Original Article
ISSN (Print) : 0971-6580
Toxicology International, *Vol 25(1), 26-39, January-March 2018, DOI: 10.18311/ti/2018/v25i1/22527* *ISSN (Online) : 0976-5131*

Significant Analysis of Microarray (SAM) to Identify Synergistic Effect of RV and NGF in Repairing Damaged Neuronal Cells

Ruchi Yadav and Prachi Srivastava*

Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow – 226028, Uttar Pradesh, India; psrivastava@amity.edu, p.srivastava2@gmail.com

Abstract

Neurodevelopmental disorders include diseases that are related with genetic disorders and are caused due to stress condition or environmental toxins during pre or post natal condition. In recent years, there is considerable research in neurodevelopmental disorders and examining therapeutic role of resveratrol as potential antioxidant. There is less information about the genes that are mutated or altered during neurodevelopmental phase and cause neuronal developmental disorders. Current researches are evidentially showing the therapeutic potential of Resveratrol (RV) and Nerve Growth Factor (NGF) against neuronal diseases. In current study Microarray experiment was designed to identify the genes that are altered when Mesenchymal Stem Cells (MSC) were exposed to Monocrotophos (MCP). MSCs were also coexposed with resveratrol and nerve growth factor to study the synergistic effect of NGF with RV. Computational analysis of microarray data was carried out through different software's and bioinformatics tools to identify genes that are expressed in different samples across microarray experiment. Statistical methods like T-test, SAM analysis and clustering techniques were performed between different samples using MeV software. Through Significant Analysis of Microarray (SAM) method we identified positive and negative significant genes with respect to current study. Clustering method was used to cluster genes associated with neuronal disease genes. Key genes that were predicted on the basis of t-test and SAM analysis are DNMT1, PGAP1, RDX and PEX26 genes, these genes have noticeable function in different classes of neuronal diseases like cerebellar ataxia, deafness, narcolepsy, mental retardation and zellweger spectrum disorder etc. Cluster analysis we identified genes such as ATP6V0D1, TESMIN, TRIM22, NAPEPLD, CDK7 and PKM, these genes have important function in cell growth, cell proliferation, protein synthesis, cell cycle regulation, and cell signaling. Study suggests that exposure of damaged neuronal cells to RV and NGF enhances the expression of neuronal repair genes, thus signifies the neuroprotectant and synergistic activity of RV and NGF. "The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO Series accession number GSE121261 **(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE121261)."**

Keywords: Microarray Analysis, Nerve Growth Factor, Neuronal Damage, Neuronal Diseases, Resveratrol, SAM

1. Introduction

NGF is a highly studied molecule and its function is discovered mainly in differentiation and survival of sensory neurons along with development of brain[1]. Till date different experiments has been done to evaluate the potentiality of NGF as neuronal repair[2]. Researchers have also explored the other dimension of NGF to demonstrate its efficiency in neurodevelopmental disorders[3]. Neurodevelopment disorder consists of wide spectrum of abnormal neurological conditions like difficulty in learning, speech, writing, attention deficit, intellectual deficit and abnormal behavior[4]. Different neuronal disorders can be identified in its early phase of

*Author for correspondence

development at birth. Due to lack of information about gene expression in neuronal developmental disorders, in its initial phase of embryo development many diseases cannot be diagnosed at early phase of progress[5]. To understand the causes of many neuronal disease, neurodevelopment disorders study is a thrust area of research for neuroscientists[6]. Experiments have been done to identify genes and establish gene network, in relation with different neurological diseases[7]. Effect of Nerve Growth Factor (NGF) as repairing agent against damaged neurons has been shown in many neurodevelopmental disorders[8]. Studies have shown that NGF and Brain-Derived Neurotropic Factor (BDNF) have important function in neuron development, differentiation, growth and function in early and adult stage of brain development[9]. Experiments have been done on schizophrenia disease, to highlight the importance of NGF in restoring function of damaged neurons[10]. Loss of NGF is found in many diseases like depression, schizophrenia etc which shows which reveal its importance in said disorders[11]. In current course of study we have identified the synergistic effect of NGF and Resveratrol in repairing damaged neuronal cells at developmental phase.

Resveratrol is a well-known natural phytochemical showing its therapeutic values against many diseases including neuronal disorders[13]. Resveratrol is a natural stilbene formed in many plants and mostly found in red-wine extracts, has potential neuroprotectiverole[14]. Experiments have been done on different neurological diseases like Alzheimer's disease, Parkinson disease, Huntington disease, and Epilepsy to prove the neuroprotective mechanism of resveratrol[15]. Current researches are showing the potentiality of resveratrol as good neuroprotectant with antioxidant properties. Neurodevelopmental disorders are class of neuronal disease which is caused due to impairment of brain neurons during brain development[16]. These disorders also include diseases that are related with genetic disorders and are caused due to stress condition or environmental toxins during pre or post natal condition[17]. *In Vivo* and *In-Vitro* experiments have been done to examine the effect of alcohol, different infection, and malnutrition including other stress condition like alteration in metabolic conditions on neurodevelopmental disorders[18].

