biological and physical chemical reactions at the atomic level is through molecular modelling techniques.

Molecular docking

The three-dimensional structure of the Down syndrome target proteins [PDB ID:1PT6 (ITGA1), 5BP0 (FN1), 1Q7D (COL1A1), 5HJ2 (ITGA2), 7UDH (ITGA2B)] was obtained from the Protein Data Bank (PDB). The protein structure was prepared for docking using PyRx, which includes the addition of polar hydrogen atoms, assignment of partial charges, and removal of water molecules. Molecular docking is a widely used technique in structure-based drug design because it effectively predicts how small molecule ligands bind and conform to their target binding sites (Blessy & Sharmila, 2015; Blessy *et al.,* 2023; Blessy *et al.,* 2019). A receptor grid box was generated around the active site of the proteins to define the search space for ligand binding. Grid parameters, such as grid dimensions and spacing, were optimised based on the size and shape of the binding pocket (Table 1)**.** In total seven phytochemicals Curcumin, resveratrol, silymarin, epigallocatechin gallate, luteolin, apigenin, genisteinare sourced from and retrieved from the PubChem database (Kim *et al.,* 2019). The phytochemicals were prepared in PDBQT format, ensuring the correct protonation states using PyRx (Dallakyan & Olson, 2015). In PyRx, the docking algorithm primarily used is based on AutoDock Vina. The Seven phytochemicals were docked against the DS target proteins 1PT6, 5BP0, 1Q7D, 5HJ2, 7UDH.

Results and Discussion

To gain a preliminary understanding of the mechanisms behind down syndrome, 60 patients (35 down syndrome patients and 25 normal patients) were chosen for further investigation and their corresponding id's are SRR26135254, SRR26135248, SRR26135238, SRR26135236, SRR26135244, SRR26135240, SRR26135252, SRR26135246, SRR26135235, SRR26135241, SRR26135239, SRR26135237, SRR26135243, SRR26135234, SRR26135236, (Figure 1 and 2). The volcano plot was used to investigate the differentially expressed genes between healthy and DS samples from limma-voom. The volcano plot demonstrates the expressed fold change of genes in normal and DS patient samples calculated with the degree of statistical significance in different samples (Figure 3) A total of 1110 DEG's with a threshold criterion of log FC greater than and equal to 2.5 and p-value less than 0.05 as the cut-off point were diagnosed. Among them, 555 upregulated and 555 downregulated. The Gene ontology resources showed fold enrichment pathways for up and downregulated genes.

The integrated pathways from KEGG database for differentially expressed genes comprise of pathways in DS, insulin signalling, metabolic pathways, coagulation pathway, neuroinflammation and immune response, vascular Development (Figure 4). The PPI between DEG's were derived from the STRING database. The PPI network was represented in the form of nodes and edges, where each node represented a protein product of single-gene and edges represented the protein-protein association. The network backbone of identified

upregulated genes consisted of 57 nodes and 143 edges with an estimated clustering coefficient 0.242 (Figure 5). Similarly the network backbone of identified downregulated genes consisted of nodes and edges with an estimated clustering coefficient of 0.404. After string analysis, Cytoscape was used to visualise and identify the PPI network. MCODE plugin was used to identify the hub genes, and the parameters of DEG clustering and scoring were as follows: for cluster 1 MCODE score=9.750, Degree Cut-off=2, Node Score Cut-off=0.2, k-score=2, and Max. Depth=100. It consists of 55 nodes and 1327 edges (Figure 6).

