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Article: **Analyzing miR-106b-5p and miR-93-5p as Promising Diagnostic Markers for Autism Spectrum Disorder**

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Analyzing miR-106b-5p and miR-93-5p as Promising Diagnostic Markers for Autism Spectrum Disorder

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Abstract

Autism spectrum disorder (ASD) is a complex group of both neurological and developmental disorders encompassing perturbations in verbal and non-verbal communication, social skills, as well as repetitive and restricted behaviour, activity, or response. The pathogenesis of the disorder is still unknown, yet several studies have documented the involvement of both genetic and environmental factors in its onset. Intense efforts have been made to identify reliable biomarkers to aid in early diagnosis. MicroRNAs (miRNAs) are the regulatory noncoding regions of ribonucleic acid (RNA) that can alter the expression of a gene through posttranscriptional mechanisms. In this study, an in-silico technique was used to identify two novel biomarkers, namely miR-106b-5p and miR-93-5p. The analysis identified that these diagnostic biomarkers are associated with ASD and can aid in its early treatment since miRNAs show a significant part in the progression and function of the central nervous system (CNS).

Keywords: autism spectrum disorder (ASD), biomarker, *in-silico* techniques, microRNAs (miRNAs)

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Introduction

Autism spectrum disorder (ASD) is a complicated and diversified neurological disorder that influences the developmental paths in many key behavioural domains, such as social interactions, psychological features, repetitive behaviour patterns, and language impairment [1]. In the recent past, the prevalence of ASD rapidly rose to 1:37 children in the U.S [2]. Literature reports that ASD influences approximately 1-2% of the population and the ratio of males to females is 4-5:1[3]. The severity of autistic characteristics along with the occurrence of comorbid illnesses, such as anxiety, intellectual incapacity, epilepsy, and gastrointestinal disorders, substantially varies between autistic individuals [4].

Currently, the assessment of ASD is being done under standards and rules set by the Diagnostic and Statistical Manual of Mental Disorders (5^{th} edition) (DSM-5). Although skilled clinicians can diagnose ASD even in 24-month-old toddlers, the typical age at which the disorder is diagnosed remains significantly high and can reach up to 4 years [2]. So far, the aetiology and pathological processes of ASD have not been interpreted fully. It was reported in many studies that ASD is a multifactorial disorder with various risk factors such as genetic, epigenetic, and environmental factors [5, 6].

Currently there are very limited options available for the treatment of ASD, though the early intervention was reported to result in rapid improvement in the overall mental health of autistic patients [7]. Early intervention strategies can only be offered if the diagnosis is made well in time, especially for neurodevelopmental disorders such as ASD. For this reason, it is essential to avoid delayed diagnosis; however, the absence of liable and specific biomarkers makes the early diagnoses of ASD a demanding /challenging task. Thus, for an efficient early diagnosis of ASD, a reliable and stable biomarker is required [8]. In the field of genetics, there is an economical and effective model that can be used to diagnose ASD. In this model, microRNA analysis resulted in encouraging outcomes, which can be used to distinguish ASD children from their typically developing peers [9]. MicroRNAs (miRNAs) are 70% expressed in CNS and regulate almost two-third of human mRNAs [10]. The miRNA expression patterns are varied depending from organs ranging from brain, saliva, blood and olfactory

precursor cells of patients effected with ASD. At present, several identified miRNAs show potential involvement in ASD [11]. According to previous studies, miRNAs are strongly associated with the pathogenesis of ASD [12].