Neurodevelopmental disorders like epilepsy, autism, intellectual disability has been of great interest for neuroscientist as their prevalence is a social problem on society and its prevention or control is of great demand[19]. Number of genes has been identified like delta-2-protocadherin (*δ2-PCDH*) family gene it is reported mutation in this gene causes neurodevelopmental diseases. *PCDH* genes is involved in development of axons, dendrites, synapsis and regulating signal in brains, loss in function of this gene may lead to development of autism spectrum disorders and other diseases[20]. Fragile X Mental Retardation Protein (FMRP) mutation or loss of this protein causes defects in spine development and also indirectly effect the function of other genes that are involved in neurogenesis[21]. RNA splicing factor *RBFOX1* is involved in regulating large number of gene network that have function in neuronal development, under expression of this gene is shown in many diseases like autism, epilepsy and cognitive deficit[22].

Though in current genomic era we are consistently working on the expression analysis of genes in concern to different disorders but still less is known about genes that are mutated or altered during neurodevelopmental phase and cause neuronal developmental disorders[23]. Neurodevelopmental disorders are very complex and mostly co-exist with different other disorders proves their complexity to explore their mechanism of expression. Large scale gene networks are involved in these diseases; hence a genome wide experiment is required to study gene and gene interactions, which are involved in neurodevelopmental disorders. To overcome this problem microarray experiment is widely used to study gene expression and interaction between genes that are expressed in neurodevelopmental phase of neuronal injury.

Microarray is a powerful experiment when it comes to understand the complex mechanism of disorders at expression levels of genes including neurological disorders[24]. To understand the mechanism of neuronal diseases and to identify genes that are expressed or co-expressed during neuronal disorders, high throughput experiments like microarray experiment is designed[25]. Microarray experiment gives the snapshot of gene expression in single run of a microarray experiment[26]. Microarray database analysis of ArrayExpress at EMBL[27] and Gene Expression Omnibus (GEO) database[28] at NCBI shows progress in microarray experiment to identify genes responsible for different neurodevelopmental disorders like schizophrenia, Rett syndrome, brain disorders and Autism[29]. MeCP2 (methyl-CpG binding protein 2) gene have been identified to be involved in schizophrenia and regulates other neuronal expression in hypothalamus[30]. Use of microarray experiments in neuroscience has been

extended in the field of neurodevelopmental disorders research, to identify potential drug target, effect of drug on neuronal diseases, therapeutic role of natural antioxidants[31]. Scientist has also discovered the scope of microarray experiment to enhance the therapy of neuronal diseases according to patients' genetic makeup[32]. In current research work, we have used microarray experiment analysis data, to evaluate the combined action of Resveratrol and Nerve growth factor in repairing damaged neuronal cells.

2. Materials and Methods

2.1 Microarray Experiment Sample Probes

Microarray experiment wasdesigned to identify gene alteration when Mesenchymal Stem Cells (MSC) were exposed to Monocrotophos (MCP) and Resveratrol (RV). MSCs were also coexposed with Resveratrol and Nerve Growth Factor (RV+NGF) to study the synergistic effect of RV with NGF. Microarray experiment was extended to identify the effect of pre and post exposure of RV and NGF on MSC.Total ten samples were prepared 1) Control sample (MSC), 2) RV, 3) NGF, 4) RV+NGF, 5) pre exposed RV , 6) pre exposed NGF, 7) pre exposed RV+NGF, 8) post exposed RV, 9) post exposed NGF, 10) post exposed RV+NGF. These samples were hybridized to Affymetrix chip Prime View platform. Microarray gene expression data was acquired and computational analysis was done to examine expression of gene in different sample conditions, coexpression, pathway analysis, and functional annotation. Part of the microarray data has been analyzed to identify genes expressed in response to monocrotophos and previously published[33]. Major aim of current analysis was to identify the genes that show enhanced expression when RV was coexposed with NGF.

2.2 Microarray Data Analysis

Microarray analysis has capacities to identify genes under study. Microarray feature probes or microarray experiment sample probes can be grouped to answer biological question setup during experiment. Methods and strategies that are used to analyze microarray data have a major effect on interpretation of results[34]. Number of computational tools are available that can be used to identify important genes from experiment. In this paper we have developed protocol to identify significant genes that are expressed in different sample condition. Different software's were used like R and Bioconductor[35], MeV[36], DAVID tool[37] to analyze and annotate microarray data.

Computational analysis of microarray data was done to identify the effect of RV and NGF in repairing damaged neuronal cells caused by exposure of MSC with MCP. Figure 1 shows the accumulation of different comparison statistics between samples to identify significant genes that are expressed in different samples. Two groups were made to identify genes, which show significant increase in level of gene expression, when MSCs were exposed to RV and NGF. R and Bioconductor, Affy package is used to write gene expression file using ten sample file and Prime view microarray chip file. Gene Expression file is used for statistical analysis like t-test, SAM[38] etc., between different samples. Comparative analysis is done to identify genes that are significantly expressed when MSC are co-exposed with RV and NGF.