For cluster 2 MCODE score=9.750, Degree Cut-off=2, Node Score Cut-off=0.2, k-score=2, and Max. Depth=100. It consists of 15 nodes and 105 edges (Figure 7). For cluster 3 MCODE score=9.750, Degree Cut-off=2, Node Score Cut-off=0.2, k-score=2, and Max. Depth=100. It consists of 21 nodes and 71 edges (Figure 8). Using a cluster analysis of filtering nodes, 10 hub node genes were identified. The hub genes were FN1, ITGA1, ITGA2, ITGA2B, COL1A1, ESAM, ITGA6, ITGB7, CLDN20, CLDN23 (Figure 9). In the current study molecular docking was performed using PyRxsoftware. The DS target proteins [PDB ID: 1PT6 (ITGA1), 5BP0 (FN1), 1Q7D (COL1A1), 5HJ2 (ITGA2), 7UDH (ITGA2B)] were chosen for docking (Table 1). The seven phytochemicals: curcumin, resveratrol, silymarin, epigallocatechin gallate, luteolin, apigenin, genistein were docked against the five target proteins of DS. The Epigallocatechin shows better binding toward the active site of 1PT6 with the docking score of -7.7 kcal/mol. The phytochemical resveratrol shows better binding toward the active site of 1PT6 with the docking score of -6.3 kcal/mol. The phytochemical silymarin, shows better binding toward the active site of 5BP0 with the docking score of -7.3 kcal/mol. The phytochemical apigenin shows better binding toward the active site of 1PT6 with the docking score of -6.9 kcal/mol. Luteolin, Curcumin, and genistein show binding free energy of -7.3,-6.6 kcal/mol, -7.7 kcal/mol with 1PT6 respectively (Table 2).

In this study, a comprehensive analysis was conducted to understand the molecular mechanisms underlying DS by examining differential gene expression between DS patients and normal samples. Using a volcano plot derived from limma-voom, a total of 1110 differentially expressed genes (DEGs) were identified, with a stringent threshold of a log fold change (FC) greater than or equal to 2.5 and a p-value less than 0.05. Among these, 555 genes were upregulated, while 555 were downregulated.

Gene Ontology and KEGG pathway analyses revealed that these DEGs are involved in critical pathways related to DS such as the insulin signalling and metabolic pathways, coagulation pathway, neuroinflammation and immune response, vascular development. The neuro inflammation pathway, in particular, is known to be pivotal in neurological development and progression, making it a significant focus in DS research. Additionally, the involvement of insulin signalling pathways suggests potential alterations in metabolic processes associated with DS.

To further elucidate the interactions among DEGs, we utilised the STRING database to construct PPI networks. The PPI network for upregulated genes comprised 57 nodes and 143 edges, while the network for downregulated genes included a smaller number of nodes and edges with distinct connectivity patterns. The clustering coefficients for upregulated and downregulated networks were 0.242 and 0.404, respectively, indicating a higher degree of clustering among downregulated genes.

The MCODE plugin in Cytoscape identified several key clusters within these networks. Cluster 1, with a high MCODE score of 9.750, consisted of 155 nodes and 1327 edges, suggesting a densely interconnected network. Clusters 2 and 3, though smaller, also showed significant interaction patterns, particularly around and in Cluster 2 and 3. Through cluster analysis 10 hub node genes were identified; the hub genes were FN1, ITGA1, ITGA2, ITGA2B, COL1A1, ESAM, ITGA6, ITGB7, CLDN20, CLDN23. This dense clustering underscores the complex and highly interconnected nature of the molecular changes associated with down syndrome.

Molecular docking studies using PyRx software aimed to evaluate the interaction of nine phytochemicals with down syndrome target proteins. They are 1PT6 (ITGA1), 5BP0 (FN1), 1Q7D (COL1A1), 5HJ2 (ITGA2), 7UDH (ITGA2B). The results demonstrated varied binding affinities across different target proteins. The epigallocatechin shows better binding toward the active site of 1PT6 with the docking score of -7.7 kcal/mol. The phytochemical resveratrol shows better binding toward the active site of 1PT6 with the docking score of -6.3 kcal/mol.

Table.1 Active Site of Down syndrome target proteins.

PDB ID	Active site
1PT6	Asn313, Val314, Leu282, Ser315
5BP0	Asp85, His145, Ile19, Ser75, Val74, Arg3, Val18, Asp17
1Q7D	Blind docking
5HJ2	Tyr93, Asn54
7UDH	Met285, Asn320, Leu317, Arg281

Table.2 Molecular docking of down syndrome target proteins with phytochemicals.

Docking Score (Kcal/mol)					
Phytochemicals	Protein				
	1PT6	5BP0	1Q7D	5HJ2	7UDH
Curcumin	-6.6	-6.4	-3.6	-6.1	-6.2
silymarin	-7.8	-7.3	-4.1	-6.6	-6.6
luteolin	-7.3	-6.6	-3.8	-6.6	-6.7
Epigallocatechin gallate	-7.7	-6.5	-4.3	-6.4	-5.8
apigenin	-6.9	-6.6	-4	-6	-6.3
genistein	-6.5	-6.3	-3.9	-5.7	-5.7
resveratrol	-6.3	-6.2	-3.6	-6.2	-6

Figure.1 Control vs Case for Up-regulated and Down-regulated Genes for down syndrome.

