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Genetic Analysis of Differentially Expressed Genes Associated with the Pathogenesis of Autism Spectrum Disorder

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ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder present at birth and affects 1 in 36 children in the US. It causes symptoms like intellectual disability, lack of communication skills, delayed development of coordination and movement, and more. ASD is a spectrum disorder, and its complexity makes early diagnosis in children difficult as there are no known biomarkers that cause ASD. This project aims to identify genetic factors that may be associated with ASD. By splitting samples in the dataset accessed from the GEO database into a test group with ASD and a control group, this study used a statistical t-test to identify the top 250 differentially expressed genes with p-values < 0.05. These genes were entered in the STRING database to create a map of gene interactions and identify top pathways and biological processes. Analysis of pathways and biological processes led to findings that the differentially expressed gene ARF6 (ADP Ribosylation Factor 6) was active in all three pathways: RAS Signaling, Phospholipase D Signaling, and Salmonella Infection Pathways and played important roles in cellular functions. The experiment validated my hypothesis by providing evidence for a significant difference in gene expressions between ASD individuals and neurotypical individuals. ARF6 was found to be down regulated and involved in pathways and biological processes associated with ASD like immune and cell signaling dysregulation. The implication is If ARF6 gene factor can be controlled to regulate ARF6 expression, the mutations which contribute to ASD can be suppressed through multiple pathways.

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder which usually presents itself at birth. It predominantly involves dysfunction of the central nervous system. According to CDC researchers' studies, 1 in 36 children in the United States had autism in 2020, which quadrupled the rate of 1 in 150 in 2000 (Maenner, et al., 2023). In fact, Professor Maureen Durkin, head of the Autism and Developmental Disabilities Monitoring Network mentioned that a significant upward trend of autism has been observed globally (Durkin, et al., 2017).

ASD is characterized as a spectrum disorder, ranging from the milder Asperger's syndrome to the more serious autistic disorder or Pervasive Developmental disorder. Its symptoms include a wide range of signs: delayed language, movement, and cognitive skills, social and communication impairment, hyperactive and inattentive behavior, unusual mental and sleeping issues, etc. Therefore, its complexity makes early ASD diagnosis much more difficult at a younger age. Currently, there are no blood tests, brain scans, or validated biomarkers for ASD diagnosis. Instead, physicians often rely on parents' descriptions of medical history, the physician's physical exam, and their own observations and/or Social Response Scale (SRS) to diagnose the condition.

Scientists have tried to search for the causes of autism for many years. Given the complexity of the disorder and the wide range of symptoms, there could be many reasons behind the neurobiological disorder. ASD can be the result of complex interactions among environmental, genetic, and immunological factors

(Mandy & Lai, 2016). While some researchers explored environmental factors such as viral infections, complications during pregnancy, medication, and pollution, others focused on genetic associations between autism and contributing genes.

Folstein and Rutter's research on 21 pairs of same-sexed twins showed that autism has strong heritability (Folstein & Rutter, 1977). Since then, a large ASD survey study conducted on identical twins, fraternal twins and siblings of 2 million Swedish families estimated that autism has a heritability index of 0.5-0.90 and varying degrees of severity due to multiple genes involved (Sandin & Lichtenstein, 2014). Recent scientific research has put efforts into the exploration of the behavioral functions and pathological mechanisms of ASD. Particularly, new evidence has emerged in the signal transduction in autism from the neural circuit, molecular and cellular perspectives. A recent study showed that there is a significant association between Autism and the mediator of cell motility 1 (Memo1), a critical gene that is needed for the tiled organization of radial glial cells (RGCs) (Nakagawa &Plestant, 2019). Each RGC forms a single stalk-like structure, then extends to the top of the cortex, and finally forms a scaffold structure. With the scaffold structure, the cortex develops a regular structure of 6 layered neurons which is necessary for neocortical development. The study found that deficiency in MEMO1 led to hyper branching of RGC processes, disruption of RGC tiling, and anomalous neuronal layering. Their findings suggest that as MEMO1 regulates RGC tiling, mutations in the MEMO1 gene and resultant cortical malformations may contribute to certain types of Autism.