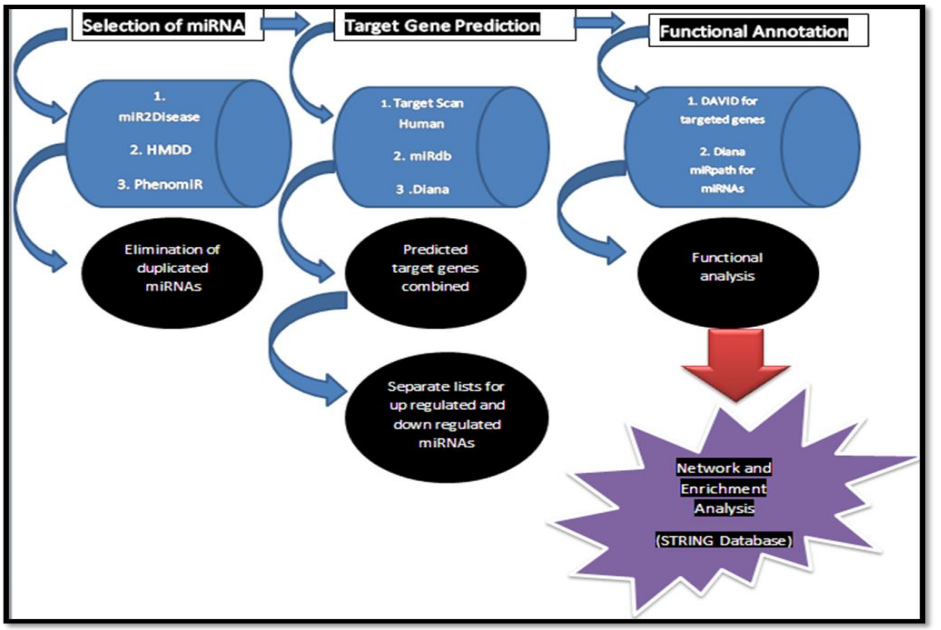
Thus, it is pertinent to identify and examine diagnostic biomarkers of ASD, since there is no standard biochemical or a molecular diagnostic criterion available for this disorder. For this purpose, this study identified and examined two miRNAs, namely miR-106b-5p and miR-93-5p, which can act as biomarkers and aid in early treatment of ASD

Methodology

To identify an efficient biomarker for ASD, we first selected the microRNAs (miRNAs) that were experimentally validated to be involved with ASD and were particularly present in blood, serum, and plasma. For this purpose, three databases, including phenomiR2 (http://mips.helmholtzmuenchen.de/phenomir/main/showmir/6809?manorder=asc&sort=pm.mir.name), HMDD (http://210.73.221.6/hmdd), and miR2disease (http://www.mir2disease.org/), were explored. Afterwards, three different lists of miRNAs were retrieved and combined to remove redundancy. A single list that showed upregulation or downregulation of miRNAs was maintained. These short-listed miRNAs were prioritized/identified and categorized based on target gene prediction by using the three software: Target Scan (http://www.targetscan.org/vert_71/), mirdb (http://www.mirdb.org/cgi-bin/search.cgi), and Diana Micro-T (http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=microT_CDS/index). Once up-regulated and down-regulated miRNAs were screened, its target prediction was performed separately, indicating that miRNAs were involved with the genes. The duplicates were deleted and the predicted target genes were enlisted if all of them showed their presence in the three predicted algorithms, which gave the putative list of target genes. Then, the known genes for ASD were obtained from the Autism Informatics portal (AutDB) (http://autism.mindspec.org/autdb/Welcome.do). Next, the predicted target genes were compared with the known genes associated with ASD through the VENNY tool (http://bioinfogp.cnb.csic.es/tools/venny/) to determine whether any of the predicted target genes were related to ASD or not. The generalized methodology is mentioned in Figure 1.

Subsequently, the functional annotation was performed on the predicted target genes and miRNAs to identify and specify/define the supposed functions of these differently expressed genes (DEGs). Functional annotation highlighted different pathways in the miRNAs so that the putative miRNAs involved in certain pathways could be identified. For this purpose, at first, the functional annotation was performed separately for/on all of the gene lists obtained from the target prediction. This was done using a clustering tool, which is available on DAVID (version 6.7) (The Database for Annotation, Visualization and Integrated Discovery) (https://david.ncifcrf.gov/tools.jsp). In this way, the predicted target gene list was made more precise. Then, this shortened gene list was put into the VENNY tool and compared to the list of known ASD genes obtained from AutDB. The overlapped genes were considered more significant and used for further study. Afterwards, the functions of up-regulated and down-regulated miRNAs were determined using Diana-mirPath (http://snf-515788.vm.okeanos.grnet.gr/). Next, the annotated functions of miRNAs and predicted target genes were compared to check if there were any overlaps. The genes which were against the miRNA annotated were saved in XLM files and were compared with the known up-regulated targeted genes using the VENNY tool to find out the predicted genes. After the comparison, the genes showed their functions in terms of pathways. A heatmap was also generated after the union of pathways.

Figure 1. Methodology used for Identification of miRNA as a biomarker for diagnosis of ASD



Pathways and network enrichment analysis was used to predict functions of selected candidate genes using STRING (version 10) (Search Tool for the Retrieval of Interacting Genes) (https://string-db.org/). Later, KEGG enrichment analysis for associated pathways was done for the up- and down- regulated target genes. Subsequently, DEGs genes were separately queried and mapped in STRING for/to generate gene network enrichment and KEGG pathway.