T-test is main statistical method performed to analyze microarray data to identify significant and non-significant genes[39,40]. T-test is done between different samples using MeV software. Two groups were made for T-test statistics, group 1 include t-test between RV samples (RV, Pre exposed RV, post exposed RV) and RV+NGF samples (RV+NGF, pre exposed RV+NGF, post exposed RV+NGF). Group 2 include t-test between NGF samples (NGF, Pre exposed NGF, post exposed NGF) and RV+NGF samples (RV+NGF, pre exposed RV+NGF,

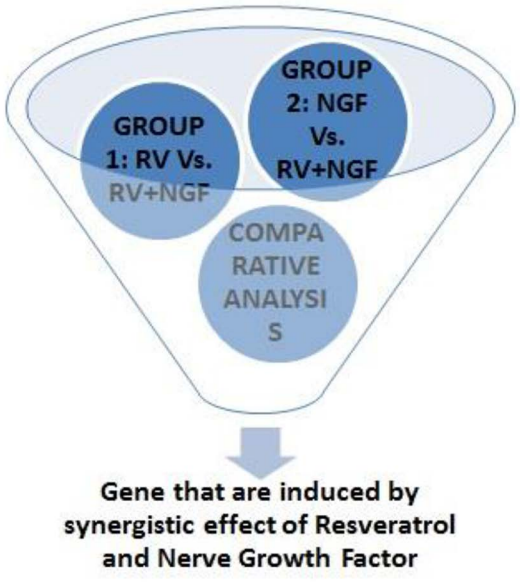


Figure 1. Comparison scheme between different samples to identify genes that are expressed in presence of RV and NGF both.

post exposed RV+NGF). Significant genes that were identified from t-test in both the groups are used further for Significant Analysis of Microarray (SAM test).

SAM is a powerful method used to identify differentially expressed genes between different samples. SAM provides an interactive method to visualize gene expression in different samples[41]. SAM analysis identifies the regulation of genes among significantly expressed genes[42]. Figure 2 shows the hierarchy of steps used to identify gene expression and gene regulation in both the groups. SAM test identifies number of positive significant genes and negative significant genes. K-means clustering is done on group 1, significant genes to cluster coexpressed genes and genes that have similar gene expression profile across all sample probes. Clustering is done only on group1 significant genes as number of significant gene is 69 as compared to only 3 genes in group 2. SAM test and clustering is done using MeV software. Clustering analysis identifies genes that are coexpressed and may belong to common pathways related with neurogenesis like differentiation or regeneration.

3. Results

3.1 T-Test

T-test is done for both the groups as mentioned earlier in methodology section. Table 1 shows the t-test between two groups and number of significant and non–significant genes found. Group 1, t-test shows that 69 genes are significantly expressed in presence of Resveratrol and group 2, t-test result shows that only 3 genes are significantly expressed in presence of NGF. T-test result between two groups shows that Resveratrol enhances the effect of NGF in neuronal development cells, since more number of genes are significantly expressed in presence of RV. List of significant gene were clustered in one group and saved as significant genes group1 and significant genes group 2.

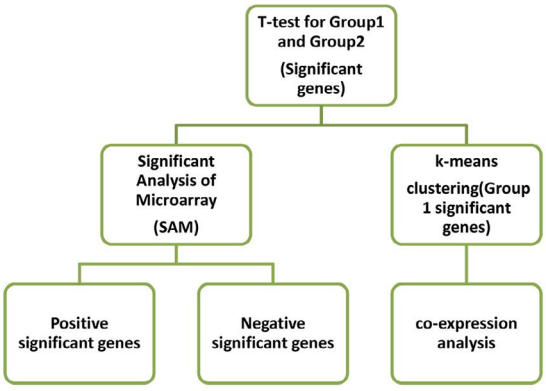


Figure 2. Protocol and steps used for microarray data analysis.These steps are used in both the groups to identify significant genesand gene regulation in different samples.

3.2 SAM Analysis of Significant Genes Identified from Group 1

SAM analysis is done on significant genes obtained from t-test in both the groups. Figure 3 shows the SAM result of group 1. In SAM graph red color shows genes that are positive significant and green color shows genes that are negative significant. SAM result of group1 shows that out of 69 significant genes 50 genes are positive significant and 19 genes are negative significant. Figure 4 shows a) positive significant genes and b) negative significant genes. These genes are annotated and functional study is done to identify crucial genes that are involved in neurodevelopment repairing mechanism and neuronal disease. Functional annotation of positive significant genes and negative significant genes are shown in Table 2 and Table 3.

Functional annotation of Affymetrix id is done using DAVID tool to identify genes that are involved in neuronal disease. Out of 50 positive significant genes, 12 genes are found that are associated with different diseases. Four genes are associated with neuronal diseases like mental retardation, Zellweger spectrum disorder, deafness etc.

Affymetrix id 11756434_a_at, DNA methyltransferase 1(DNMT1)[43,44] is expressed in diseases like Cerebellar ataxia, deafness, and narcolepsy; 11731738_a_at,post-

Table 1. Comparison statistics between samples and identification of significant genes

	T.test between samples		Significant genes	Non-significant genes
Group 1	RV, Pre exposed RV, post exposed RV	RV+NGF, pre exposed RV+NGF, post exposed RV+NGF	69	31
Group 2	NGF, Pre exposed NGF, post exposed NGF	RV+NGF, pre exposed RV+NGF, post exposed RV+NGF	3	97