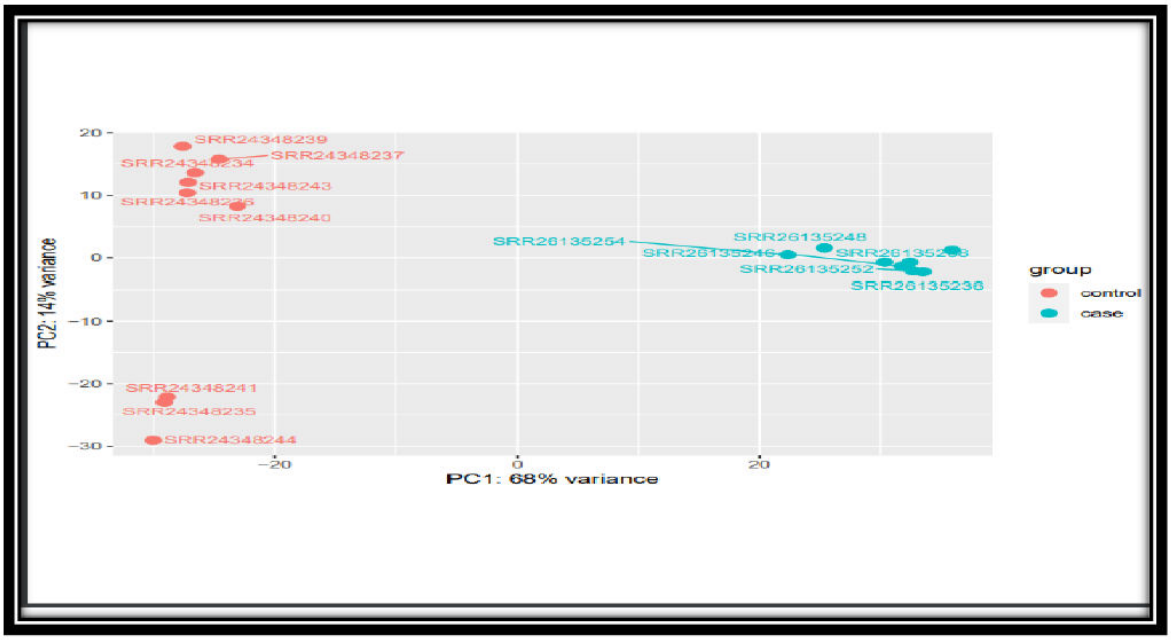


Figure.2 MA Plot for Pc (case vs control) for up-regulated and down-regulated Genes for down syndrome.