Another study on 1231 autism cases analyzed the gene which encodes MET receptor tyrosine kinase and showed a strong genetic association between autism and a common C allele in the promoter region of the MET gene (Campbell & Sutcliffe, 2006). MET signaling is critical in immune system function, and the development of the cerebral cortex and cerebellum. Hypomorphic MET signaling in the cerebral cortex and cerebellum results in neuropathologic abnormalities which are observed in the brains of autistic people. By focusing on MET as an autism candidate gene, the researchers found that the C allele causes a 2-fold reduction in MET promoter activity and therefore downregulated MET gene expression among autistic individuals.

Recent molecular genetics studies have identified several hundred risk genes that contribute to ASD. The genetic landscape of ASD likely varies from one person to another. About 5% of autism cases are the result of single-nucleotide polymorphisms (SNPs) in genes such as SHANK3, MECP2, and FMR1 (Quesnel-Vallieres & Weatheritt, 2019). Some studies have also identified rare de novo mutations of SCN1A, SCN2A, SYNGAP1, DSCAM, and TBR1 associated with certain autistic individuals (Ziats & Rennert, 2013). Additionally, there are 10% of autism cases which are caused by copy-number variations (CNVs) of disrupted protein coding, such as chromosomal duplications, large deletions, translocation, and inversions (Girirajan & Dennis, 2013). Although there are many ASD risk genes involved, they seem to converge in a limited number of biological pathways including synaptic function, transcription and translation, protein synthesis and degradation (Jiang & Lin, 2022).

Some autism-linked genes play a role in the activity-driven synaptic pathway by directly encoding synaptic scaffold proteins, neurotransmission, and dendritic spine formation (Durand & Betancur, 2007). For example, SHANK genes encode the proteins in the postsynaptic scaffolding protein family. Different mutations within the SHANK gene family including SHANK1, SHANK2, and SHANK3 may cause different synaptic effects and thus distinct symptoms which are reported by ASD individuals. Mutations in gene SHANK3 on chromosome 22q13 decrease actin accumulation in spines and therefore affect the development of dendrites and the axonal growth cone motility.

Translational signaling pathways are crucial in the regulation of gene expression and sense extracellular stimuli (i.e., growth factors, hormones), environmental stresses, and intracellular cues. The mutations in genes such as TSC1, TSC2, PTEN, FMRP involved in translational signaling pathways such as mTOR, and MAPK, cause increased protein synthesis and altered synaptic plasticity. Particularly, TSC1 acts as the regulator of the stability of TSC2, and their mutations are observed among autistic individuals with deficits in long-term memory and learning disability (Ehninger & Silva, 2011). Meanwhile, FMRP disorder is the most common

single-gene cause of autism. The proteins encoded by FMRP regulate the balance of translation in synaptic plasticity (Darnell & Driesche, 2011).

Proteins account for more than 50% of cellular dry weight. Their continuous synthesis and degradation allow old proteins to be replaced with new ones. The execution of protein synthesis and degradation involves the regulation of protein level which is essential to the cellular function and the health of the whole organism. Mutations in genes involved in the regulation of synaptic proteins such as NF1, PTEN, and synGAP1 cause an increase in translation in neuronal activities. The excessive protein synthesis is one core pathophysiological mechanism of autism (Auerbach & Osterweil, 2011). Meanwhile, gene mutations in chromatin remodeling such as MECP2, MEF2C, HDAC4, and CHD8 have been observed in a group of ASD individuals (Ebert & Gabel, 2013). Particularly, the mutation in MECP2, a protein that regulates transcription in neurons will cause a prominent level of gene expression. In the protein degradation pathway, the UBE3A gene produces E3 ligase E6-associated protein (E6AP). While the underexpression of E6AP leads to the development of Angelman syndrome (AS), the overexpression of E6AP is strongly associated with autism spectrum disorders (Khatri & Man, 2019).