Table 2. Functional annotation of genes that are positive significant in Group 1

S.No	Affymetrix Id	Gene Symbol	Protein_Acc	Gene Title	Fold Change (Unlogged)
1	11756434_a_at	DNMT1	NP_001124295	DNA (cytosine-5-)-methyltransferase 1	1.09
2	11733984_at	SLC5A8	NP_666018	solute carrier family 5 (iodide transporter), member 8	1.07
3	11759670_a_at	CEP57	NP_055494	centrosomal protein 57kDa	1.14
4	11754464_x_at	GIT1	NP_001078923	G protein-coupled receptor kinase interacting ArfGAP 1	1.06
5	11735673_a_at	ARL13A	NP_001013008	ADP-ribosylation factor-like 13A	1.08
6	11722371_x_at	TRIM22	NP_006065	tripartite motif-containing 22	1.09
7	11722183_s_at	ATP6V1C1	NP_001686	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1	1.1
8	11729576_a_at	TM9SF4	NP_055557	transmembrane 9 superfamily protein member 4	1.1
9	11758896_s_at	CCNI	NP_006826	cyclin I	1.22
10	11748532_a_at	KIAA0174	NP_055576	KIAA0174	1.09
11	11719318_a_at	GOLGA4	NP_001166184	golgin A4	1.17
12	11734263_at	ZNF780A	NP_001010880	zinc finger protein 780A	1.19
13	11730953_a_at	PEX26	NP_001121121	peroxisomal biogenesis factor 26	1.13
14	11720647_a_at	BUB1B	NP_001202	budding uninhibited by benzimidazoles 1 homolog beta (yeast)	1.29
15	11750466_a_at	SLC41A3	NP_001008485	solute carrier family 41, member 3	1.12
16	11750418_at	UPF0639	NP_001154970	UPF0639 protein	1.2
17	11723300_at	RPS6KB1	NP_003152	ribosomal protein S6 kinase, 70kDa, polypeptide 1	1.39
18	11740417_a_at	PLA2R1	NP_001007268	phospholipase A2 receptor 1, 180kDa	1.18
19	11732720_a_at	EREG	NP_001423	Epiregulin	1.18
20	11749225_a_at	BTN2A2	NP_001184166	butyrophilin, subfamily 2, member A2	1.67
21	11751723_a_at	KRT5	NP_000415	keratin 5	1.15

S.No	Affymetrix Id	Gene Symbol	Protein_Acc	Gene Title	Fold Change (Unlogged)
22	11722235_a_at	FBXO2	NP_036300	F-box protein 2	1.14
23	11741745_a_at	ABL2	NP_001129472	v-abl Abelson murine leukemia viral oncogene homolog 2	1.12
24	11760853_a_at	ZCCHC7	NP_115602	zinc finger, CCHC domain containing 7	1.15
25	11735242_s_at	ZC3H12C	NP_203748	zinc finger CCCH-type containing 12C	1.19
26	11731738_a_at	PGAP1	NP_079265	post-GPI attachment to proteins 1	1.14
27	11756733_s_at	CTNNA1 /// LRRTM2	NP_001894	catenin (cadherin-associated protein), alpha 1, 102kDa /// leucine rich repeat transmembrane neuronal 2	1.14
28	11763414_at	STBD1	NP_003934	starch binding domain 1	1.14
29	11737063_a_at	MTL5	NP_001034745	metallothionein-like 5, testis-specific (tesmin)	1.37
30	11718195_a_at	ATP6V0D1	NP_004682	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	1.24
31	11752458_a_at	PKM2	NP_002645	pyruvate kinase, muscle	1.25
32	11725304_s_at	RAC1	NP_008839	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	1.17
33	11730699_at	PLA2G10	NP_003552	phospholipase A2, group X	1.27
34	11724457_at	HCN4	NP_005468	hyperpolarization activated cyclic nucleotide-gated potassium channel 4	1.12
35	11759725_a_at	RDX	NP_002897	Radixin	1.04
36	11743772_a_at	LZTFL1	NP_065080	leucine zipper transcription factor-like 1	1.16
37	11717845_a_at	CDK7	NP_001790	cyclin-dependent kinase 7	1.18
38	11740686_x_at	NAPEPLD	NP_001116310	N-acyl phosphatidylethanolamine phospholipase D	1.2
39	11751718_a_at	C22orf24	NP_056187	chromosome 22 open reading frame 24	1.1
40	11716797_s_at	LBR	NP_002287	lamin B receptor	1.21
41	11762262_a_at	NUB1	NP_057202	negative regulator of ubiquitin-like proteins 1	1.4
42	11726612_a_at	SLC22A6	NP_004781	solute carrier family 22 (organic anion transporter), member 6	1.16

S.No	Affymetrix Id	Gene Symbol	Protein_Acc	Gene Title	Fold Change (Unlogged)
43	11726084_a_at	FAM69B	NP_689634	family with sequence similarity 69, member B	1.16
44	11755970_a_at	CHRNB3	NP_000740	cholinergic receptor, nicotinic, beta 3	1.12
45	ERCC-00018-01_at	NA	NA	NA	1.1
46	11753754_x_at	SNCG	NP_003078	synuclein, gamma (breast cancer-specific protein 1)	1.19
47	11763168_s_at	ALG13	NP_001034299	asparagine-linked glycosylation 13 homolog (S. cerevisiae)	1.37
48	11732343_at	ZNF33A	NP_008885	zinc finger protein 33A	1.19
49	11735250_a_at	HECW1	NP_055867	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	1.14
50	11753613_x_at	LCN10	NP_001001712	lipocalin 10	1.18

Table 3. Functional annotation of 19 genes that are negative significant in Group 1.