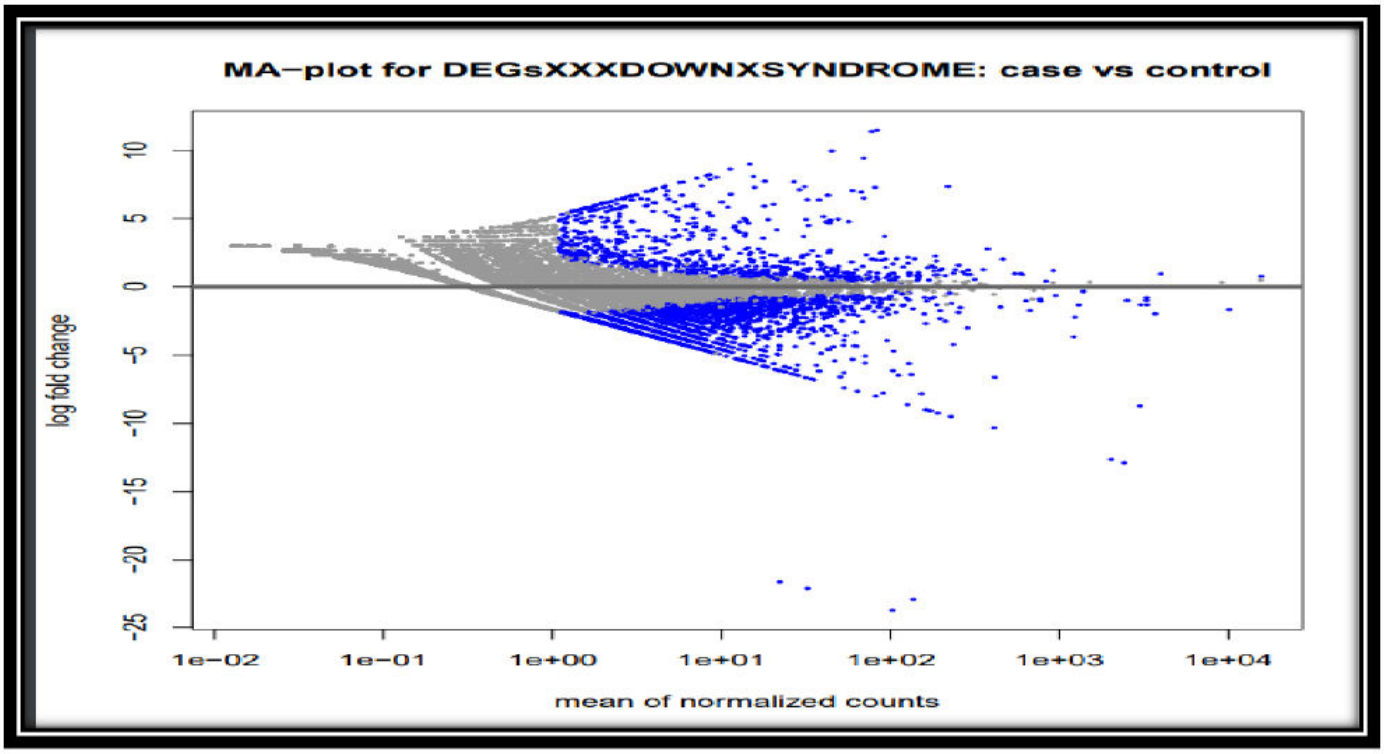


Figure.3 Dispersion estimates for up-regulated and down-regulated Genes for down syndrome.