Research on the pathogenesis of ASD in past studies associates ASD with biological processes such as synaptic function, transcription and translation, protein synthesis and degradation, and cell signaling dysregulation. Based on this background research, I theorized that potential biomarkers may be active genes and genetic factors highly involved in gene pathways regulating these specific ASD-associated biological processes. In this study, I hypothesize that if the human body contains genes and gene pathways that help regulate or suppress these biological processes, then these genes are more likely to be up or downregulated (differentially expressed) in individuals with autism spectrum disorder compared to neurotypical individuals. First, the GEO dataset was analyzed to identify the top genes whose gene expressions were significantly different between the test group and the control group. Analysis of those significant genes using STRING and KEGG database led to the discovery of a particular gene that was downregulated and active on three important pathways: RAS Signaling Pathway, Phospholipase D Signaling Pathway, and Salmonella Infection Pathways.

Materials and Methods

The online GSE111176 dataset from the Gene Expression Omnibus (GEO), a public database for gene expression profiling of microarray or RNA sequence data was used for this research study. This dataset contains Homo Sapien gene expression data profiled by array from the study "Transcriptional organization of autism spectrum disorder" conducted by the University of California San Diego. The dataset was divided into a control group and a test group with ASD. Then a statistical t-test was performed on the data with the GEO2R package. This test generated a table for the top 250 differentially expressed genes with p-value <0.05. Significant biological pathways were assessed and identified by utilizing three public resources: the Gene Cards Database from the Crown Human Genome Center, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) Database from Global Biodata Coalition, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database from the Kyoto University. The analysis of gene interactions and pathway maps led to the discovery of common genes active in all significant pathways. This information helped locate the common genes inside the GEO2R data table and extract the logFC value to determine if it is more likely to be up or downregulated in the ASD test group in comparison to the control group. Meanwhile, outside research on the significant pathways, shared genes, and biological processes was performed to provide context for the results.

Results

The goal of this project was to identify genetic factors potentially associated with autism spectrum disorder. This way, knowledge of discrete relationships between genetic activation, expression, and the execution of pathways causing the symptoms of autism spectrum disorder could be established and used to inform and develop improved treatments. To test our scientific question of whether relationships could be established between certain genes, gene pathways, and biological processes responsible for the ASD condition, multiple types of statistical analysis and data modeling were done on the Gene Expression Omnibus (GEO) dataset, a public database for gene expression profiling.

The Volcano plot generated from the dataset using the GEO2R package (figure 1) clearly indicated the distribution of significant genes according to the p-value. There were many genes that were significantly different in gene expressions between the experimental group and the control group. The genes shown in red color were upregulated and suggested that the gene expressions of those genes were significantly higher in the ASD experimental group than in the control group. On the contrary, genes with lower gene expressions in ASD samples were indicated in blue color. Since this dataset showed many particular genes that demonstrated significant differences between the experimental group and the control group, those genes were selected for further analysis within this study.

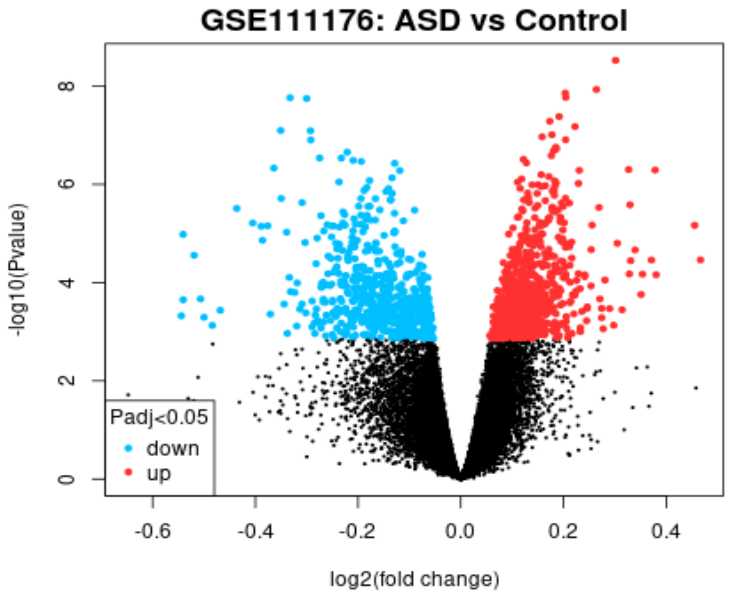

Figure 1 Volcano plot: a scatterplot showing the magnitude of fold change versus statistical significance, and the distribution of genes. Genes in red and blue color are the most biologically significant genes.