Results

Lists of 28, 28, and 41 miRNAs from miR2disease, phenomiR, and HMDD were obtained, respectively. The three lists were combined and their duplication was removed. The final list contained 46 miRNAs in total. The list of miRNA retrieved from different databases is given in Table 1.

Table 1. List of miRNA Short Listed from Various Databases

Sr No.	miRNA ID	Source Database	Detection method	Expression Pattern
1	hsa-miR-15a	miR2Disease PhenomiR	DNA Microarray	Increased expression

Sr No.	miRNA ID	Source Database	Detection method	Expression Pattern
	hsa-miR 15b	HMDD miR2Disease PhenomiR	DNA Microarray	Increased expression
2		HMDD		
3	hsa-miR-21	miR2Disease PhenomiR	DNA Microarray	Increased expression
4	hsa-miR-212	HMDD miR2Disease PhenomiR	DNA Microarray	Increased expression
5	hsa-miR-431	HMDD miR2Disease PhenomiR	DNA Microarray	Increased expression
6	hsa-miR-432	HMDD miR2Disease PhenomiR	DNA Microarray	Decreased expression
7	hsa-miR-484	HMDD miR2Disease PhenomiR	DNA Microarray	Increased expression
8	hsa-miR-539	HMDD miR2Disease PhenomiR	DNA Microarray	Decreased expression
9	hsa-miR-598	HMDD miR2Disease PhenomiR	DNA Microarray	Increased expression
10	hsa-miR-652	HMDD miR2Disease PhenomiR	DNA Microarray	Decreased expression
11	hsa-miR-93	HMDD miR2Disease PhenomiR	DNA Microarray	Decreased expression
12	hsa-miR-95	HMDD miR2Disease PhenomiR	DNA Microarray	Increased expression

Sr No.	miRNA ID	Source Database	Detection method	Expression Pattern
13	has-miR-106b	miR2Disease PhenomiR HMDD	DNA Microarray	Decreased expression
14	hsa-miR-181d	miR2Disease PhenomiR HMDD	DNA Microarray	Decreased expression
15	hsa-miR-23a	miR2Disease PhenomiR HMDD	DNA Microarray	Increased expression
16	hsa-miR-27a	miR2Disease PhenomiR HMDD	DNA Microarray	Increased expression
17	hsa-miR-148b	miR2Disease PhenomiR HMDD	DNA Microarray	Increased expression
18	hsa-miR-106a	miR2Disease PhenomiR HMDD	DNA Microarray	Decreased expression
19	hsa-miR-146b	miR2Disease PhenomiR HMDD	DNA Microarray	Decreased expression
20	hsa-miR-193b	miR2Disease PhenomiR HMDD	DNA Microarray	Decreased expression
21	hsa-miR-381	miR2Disease PhenomiR HMDD	DNA Microarray	Decreased expression
22	hsa-mir-146a	miR2Disease PhenomiR HMDD	qPCR	Increased expression
23	hsa-miR-132	miR2Disease PhenomiR HMDD	DNA Microarray	Increased expression

Sr No.	miRNA ID	Source Database	Detection method	Expression Pattern
24	hsa-miR-140	miR2Disease PhenomiR HMDD	DNA Microarray	Decreased expression
25	hsa-miR-320a	miR2Disease PhenomiR	DNA Microarray	Decreased expression
26	hsa-mir-129-1	PhenomiR HMDD	qPCR	Increased expression
27	hsa-mir-7-1	PhenomiR HMDD	qPCR	Increased expression
28	hsa-miR-550	miR2Disease	DNA Microarray	Decreased expression
29	hsa-miR-128a	miR2Disease	DNA Microarray	Increased expression
30	hsa-miR-129	miR2Disease	DNA Microarray	Increased expression
31	hsa-miR-7	miR2Disease	DNA Microarray	Increased expression
32	hsa-mir-128-1	PhenomiR	qPCR	Increased expression
33	hsa-mir-550-1	PhenomiR	qPCR	Decreased expression
34	hsa-mir-129-2	HMDD	qPCR	Decreased expression
35	hsa-mir-181b-1	HMDD	qPCR	Decreased expression
36	hsa-mir-23b	HMDD	qPCR	Increased expression
37	hsa-mir-363	HMDD	qPCR	Decreased expression
38	hsa-mir-486	HMDD	qPCR	Decreased expression
39	hsa-mir-550a-1	HMDD	qPCR	Decreased expression