S.No	Affymetrix Id	Gene Symbol	Protein_Acc	Gene Title	Fold Change (Unlogged)
1	11758098_x_at	TPT1	NP_003286	tumor protein, translationally-controlled 1	0.883134
2	11746973_x_at	POR	NP_000932	P450 (cytochrome) oxidoreductase	0.81675
3	11723729_at	KREMEN1	NP_001034659	kringle containing transmembrane protein 1	0.780217
4	11731658_at	TRAM1L1	NP_689615	translocation associated membrane protein 1-like 1	0.828032
5	11749401_a_at	TRIT1	NP_060116	tRNA isopentenyltransferase 1	0.824632
6	11726032_a_at	CSNK1G3	NP_001026982	casein kinase 1, gamma 3	0.867835
7	11746854_a_at	SPINT1	NP_001027539	serine peptidase inhibitor, Kunitz type 1	0.840898
8	11718198_at	LHFP	NP_005771	lipoma HMGIC fusion partner	0.805235
9	11749703_a_at	SIRT1	NP_001135970	sirtuin 1	0.802939
10	11722237_at	CNO	NP_060836	cappuccino homolog (mouse)	0.890326
11	11733164_a_at	TTC29	NP_114162	tetratricopeptide repeat domain 29	0.823783
12	11719558_at	SYS1	NP_001093261	SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)	0.84371
13	11725888_at	C19orf59	NP_777578	chromosome 19 open reading frame 59	0.808693
14	11735477_a_at	CHST6	NP_067628	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6	0.842747
15	11735662_a_at	P2RY2	NP_002555	purinergic receptor P2Y, G-protein coupled, 2	0.863142
16	11721972_at	PHF16	NP_001070913	PHD finger protein 16	0.871246
17	11762693_at	NA	NA	NA	0.832408
18	11747631_x_at	XAF1	NP_059993	XIAP associated factor 1	0.848728
19	11757127_at	SNORA23	NA	small nucleolar RNA, H/ACA box 23	0.794084

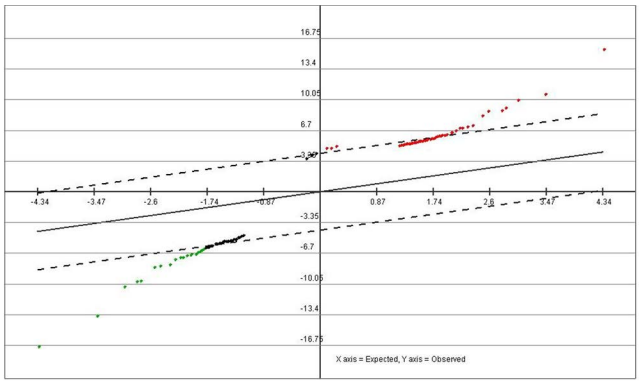


Figure 3. SAM test to identify positive and negative regulation of genes in group 1 significant genes.

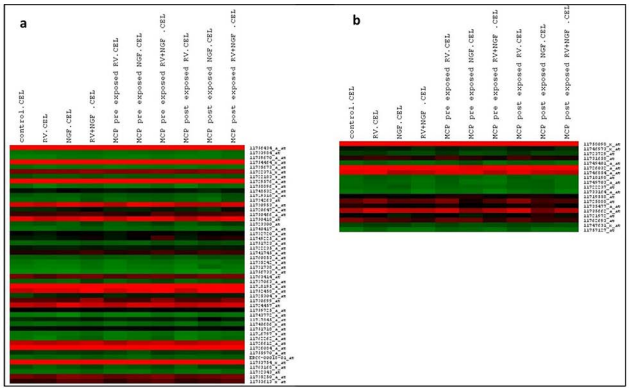


Figure 4. SAM result of group 1 significant genes. **(a)** Positive significant genes**. (b)** Negative significant genes.

GPI attachment to proteins 1(PGAP1)[45] is associated with Mental retardation;11759725_a_at, radixin (RDX) [46]involved in Deafness; 11730953_a_at, peroxisomal biogenesis factor 26(PEX26)[47] is expressed in Peroxisome biogenesis disorder 7A (Zellweger).

Some genes are found to be associated with various class of cancer, affymetrix id11741745_a_at, ABL proto-oncogene 2; is expressed in non-receptor tyrosine kinase (ABL2)[48] Leukemia, acute myeloid and eosinophilia; 11720647_a_at, BUB1 mitotic checkpoint serine/threonine kinase B(BUB1B)[49] is involved in Colorectal cancer; 11763168_s_at ALG13, UDP-N-Acetylglucosaminyltransferase subunit (ALG13)[50]is highly expressed in Congenital disorder of glycosylation.

Other genes with Affymetrix ids 11759670_a_at, centrosomal protein 57(CEP57)[51]; 11724457_at hyperpolarization activated cyclic nucleotide gated potassium channel 4(HCN4)[52];11751723_a_at keratin 5(KRT5) [52]; 11716797_s_at, Lamin B Receptor (LBR)[53]; 11743772_a_at, Leucine Zipper Transcription Factor Like 1(LZTFL1)[54]; are expressed in different syndromes like Mosaic variegated aneuploidy syndrome 2, Sick sinus syndrome 2, Brugada syndrome 8, Epidermolysis bullosa simplex, Dowling-Meara type Pelger-Huet anomaly, Greenberg skeletal dysplasia, Reynolds syndrome, and Bardet-Biedl syndrome resp.