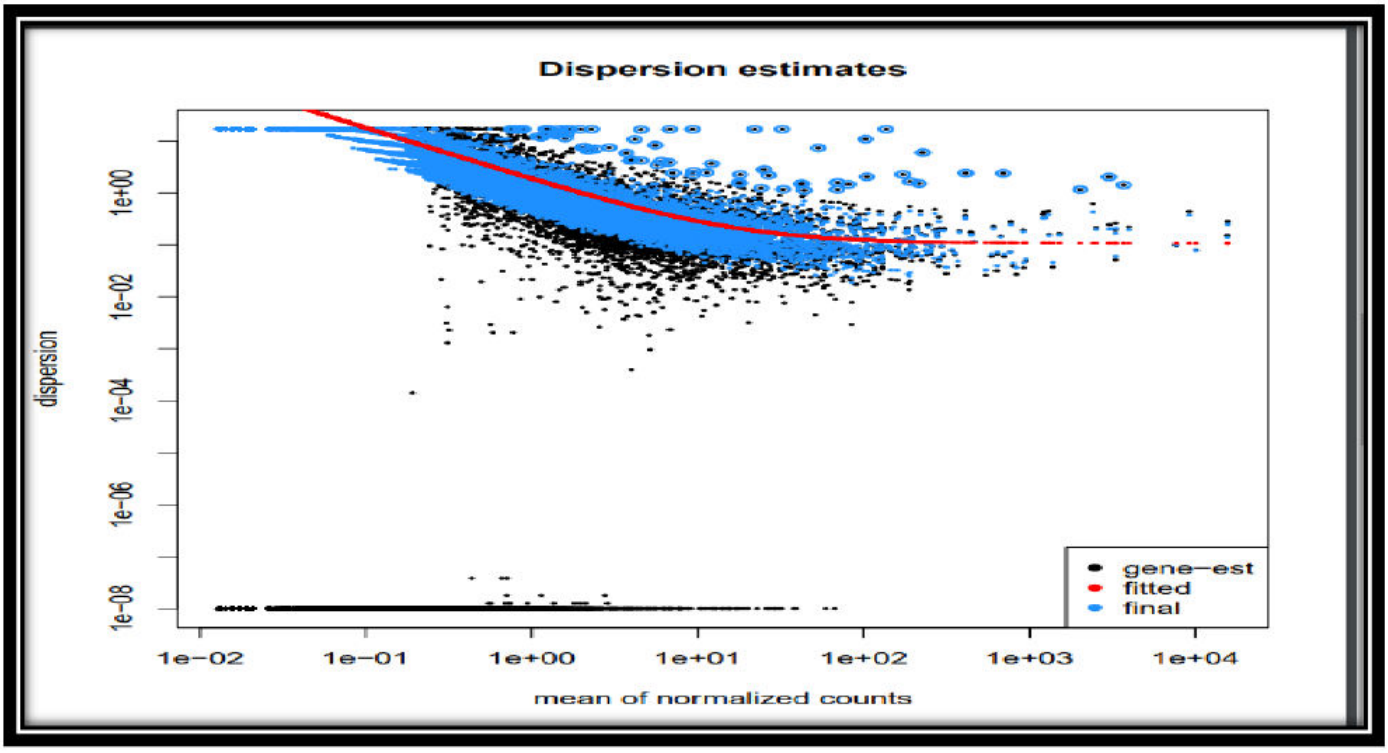


Figure.4 Enrichment scores of significant biological pathways for down syndrome.

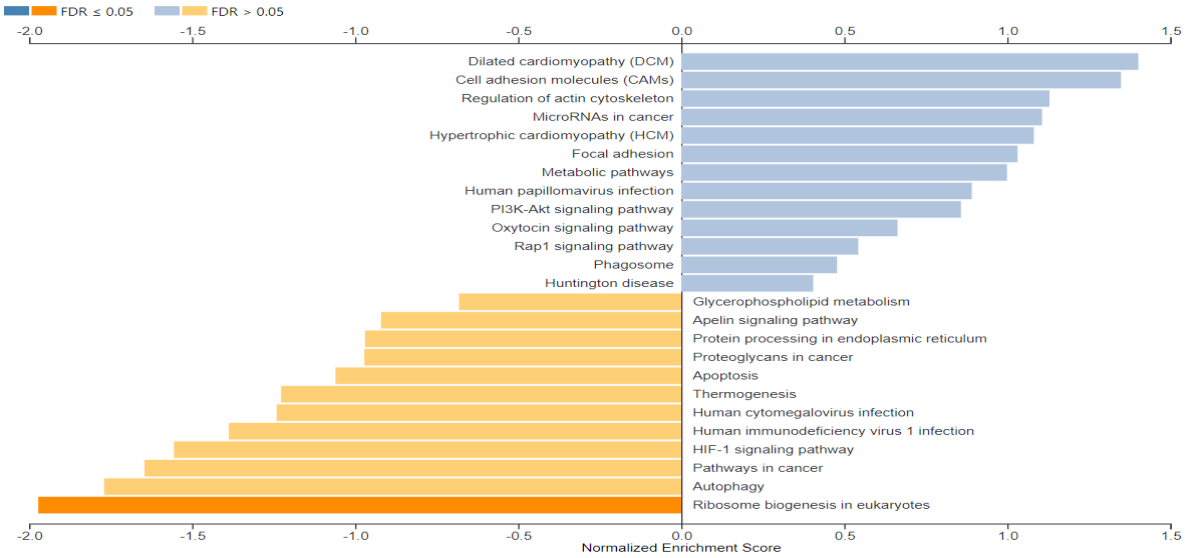


Figure.5 PPI network backbone of identified upregulated genes consisted of nodes and edges using string for down syndrome.