The top 250 genes which are significantly expressed in ASD experimental group were entered into the STRING Database. The STRING analysis in Table 1 showed three significant KEGG pathways: the RAS Signaling Pathway represented with blue nodes, the Phospholipase D Signaling Pathway with red nodes, and the Salmonella Infection pathways with green nodes as shown in Figure 2, which was a portion of the STRING map that focused on only three KEGG pathways. It was observed from Figure 2 that a particular gene, ARF6 was active on all three pathways. While Figure 3 showed part of the Ras Signaling Pathway Map, Figure 4 displayed a portion of the Phospholipase D (PLD) Signaling Pathway Map, and Figure 5 presented part of the Salmonella Infection Pathway Map. It was clear that ARF6 gene was shown in all three figures and played a

role in three pathways. The ARF6 gene, ADP Ribosylation Factor 6, belongs to the human ARF gene family and encodes guanine nucleotide-binding proteins that involve in the ADP-ribosyltransferase activity of cholera toxin, vesicular trafficking, and phospholipase D. The statistical test result indicated that the logFC of ARF6 was -0.16997 with an adjusted p-value of 0.0268. This means the ARF6 expression for the ASD groups was downregulated compared to the gene expression of the control group. Thus, this collected correlative evidence provided compelling evidence of an association between differential expression of the gene ARF6 and biological pathways responsible for ASD development.

Table 1. STRING analysis

	Biological Process (Gene Ontology)			
GO-term	Description	Count in Network	Strength	False Discovery Rate
GO:0031392	Regulation of prostaglandin biosynthetic process	3 of 10	1.39	0.0408
GO:0045059	Positive thymic t cell selection	3 of 11	1.35	0.046
GO:0001916	Positive regulation of t cell mediated cytotoxicity	4 of 25	1.12	0.0363
GO:0001914	Regulation of t cell mediated cytotoxicity	5 of 33	1.09	0.0123
GO:0043368	Positive t cell selection	4 of 27	1.08	0.0429
	Molecular Function (Gene Ontology)			
GO-term	Description	Count in Network	Strength	False Discovery Rate
GO:0002020	Protease binding	10 of 138	0.77	0.0209
GO:0005488	Binding	190 of 12516	0.09	0.00051
	KEGG Pathways			
Pathway	Description	Count in Network	Strength	False Discovery Rate
hsa05132	Salmonella infection	13 of 209	0.71	0.0011
hsa04072	Phospholipase D signaling pathway	9 of 147	0.7	0.0201
hsa04014	Ras signaling pathway	11 of 226	0.6	0.0201