Result shows that these genes are expressed in neuronal cells when exposed to MCP. RV has significant effect on these genes, as SAM result suggests that these genes are positively significant when MSC are co-exposed to RV and NGF. Fold change values of these genes ranges from 1.09-1.2 this suggest that genes expression has been significantly suppressed when MSC are treated with both RV and NGF. Further these genes are clustered to identify other genes that are co-expressed with neuronal disease genes, and functional analysis of clustered genes is done. Clustering result is discussed further in this paper.

Functional study of negative significant genes shows that two genes, 11735477_a_at, carbohydrate sulfotransferase 6(CHST6)[55]is expressed in Macular corneal dystrophy; and affymetrix id 11746973_x_at, cytochrome p450 oxidoreductase (POR)[56], is expressed in Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis. SAM analysis shows that these genes are significantly down regulated when MSC are coexposed with RV and NGF. FC value of these genes is 0.8 which shows that these genes are significantly down regulated due to RV and NGF.

3.3 SAM Analysis of Significant Genes Identified from Group 2

To identify genes that are regulated by NGF significant analysis is done for group 2. SAM test is also done on significant genes obtained from group2 t-test. Total 3 genes were identified that are significant .SAM result shows that 2 genes are positive significant and 1 gene is negative significant. SAM graph (Figure 5) shows number of positive (red) and negative significant genes (green). Affymetrix ids of positive and negative significant genes (Figure 6). Functional annotation Affymetrix id is done to study function of these genes. Table 4 shows detail of affymetrix id and genes that are positive significant and Table 5 shows detail of genes that are negative significant.

Two genes ZNF33A[57] and PLA2G10[58] with affymetrix id 11732343_at and 11730699_at, are-identified that are positive significant when MSC are

exposed to NGF. ZNF33A gene is associated with autosomal recessive disease (Xeroderma pigmentosum) and PLA2G10 is found to be over expressed in asthma, adenocarcinoma, and cancer. Comparative analysis of positive significant genes of group 1 and group 2 verifies that RV and NGF have synergistic effect on regulating gene expression since more number of genes are expressed when MSC are coexposed with RV and NGF both. 50 genes shows alteration in gene expression when MSC are treated with RV and NGF both but only 2 genes shows regulation MSCare exposed with NGF alone. This proves that RV and NGF have combined effect on repairing damaged neuronal cells during neuronal development.

Only one gene i.e., tetratricopeptide repeat domain 29(TTC29)[59]with affymetrix id 11733164_a_at; shows negative regulation when MSC are exposed to NGF. Tetratricopeptide repeat domain 29(TTC29) is associated with diseases like Coronary Artery Disease, cardiovascular immune and metabolic disorders. Comparative study of group 2 negative significant genes with group1 negative significant genes substantiates that RV have synergistic effect with NGF.

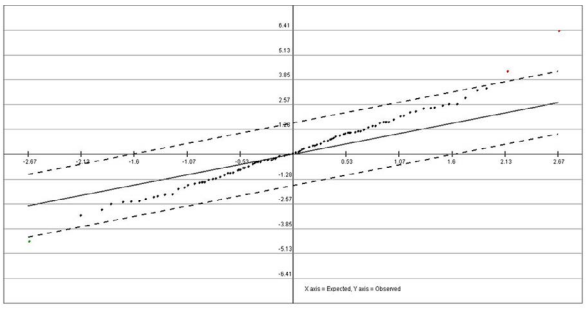


Figure 5. SAM test to identify positive and negative regulation of genes in group 2 significant genes.

3.4 Clustering Analysis

After SAM analysis and identification of genes that are important in neurodevelopment, clustering of 50 positive significant genes identified from SAM test of Group1 is done to identify genes that are closely related to one another and have same biological function. Clustering analysis also reveals information about genes that are co-expressed and belong to same biological pathway'. K-mean cluttering is done to group 50 positive significant genes.10 clusters were formed and these clusters are shown in Figure 7 (C1-C10). For easy representation of clusters and clustering analysis, clusters are labeled as C1-C10.

Clustering analysis is done to identify genes that are associated with affymetrix id 11756434_a_at, DNA methyltransferase 1(DNMT1); 11731738_a_at, post-GPI attachment to proteins 1(PGAP1); 11759725_a_at, radixin (RDX); and 11730953_a_at, peroxisomal biogenesis factor 26(PEX26), are studied to identify genes that are co-expressed with above genes and belongs to same cluster.