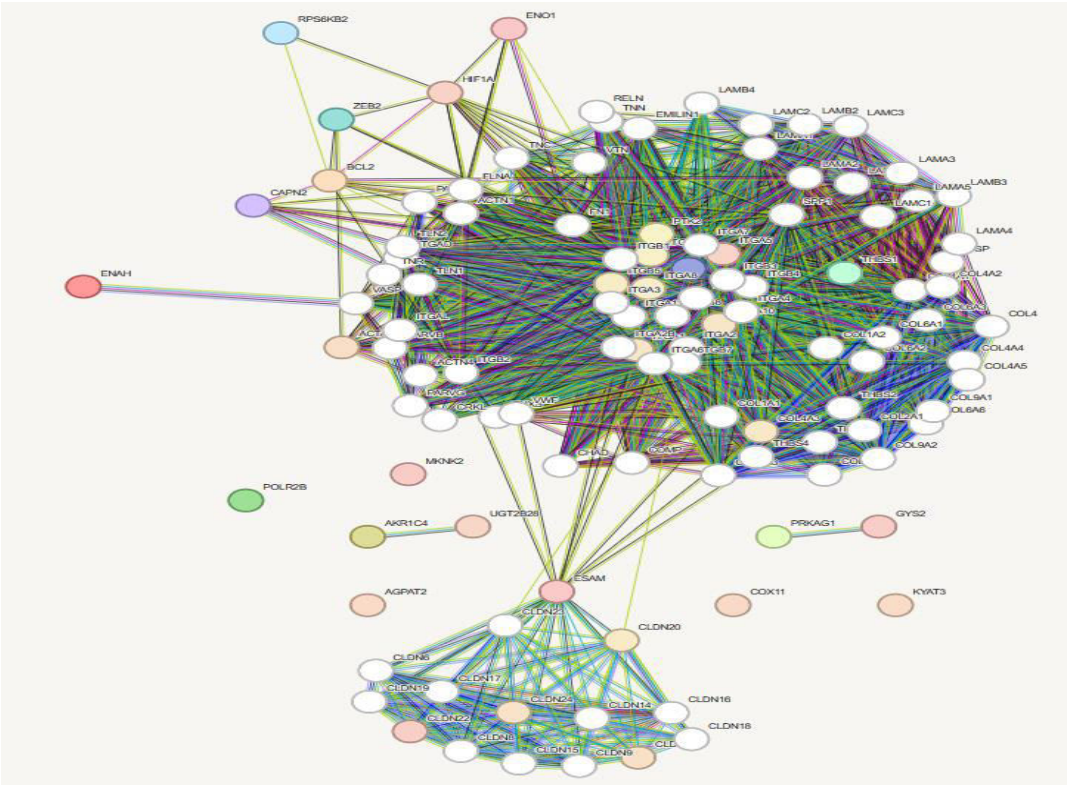


Figure.6 First Gene module was inferred which contains 55 nodes and 1327 edges for down syndrome.