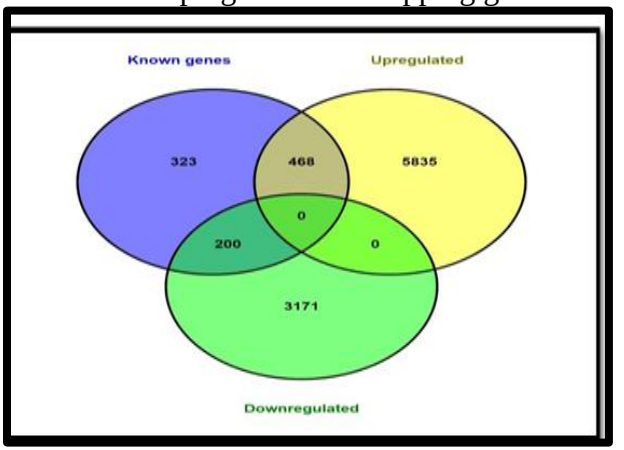
Sr No.	miRNA ID	Source Database	Detection method	Expression Pattern
40	hsa-mir-550a-2	HMDD	qPCR	Decreased expression
41	hsa-mir-663a	HMDD	qPCR	Increased expression
42	hsa-mir-7-2	HMDD	qPCR	Decreased expression
43	hsa-mir-7-3	HMDD	qPCR	Decreased expression
44	hsa-mir-92a-1	HMDD	qPCR	Decreased expression
45	hsa-mir-92a-2	HMDD	qPCR	Decreased expression
46	hsa-miR-132	HMDD	qPCR	Decreased expression

A raw total of 25454 genes were found after utilizing the software programs that predicted the 3-target genes. Moreover, 15780 duplicates were found and eliminated, and the remaining genes were only kept if all of them were present in the three predicted algorithms, which gave the putative list of target genes. Thus, 9674 unique genes were obtained and sorted, where 3371 were down-regulated and 6303 were up-regulated miRNA target genes. The redundancy was removed in the short-listed miRNAs, which were prioritized/identified and categorized based on the target gene list and statistical significance of the target gene. Following that, 12 miRNAs were excluded due to the failure of the three target prediction tools employed in this work to predict targets, resulting in a list of 33 miRNAs.

Next, the predicted target genes were compared with 991 known genes associated with ASD, retrieved through AutDB, to check whether any of the predicted target genes were related to ASD or not. 468 up-regulated and 200 down-regulated genes were similar with the known genes associated with ASD (mentioned in Figure 2)

Figure 2. Representation of solely known genes linked to ASD (blue) compared to expected down (green) and up-

regulated (yellow) gene lists. There are 200 down regulated and 468 upregulated overlapping genes**.**



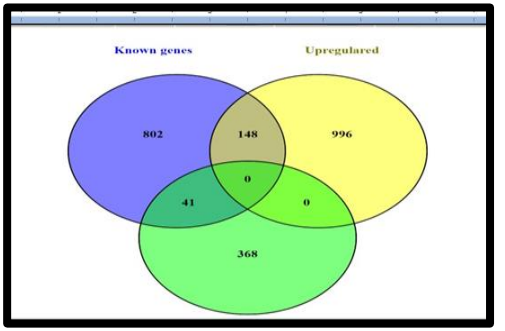
Functional annotation was done separately for each gene list obtained from the the target prediction, which was carried out using the clustering tool, available on DAVID (version 6.7).

After finding the clusters, duplicates were removed. The symbolic separation of gene in the EXCEL sheet was done by using "=LEFT (A3, FIND("",A3)-1)" formula. The VENNY tool was used to examine the trimmed gene list to a list of 991 known vulnerable genes. The overlapped genes were considered important and used for further study. 148 genes with increase expression and 41 genes with decreased expression were overlapped with the known genes. This process prioritized the gene list further. This list is given in Table 2.

Table 2. Description of Gene Cluster of Biological Process of Upregulated Targeted Genes Retrieved from DAVID

Annotation Cluster 08	**Enrichment Score 4.37**	**Count**	**p-value**	**Benjamin**
GOTERM_BP_FAT	Neuron differentiation	102	1.5E-6	3.0E-4
GOTERM_BP_FAT	Cell projection organization	87	5.0E-6	7.8E-4
GOTERM_BP_FAT	Neuron development	81	7.2E-6	1.1E-3
GOTERM_BP_FAT	Neuron projection development	65	8.5E-6	1.2E-3
GOTERM_BP_FAT	Cell projection morphogenesis	62	1.6E-6	1.9E-3
GOTERM_BP_FAT	Cell morphogenesis	82	2.6E-5	2.6E-3
GOTERM_BP_FAT	Neuron projection morphogenesis	55	2.8E-5	2.8E-3

Figure 3. After functional annotation, the known vulnerable genes connected to Autism (blue) were compared to the anticipated elevated (yellow) and downregulated (green) gene list. The known genes related with ASD were shown to coincide with 148 upregulated and 41 downregulated genes.