Genes that are co-expressed with DNA methyltransferase 1(DNMT1) are grouped in cluster 9 (C9). Researchers have shown that mutation or aberrant expression of DNMT1 gene is associated with developmental abnormalities, cerebellar ataxia, and narcolepsyetc. Clusterdetail of DNMT1 gene Figure 7 (C9). Functional study of cluster C9 affymetrix ids shows genes that are coexpressed with DNMT1 gene. 5 genes are clustered with DNMT1 gene, these genes are11718195_a_at, ATPase H+ transporting V0 subunit d1 (ATP6V0D1); 11723300_at, ribosomal protein S6 kinase B1 (RPS6KB1); 11737063_a_at, testis expressed metallothionein like protein (TESMIN); and 11722371_x_at, tripartite motif containing 22(TRIM22)[60]. These genes are involved in cell growth, proliferation, and protein synthesis. This suggests that RV enhances expression of these genes

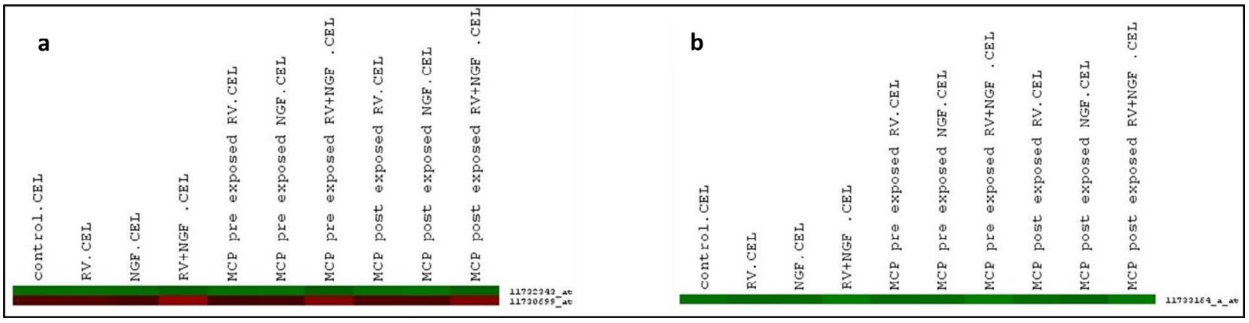


Figure 6. SAM result of group 2 significant genes. **(a)** Positive significant genes. **(b)** Negative significant genes.

Table 4. Functional annotation of genes that are positive significant in Group 2

S.No	Affymetrix Id	Gene Symbol	Protein_Acc	Gene Title	Fold Change(Unlogged)
1	11732343_at	ZNF33A	NP_008885	zinc finger protein 33A	1.19089
2	11730699_at	PLA2G10	NP_003552	phospholipase A2, group X	1.293834

Table 5. Functional annotation of genes that are negative significant in Group 2

S.No	Affymetrix Id	Gene Symbol	Protein_Acc	Gene Title	Fold Change(Unlogged)
1	11733164_a_at	TTC29	NP_114162	tetratricopeptide repeat domain 29	0.811899

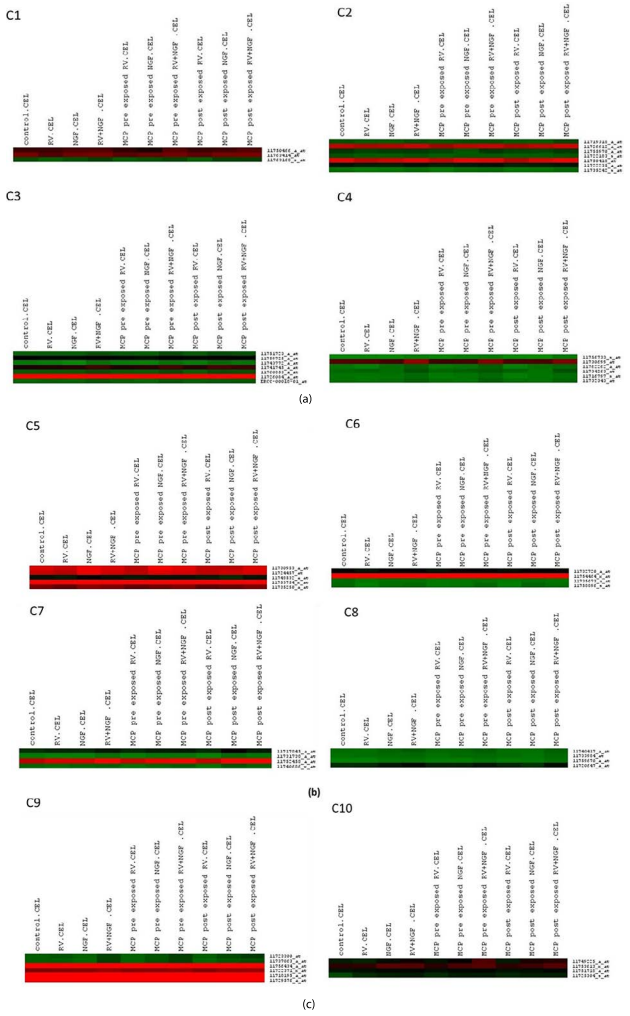


Figure 7. Clustering result of group 1 significant genes, ten clusters were labeled as C1-C10.

that have a function in repair mechanism of damaged neuronal genes.

Genes that are clustered with post-GPI attachment to proteins 1(PGAP1), 11731738_a_at, Figure 7 (C7). Defects in expression of PGAP1 gene is associated with mental retardation and autosomal disorder[61]. Functional study of cluster 7 genes, shows important genes that have function in neuronal signaling .Cluster 7 includes genes,11740686_x_at, N-acyl phosphatidylethanolamine phospholipase D(NAPEPLD); 11717845_a_at, cyclin dependent kinase 7(CDK7); 11752458_a_at, Pyruvate Kinase Muscle (PKM). These genes are involved in biosynthesis, cell cycle progression, transcription initiation, and glycolysis[62].