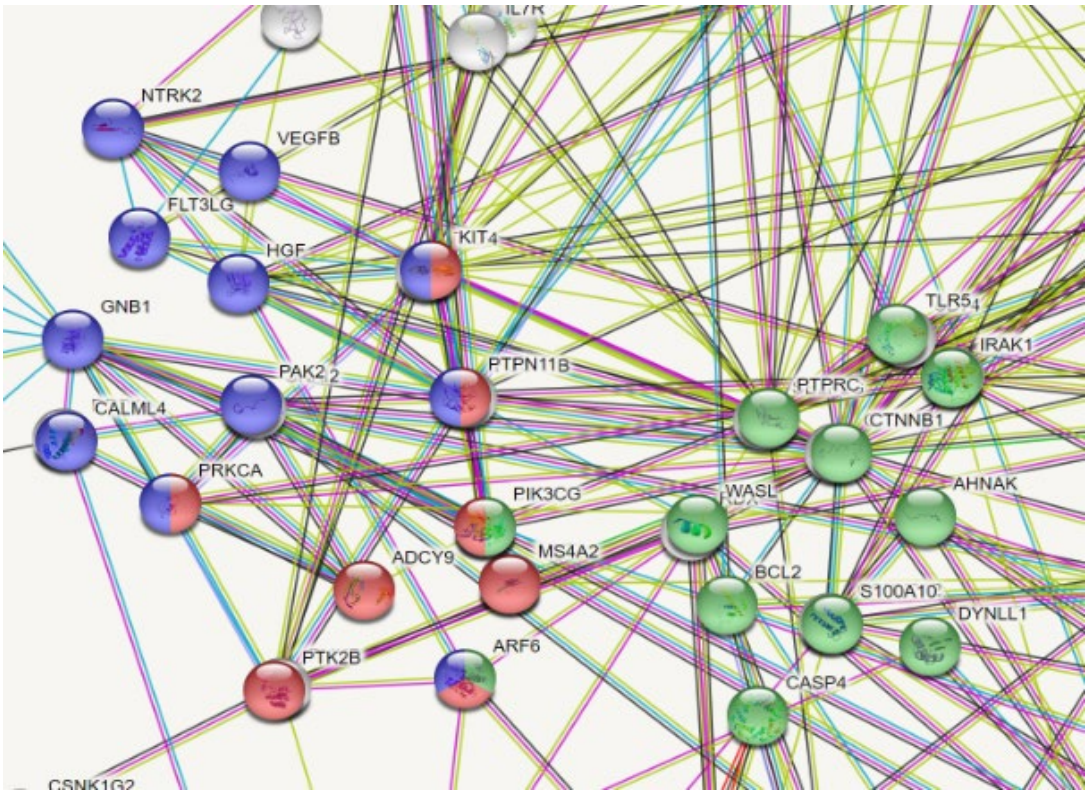

Figure 2. STRING map showing the protein-protein interactions including direct (physical) and indirect (functional) associations.

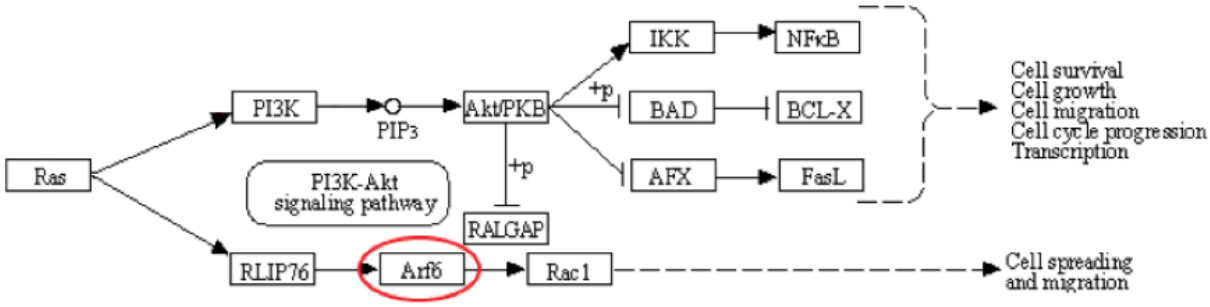

Figure 3. Ras Signaling Pathway Map shows that Arf6 is an active gene in the pathway.