The list of genes that overlap with known genes is given in Table 3.

Table 3. List of Genes Overlapped with Known Genes

LIST OF GENES OVERLAPPING WITH KNOWN GENES								
List of downregulated genes		**List of upregulated genes**						
ANKRD11	ARX	ANXA1	MAP2	POU3F2	DMD	HIVEP3	CD44	RIMS1
DLX6	DRD1	APC	MAPK1	PRICKLE2	RANBP17	ITGA4	GRIN2B	FOXP1
ERMN	YWHAE	AR	MBD1		SLC6A1	JARID2	PTGS2	SMAD4
TSC1	SMC1A	ARID1B	MBD3	PRUNE2	ACTN4	RAC1	PTGER3	SLC12A5
YY1	NEFL	ASH1L	MDGA2	PSMD12	IFNG	NF1	SLIT3	CTCF
OTX1	BBS4	ATRX	MED13	PTEN	AFF2	CAMK4	CTTNBP2	CUX1
P2RX4	DRD2	ATXN7	MFRP	NLGN3	NSD1	GRIN2A	CLASP1	SLC1A2
PCDH8	XIRP1	AVPR1B	MIB1	RFX3	HCFC1	DMXL2	CREBBP	ADRB2
PLXNA3	SLC6A4	BCL11A	MTF1	TBL1XR1	PTPRC	SHANK3	EXT1	TAF1
PLXNB1	NRP2	BCL2	MTX2	TBR1	NELL1	CHD8	GRIA1	TBL1X
APP	CECR2	BDNF	CEP290	TCF7L2	CAMK2B	EPC2	RB1CC1	ANK3
DNER	EGR2	BRAF	NRG1	THBS1	GPX1	ERBB4	SATB2	GSK3B
FLT1	WNT1	CACNA1A	NTRK2	TNRC6B	SIN3A	RPS6KA3	PAH	NBEA
THRA	SHOX	CACNA1B	PAFAH1B1	TOMM20	EP300	DCX	STX1A	CAMK2A
HTR2A	NR2F1	CACNA1E	SMARCA2	TSC2	ROBO2	NR1D1	NRXN3	CADM1
ARHGAP33	DLG1	CHD7	PAX5	TYR	RORA	FGD1	AFF4	EIF4E
DPYD	EPS8	CHD2	PAX6	WNK3	SIN3A	HERC2	CDKN1B	SETD2
HDAC4	TUBGCP5	KCNQ3	PEX7	XPO1	GRIN1	SOX5	GRM4	ROBO1
TERF2	PRKDC	KIF5C	PLCB1	ZNF462	CHD1	NRXN1	PTK7	SHANK3
ITPR1		LMX1B	POLA2	DDX11	CTNNB1	JMJD1C	NAA15	SIK1
		LRP2	PON1	DLGAP1	HUWE1	NLGN1	MYT1L	CUL3
		MAOA	POT1	DLX2				SIK1

Then functions of DEGs miRNAs were determined through Diana-mirPath. Afterwards, functional annotation of miRNAs, acquired from Diana-mirPath, were linked with functional annotation of the predicted target genes using DAVID. This was done to observe if there were any overlaps. Next, the genes which were against the miRNA annotated were saved in XLM files and were compared with the known up-regulated targeted genes to find the predicted genes. For significant and efficiency of the pathways, a heatmap was generated after the union of pathways, where a total of 13 and 14 pathways were shown. These pathways are given in Figures 4 and 5. The pathways involved in down-regulated and up-regulated miRNAs are shown in Table 4.

Table 4. Pathways Involved in Down Regulated and Upregulated miRNAs

Pathways involved in down regulated miRNAs	**Pathways involved in Up regulated miRNAs**
• Proteoglycans in cancer • Pathways in cancer • nicotine addiction • TGF-beta signaling pathway • Signaling mechansims that control stem cells pluripotency. • Hippo signaling pathway • fatty acid metabolism • Fatty acid biosynthesis • Thyroid hormone signaling pathway • Glioma • Melanoma • Lysine degradation • Axon guidance • Adherens junction	• Lacto and neolacto series glycosphingolipid biosynthesis • Hippo signaling pathway. • Proteoglycans in cancer. • Lysine degradation. • Glioma. • Signaling pathways regulating pluripotency of stem cells. • TGF-beta signaling pathway • Morphine addiction. • Amphetamine addiction. • ECM- receptor interactions • fatty acid degradation • N-Glycan biosynthesis. • Metabolism of xenobiotic by cytochrome p450

This further proved that the the functions of miRNAs and their target genes are directly associated. Identified genes are also

convoluted in nerve signalling, and the pathways discussed also have pathways involved in nerve signalling such as axon guidance and other linked pathways.