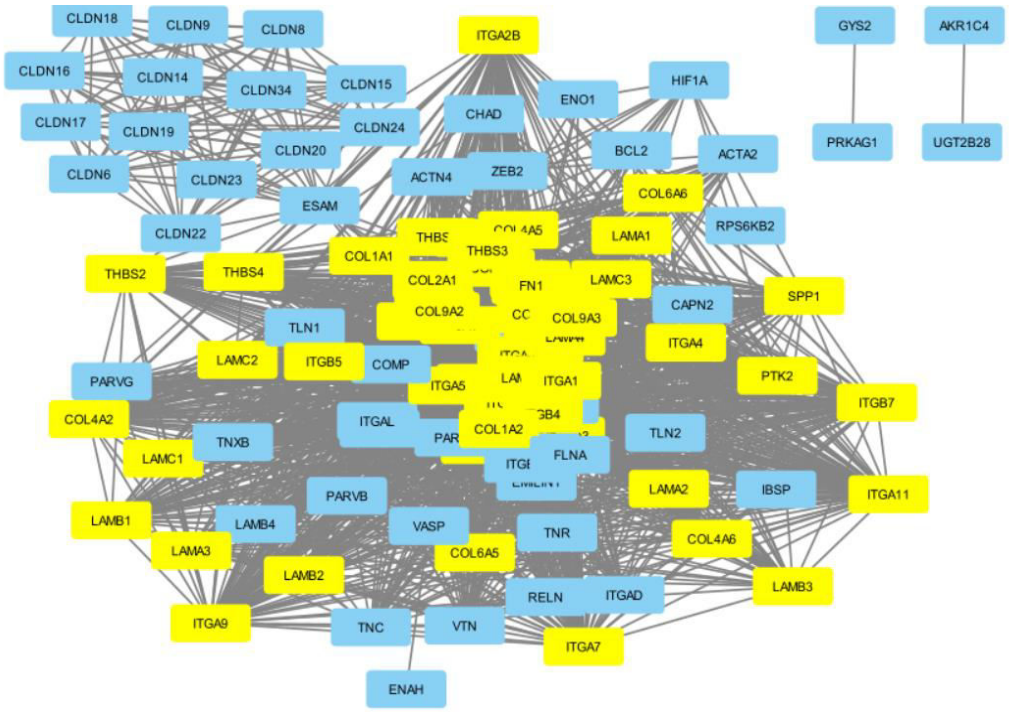


Figure.7 First Gene module was inferred which contains 15 nodes and 105 edges for down syndrome.

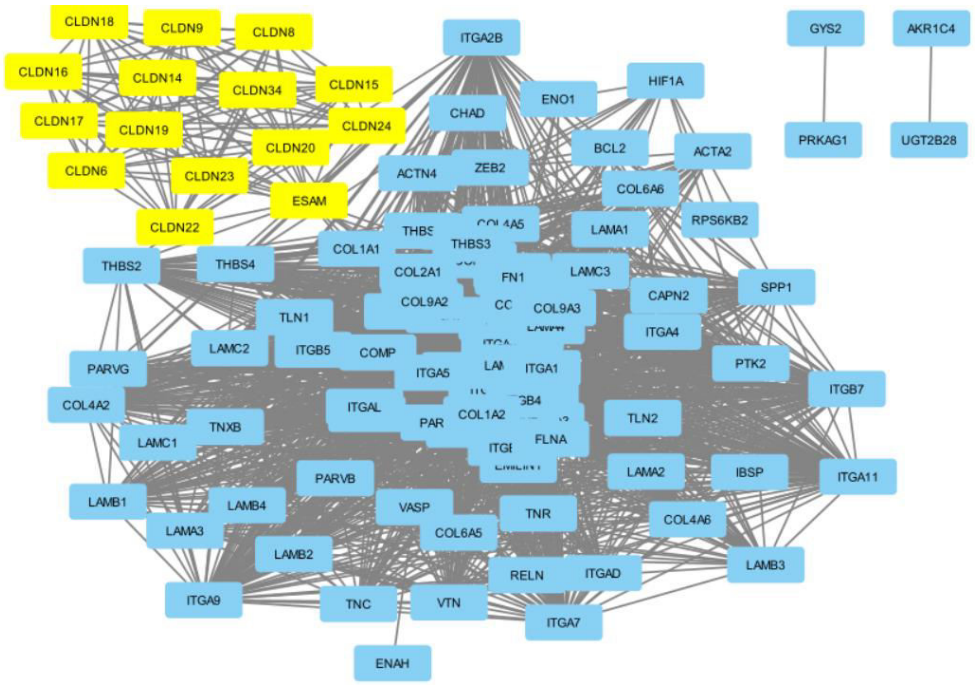


Figure.8 First Gene module was inferred which contains 21 nodes and 71 edges for down syndrome.