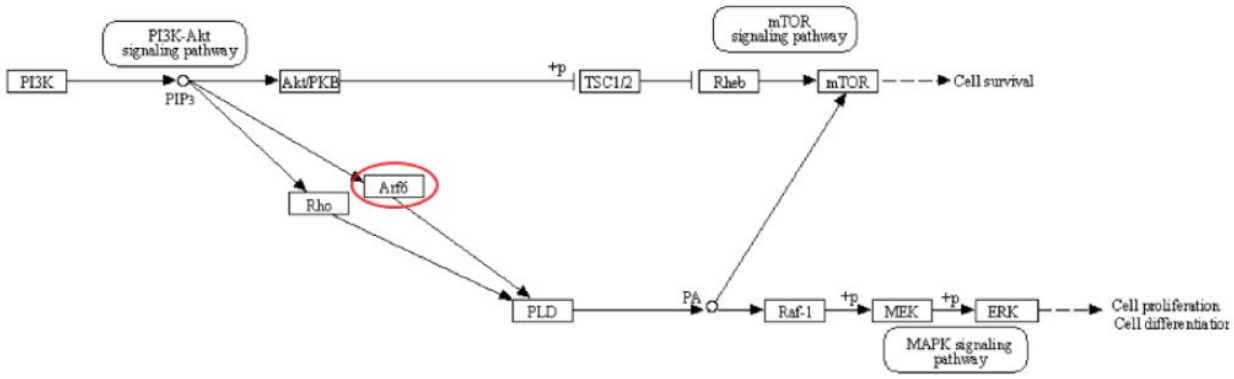

Figure 4. Arf6 is an active gene on the Phospholipase D (PLD) Signaling Pathway

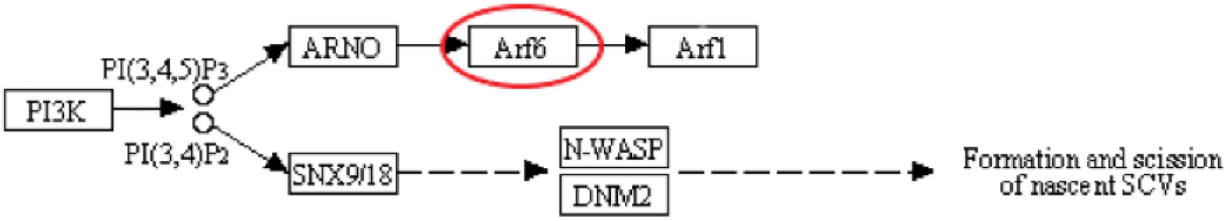

Figure 5. As shown in Salmonella Infection Pathway Map, Arf6 gene is also active on this pathway.

Discussion

The top differentially expressed genes from the test group interacted most actively within the 3 pathways. Any correlation discovered between ASD and the pathways may be influenced by the dataset chosen (GSE111176), its sample size and the profiles of the sampled individuals whose gene expressions were analyzed. The results suggested that ARF6 gene mutations and multigene interactions within three pathways were correlated with ASD: the Ras Signaling pathway, Phospholipase D Signaling Pathway, and the Salmonella Infection pathway. RAS signaling pathway is involved in transmitting signaling within cells and controls many downstream processes such as cellular proliferation, growth, survival, gene expression, and t-cell receptor signaling. RAS mutation may cause the production of permanently activated Ras proteins. RAS proteins exist in two states: GTP-bound active state and GDP-bound inactive state. While RAS proteins bind to their effectors and induce downstream signaling during the active state, the RAS GDP causes signaling to cease during the inactive state. The exchange between these two states is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). RAS superfamily is made up of 5 families: RAS (Rat Sarcoma), Rho (Ras Homologue), Rab (Ras-like protein from rat brain), Rad (Ras associated with diabetes), and ARF (ADP-ribosylation factor). The ARF family is further divided into three groups: group 1 with ARF1-3, group 2 with ARF4-5, and group III with ARF6. ARF6 is crucial to maintain the proper level of excitatory and inhibitory receptors to support the normal learning process. ARF6 mutation is known to possibly contribute to diseases including mental retardation, seizure syndrome, cholera, and deafness, etc (Kanamarlapudi & Salman, 2022). The previous study also showed evidence that an imbalance between excitation and inhibition in synaptic transmission may be behind some cases of ASD (Levy & Umanah, 2019) and that mutations affecting Ras signaling underline syndromes with elevated risk for ASD (Vasic et al., 2021).