Figure 4. Functional Annotations of Up-regulated miRNAs and their Involvement in Different Pathways, using Diana mir Path Showing the Heat Map

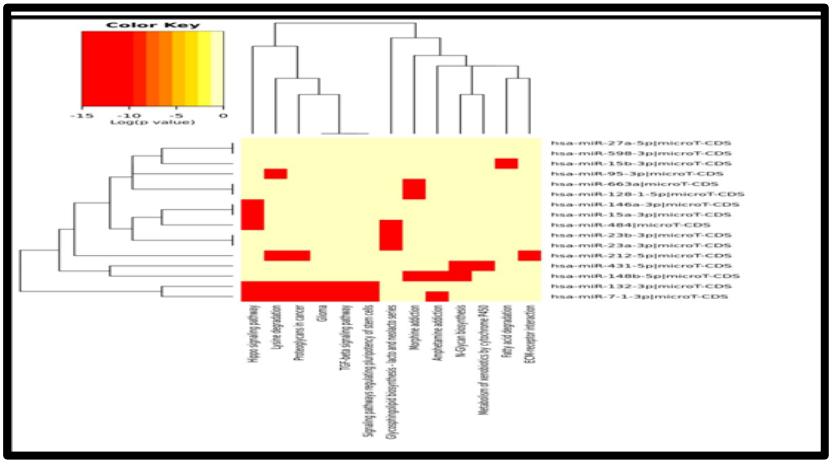
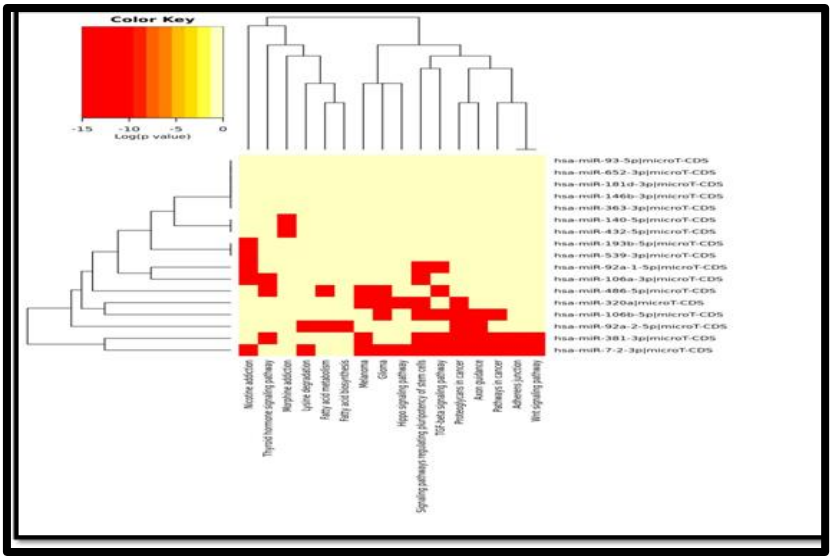


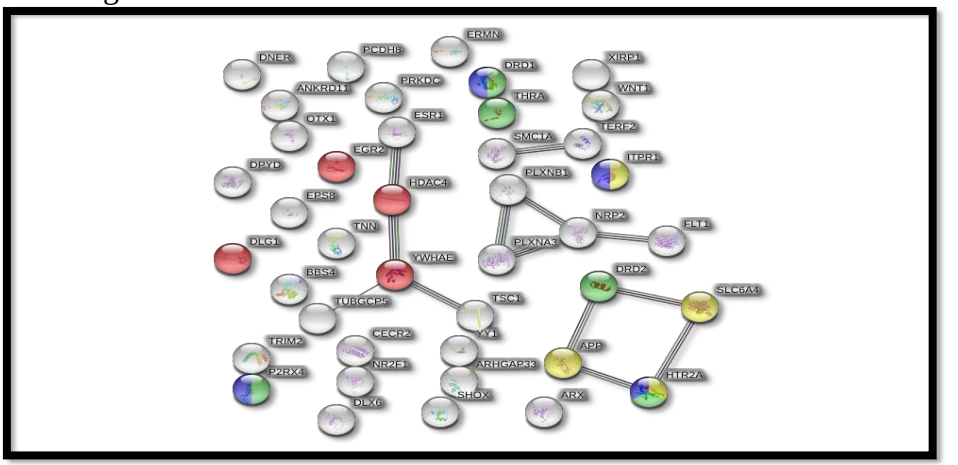
Figure 5. Functional Annotations of downregulated miRNAs and their Involvement in Different Pathways, using Diana mirPath Showing the Heat Map