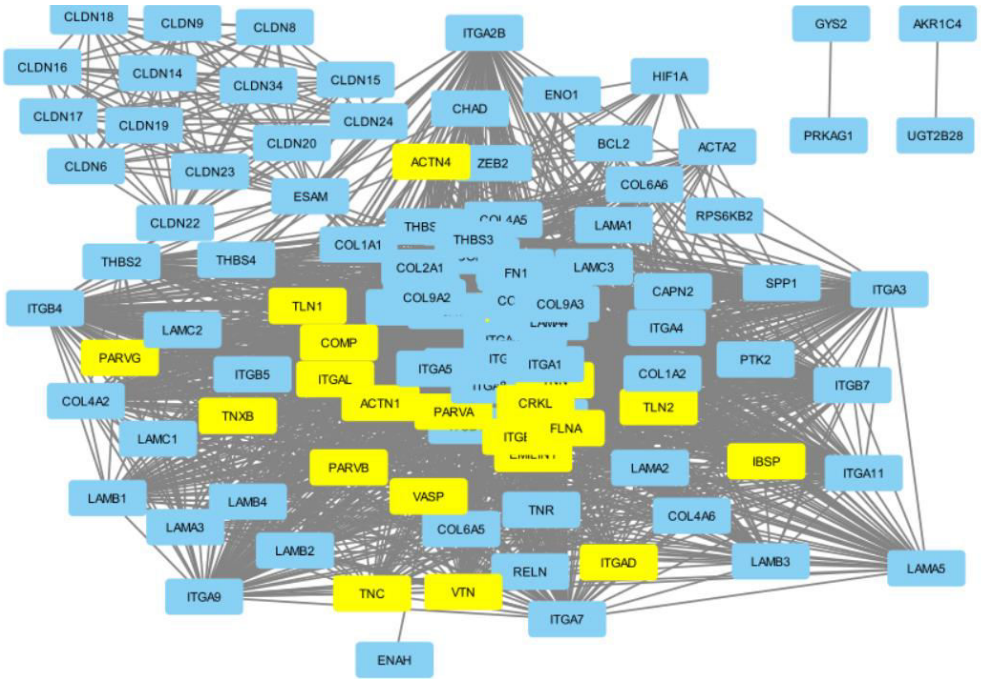
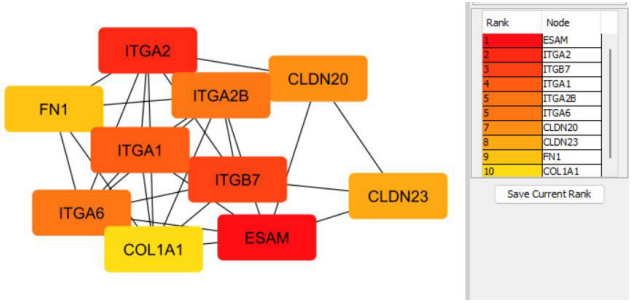


Figure.9 Top 10 hub genes were inferred using cytohubba for down syndrome.



The phytochemical silymarin, shows better binding toward the active site of 5BP0 with the docking score of -7.3 kcal/mol. The phytochemical apigenin, shows better binding toward the active site of 1PT6 with the docking score of -6.9 kcal/mol. Luteolin, Curcumin, genistein show binding free energy of -7.3, -6.6 kcal/mol, -7.7 kcal/mol with 1PT6 respectively. These findings suggest that these phytochemicals could have potential therapeutic effects in down syndrome by interacting effectively with specific target proteins.

The integration of gene expression analysis, PPI network construction, and molecular docking provides a multifaceted understanding of the molecular mechanisms driving down syndrome. The identification of key pathways and interactions, along with the promising results from phytochemical docking studies, suggests that targeted therapeutic approaches involving these compounds could be beneficial for drug design for DS.

Additionally, further exploration of the specific roles of the hub genes and their interactions within the identified clusters could provide deeper insights into the molecular underpinnings of DS and guide the development of novel therapeutic strategies. Our study highlights the importance of a multi-approach strategy in understanding down syndrome mechanisms and emphasises the potential of natural compounds in DS therapy.

The present study has significantly advanced the

understanding of DS by integrating differential gene expression analysis, PPI network construction, and molecular docking studies. By analysing a dataset from 60 patients, we identified 1110 differentially expressed genes, revealing crucial involvement of pathways such as neuroinflammation and immune response. The PPI network analysis highlighted key hub genes including FN1, ITGA1, ITGA2, ITGA2B, COL1A1, ESAM, ITGA6, ITGB7, CLDN20, CLDN23 and uncovered densely interconnected clusters, revealing on the complex molecular interactions in DS. Molecular docking studies further identified several phytochemicals with potential therapeutic effects. Overall, these findings underscore the importance of a comprehensive, multi-approach strategy in elucidating DS mechanisms and highlight promising natural compounds that could inform future therapeutic developments.

List of Abbreviation

PDB = Protein Data Bank
DEG = differential gene expression
PPI = protein-protein interaction
log2FC = logchange

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Disclosure Statement

No potential conflict of interest was reported by the author(s).

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Author Contributions

S. Yogigiridhar: Investigation, formal analysis, writing—original draft. J. Jino Blessy: Validation, methodology, writing—reviewing. Shalini Urumaiya:—Formal analysis, writing—review and editing. D. Jaswanthi: Investigation, writing—reviewing.

Data Availability

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

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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