As a member of the phospholipase superfamily, Phospholipase D (PLD) is present in various organisms including animals, viruses, and bacteria, and is a class of enzymes that produces phosphatidic acid (PA)

as an intracellular signaling species. It is activated by binding with growth factors, hormones, cytokines, and neurotransmitters, etc. PLD controls the regulation of intercellular signaling, tumorigenesis, cell proliferation and survival, and metabolic pathways through generating phosphatidic acid (PA), which emerges as a new therapeutic target for Alzheimer's and other brain disorders. In the Phospholipase D signaling pathway, PA works as a secondary messenger to transform external stimuli into physiological responses and plays a leading role in the fundamental cellular processes including cell proliferation, cell survival, cytoskeleton organization, and intracellular protein trafficking. However, the PLD activity is controlled by different effectors including small G proteins such as Rho, Ras, and ARF6. In fact, ARF6 was first identified as the direct regulator of PLD (Azreq & Valerie, 2010). The experimental results indicate that ARF6 gene expression is downregulated in ASD experimental group compared to the control group. ARF6's downregulation may cause irregular PLD activity, which needs to be further investigated.

Salmonella infection is a kind of bacterial disease caused by contaminated water and food. Salmonella bacteria cause intracellular infections by triggering host cell membrane ruffling and invasion by weakening cellular ARF guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) which regulate the cycle of GDP activation (GTP binding) and GTP inactivation (GTP hydrolysis) of ARF6 proteins. Previous studies showed that the cycle of activation and inactivation of ARF6 facilitates the Salmonella invasion through Salmonella cytoskeleton remodeling and controls the distinct steps in the cellular signaling underlying the invasion (Davidson & Humphreys, 2015). Particularly, fast-cycling ARF6 causes depletion of ARF6 GAPs in the cells which plays a role in the Salmonella invasion process. The interplay between GEFs and GAPs of ARF6 proteins allows Salmonella bacteria to drive macropinocytosis to the cells where the pathogen invasion is established. While one study showed that ARF6 was present on the Salmonella-induced host cell membrane ruffles during the infection (Adam et al., 2005), another study revealed that many children with ASD report gastrointestinal immune problems (Van et al., 2019).

Overall, ARF6 gene has many crucial functions and is critical in host-pathogen interactions. First, ARF6 plays a vital role in intracellular vesicular trafficking function and regulation of the cycle of GTP binding and hydrolysis. ARF6 has effects on a diverse set of effectors through activating the lipid-modifying enzymes phospholipase D (PLD) and phosphatidylinositol 4-phosphate 5-kinase (PIP5K), which accelerates the production of phosphatidic acid (PA) and phosplatidylinositol1-4, 5-bisphosphate (PIP2) (Acker et al., 2019). ARF6 also stimulates actin polymerization and cooperates with Rab35-GTP as antagonist in endocytic recycling process. Meanwhile, the cycle of RF6 GTP and GDP regulates the direction of transport along microtubules. Therefore, ARF6 has an impact on health and disease through exercising its roles in cell division, cell migration and cell spreading, and affecting signaling in many pathways. Since ARF6 can be hijacked by pathogens to enter host cells and invade the immune system, blocking ARF6 in particular processes can be a potential therapeutic intervention to inhibit the invasion and survival of pathogens. However, ARF6 have essential roles in many cellular processes, further research into the details of specific process and pathways which lead to ARF6 activation may provide new opportunities for intervention.

Conclusion

This project's goal is to identify genetic factors that may be associated with autism spectrum disorder. The experiment involving the GEO2R analysis of dataset GSE111176 led to the discovery of the top 250 differentially expressed genes between the test group with ASD and the control group. Investigation of these particular genes by using the STRING map and KEGG pathways reveals that ARF6 genes are active within 3 pathways: Ras Signaling, Phospholipase D Signaling, and Salmonella Infection. These genes are critical for intracellular functions such as cell survival, development, proliferation, cytokinesis, and more. The experiment supported my hypothesis as the statistical data indicated that there exist differentially expressed genes such as ARF6 which have downregulated expression levels in the test group with ASD compared to the control. My discovery of

ARF6's role in ASD and how their functions are interrelated to the 3 pathways suggests that genes like ARF6 and their gene expressions are potentially important factors in the pathogenesis of autism spectrum disorder. Future research expanding on these findings could include discovering immunotherapies to protect pathways and genes from pathogens that harm the immune system.

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