Later, KEGG pathway enrichment analysis was done for DEGs lists by using STRING database. Based on the results, the down-regulated variants were shown to be involved in the Gap junction, namely calcium signalling pathway, serotonergic synapse and neuroactive ligand-receptor interactions. Significant pathways for/of up-regulated gene list had long-term potentiation, such as went signaling pathway, prostate cancer, neurotrophin signaling pathway, calcium signaling pathway, glutamatergic synapse, dopaminergic synapse, melanogenesis, amyotrophic lateral sclerosis, colorectal cancer, amphetamine addiction, adherence junction, HIF-1 signaling pathway, cholinergic synapse, pathways in cancer, ErbB signaling pathway, thyroid hormone signaling pathway, circadian entrainment, nicotine addiction, alcoholism, microRNAs in cancer, endometrial cancer, focal adhesion, mTOR signaling pathway, proteoglycans in cancer, renal cell carcinoma, Epstein-Barr virus (EBV) infection, MAPK signaling pathway, serotonergic synapse, Rap1 signaling pathway, TGF-beta signaling pathway, FoxO signaling pathway, cocaine addiction, thyroid cancer, Huntington disease, Long term depression, retrograde endocannabinoid signaling, hepatitis B, glioma, chronic myeloid leukemia, P13K-AKT signaling pathway, arrhythmogenic right ventricular cardio myopathy, tuberculosis, axon guidance, Type II diabetes mellitus, adrenergic signaling in cardiomyocytes, neuroactive ligand-receptor

interaction, oxytocin signaling pathway, oocyte meiosis, pancreatic cancer, Ras signaling pathway, cell cycle, alzheimer's disease, influenza A, leukocyte transendothelial migration, leishmeniasis, viral carcinogenesis, natural killer cell mediated cytotoxicity, bladder cancer, insulin signaling pathway, cell adhesion molecules, GABAergic synapse, small cell lung cancer, GnRH signaling pathway, regulation of actin cytoskeleton, chemokine signaling pathway, systemic lupus erythematosus, lysine degradation, VEGF signaling pathway taste transduction, phenylalanie metabolism, basal cell carcinoma, acute myeloid leukemia, shigellosis, herpes simplex infection, HTLV-1 infection, p53 signaling pathway, osteoclast differentiation, tight junction, vascular smooth muscle contraction, B cell receptor signaling pathway, melanoma, thyroid hormone synthesis, and gastric acid secretion. Moreover, STRING analysis revealed 13 interactions for down-regulated and 231 for up-regulated miRNA target genes as shown in Figure 6 and Figure 7, respectively. Interestingly, the two genes involved in ASD susceptibility (HTR2A, SLC6A4) were shown to interact with each other.

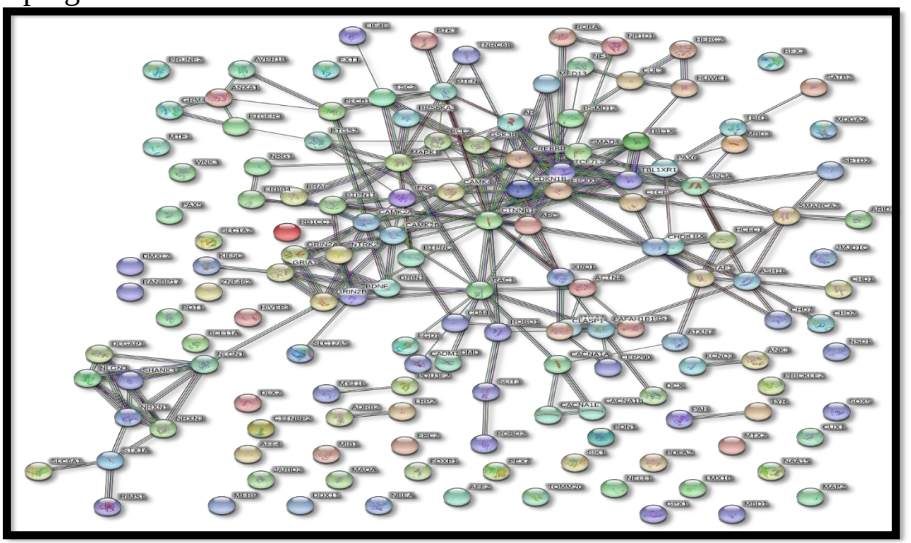
Figure 6. STRING Analysis of Pathway Enrichment and Interaction in the Downregulated miRNA Target Genes. 13 Interactions were Observed for Downregulated Genes



HTR2A gene is involved in most of the down-regulated miRNA targeted gene pathways. Serotonin (5-hydroxytryptamine, 5HT) is a conventional monoamine that is broadly involved in different physiological

processes, such as social behaviours, cardiovascular regulation, homeostasis, gastrointestinal functions, circadian rhythms, cognition, and mood swings. Serotonin signalling is also responsible for the regulation of many neurodevelopmental processes. Evidence also proposes that serotonin might play an important role in the evolving programming of childhood as well as adult onset mental conditions [13]. Previous studies claim that seven candidate genes (TPH1, ITGB3, SLC6A4, HTR1A, HTR1D, HTR2A, HTR5A), involved in the neurotransmission of the brain, serotonin pathways, and linking to autism through mapping, are responsible for the aetiology of autism [14]. Furthermore, increased levels of platelets serotonin (5-HT) were found in ASD patients. In autistic disorder patients, the serotonin transporter gene (SLC6A4) is a potential gene involved in neurochemical and neuroendocrine reactions, also the effectiveness of potential serotonin transporter inhibitors in minimizing ritualistic aggression behavior [15]. Brain and peripheral serotonin homeostasis were also found to be disrupted in ASD individuals. Serotonin receptor 2A (HTR2A) is involved in regulating central and peripheral serotonin homeostasis. It also modifies expression in autistic subjects. It has been seen and claimed that the HTR2A gene acts as a foremost applicant gene that causes serotonin disturbance in autistic patients. In this regard, several studies have reported an association of functional SNP - 1438 G/A (rs6311) present in HTR2A promoter region with autism; however, all of these studies yielded uncertain outcomes [16].

Figure 7. STRING Analysis of Pathway Enrichment and Interaction in the Upregulated miRNA Target Genes. 231 Interactions were Observed for Upregulated Genes



MAPK1 and PLCB1 genes were identified to be the pathway enrichment genes. Out of the total 85 pathways, these two genes show involvement in 30 and 40 pathways, respectively.

Mitogen-activated protein kinase 1, or MAP kinase 1, is a serine/threonine kinase that plays an important role in the MAP kinase signalling cascade. The two MAPKs that play a crucial role in the MAPK/ERK cascade are MAPK1/ERK2 and MAPK3/ERK1. They are also part of signaling cascades that are initiated by activated KIT and KITLG/SCF. The MAPK/ERK cascade arbitrates various biological functions, such as the survival cell growth, adhesion, and differentiation, by regulating the transcription, translation, and cytoskeletal rearrangements depending on the cellular context of the cell. Nevertheless, previous studies suggest that MAPK signaling and calcium signaling pathways are strongly associated with ASD [16].

Activated phosphatidylinositol specific phospholipase C enzymes regulate the generation of the second messenger chemicals diacylglycerol

(DAG) and inositol 1,4,5-triphosphate (IP3). Some previous studies claim that rare copy number variants are highly responsible for ASD. These variants affect great number of genes including PLCB1. PLCBI causes gene disruptive events in ASD [17]. Chromosomal location of PCLB1is *20p12.3.*

Conclusion

Hence, it is concluded from the pathway analysis that miR-106b-5p and miR-93-5p can serve as novel biomarkers of ASD due to their intimacy in/association with all 3 predicted genes. Several previous studies identified that these miRNAs play a significant role in autism [18]**.** As discussed in the previous section, there is no blood test available to diagnose ASD, due to which its early treatment is not possible. The analysis identified two diagnostic biomarkers (miR-106b-5p and miR-93-5p) associated with ASD. These miRNAs can also aid in the early treatment of ASD since they are majorly involved in the development and function of the central nervous system (CNS). The experimental analysis of the three genes, namely HTR2A, MAPK1 and PLCB1, can be determined by identifying the above-mentioned diagnostic biomarkers (miR-106b-5p and miR-93-5p), both of which are down-regulated miRNAs. These biomarkers can be detected in blood and thus prove beneficial in the early diagnosis of this multifactorial disorder

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