Genes that are coexpressed with radixin (RDX), 11759725_a_at are clustered in C3 Figure 7 (C3); C3. Genes that has similar gene expression profile with RDX gene is 11741745_a_at, ABL proto-oncogene 2 (ABL2); 11726084_a_at, family with sequence similarity 69 member B (FAM69B); 11751723_a_at, keratin 5(KRT5); and 11743772_a_at, Leucine Zipper Transcription Factor Like 1(LZTFL1). These genes have function in tumor formation (ABL2)[63], Bardet-Biedl Syndrome and tumor suppressor (LZTFL1)[64].

Genes having similar gene expression with 11730953_a_at, peroxisomal biogenesis factor 26(PEX26); Figure 7 (C5). PEX26 is important gene it have role in proper functioning of peroxisomes, mutation or loss of expression of this gene causes peroxisome biogenesis disorder (PBD) like Zellweger Syndrome (ZWS), Neonatal Adrenoleukodystrophy (NALD), Infantile Refsum Disease (IRD), and classical Rhizomelic Chondrodysplasia Punctata (RCDP). Genes that are coexpressed with PEX26 genes are 11748532_a_at, ESCRT-III associated factor (IST1)[65]; 11724457_at, hyperpolarization

activated cyclic nucleotide gated potassium channel 4(HCN4); and 11753754_x_at, synuclein gamma (SNCG)[66]. These genes have function in vesicle budding, sinus syndrome, neurodegenerative diseases and breast cancer.

4. Discussion

Research in neurodevelopmental disorder is very important to understand the process and mechanism of diseases at its very initial phase of embryo development. Identification of genes that are expressed in fetal stage of neuronal diseases provides hope for proper medication and treatment of neurodevelopmental disorder. Different experiments have been done to identify genes and establish gene network, in relation with neurological diseases. Microarray is a powerful experiment to understand the mechanism of neuronal diseases, to identify genes that are expressed or co-expressed during neuronal disorders. NGF and RV are highly studied molecules that have a function in repairing damaged neuronal cells to during neuronal development. Here we have studied effect of RV and NGF when both molecules are co-exposed to damaged neuronal cells (MSC induced MCP). T-test statistics between different samples shows that Resveratrol enhances the effect of NGF in neuronal development cells, since more number of genes are significantly expressed in presence of RV and NGF both , as compared to exposure of NGF alone. SAM analysis predicts important genes that have function in neuronal diseases.

Cluster analysis identifies important genes that are associated DNMT1, PGAP1, RDX and PEX26 genes, these genes have function in different class of neuronal diseases. Key genes that are predicted on the basis of clustering are ATP6V0D1, TESMIN, TRIM22, NAPEPLD, CDK7, PKM, KRT5, ABL2, LZTFL1, IST1, HCN4 and SNCG, these genes have a function in cell growth, cell proliferation ,protein synthesis, cell cycle regulation, and cell signaling. These genes are co-clustered with genes responsible for neuronal diseasesit signifies that these genes are also involved in neuronal diseases. Results shows that RV along with NGF enhances the expression of genes that have function in neuronal cell regeneration and differentiation, thus maintaining the proper functioning of neurons.

5. Conclusion

Major finding of our study is identification of genes that are expressed when MSCs are exposed to MCP and neuroprotection roles of RV and NGF. Result shows that DNMT1 (Cerebellar ataxia, deafness, and narcolepsy), PGAP1 is associated with Mental retardation, radixin (RDX) involved in Deafness, peroxisomal biogenesis factor 26 (PEX26) is expressed in Peroxisome biogenesis disorder 7A (Zellweger)[41–47] are expressed when MSC are exposed with MCP. Some genes are found to be associated with various class of cancer, ABL proto-oncogene 2 is expressed in Leukemia, acute myeloid and eosinophilia[48]; BUB1 mitotic checkpoint serine/threonine kinase B (BUB1B) is involved in Colorectal cancer[49]; ALG13 is highly expressed in Congenital disorder of glycosylation, carbohydrate sulfotransferase 6 (CHST6) is expressed in Macular corneal dystrophy; and cytochrome p450 oxidoreductase (POR), is expressed in Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis[50]. Clustering analysis reveals genes that are highly expressed in presence of RV and NGF exposed samples. In this paper we have identified genes that have a function in cell growth, cell proliferation, protein synthesis, cell cycle regulation, and cell signaling. Results show that RV along with NGF enhances the expression of genes. These genes have function in neuronal cell regeneration and differentiation, thus maintaining the proper functioning of neurons. Present work identifies genes that are expressed in neurodevelopmental disorder and genes that have repairing action in damaged neuronal cells. This study also proves synergistic effect of RV and NGF as therapeutic molecule against neurodevelopment disorders.

6. Conflict of Interest

Author declares no conflict of interest

7. References

1. Levi-Montalcini R. The nerve growth factor 35 years later. Science. 1987; 237: 1154–62. https://doi.org/10.1126/science.3306916 PMid:3306916
2. Schmidt CE, Leach JB. Neural tissue engineering: strategies for repair and regeneration. Annual Review of Biomedical

Engineering. 2003; 5: 293–347. https://doi.org/10.1146/annurev.bioeng.5.011303.120731 PMid:14527315
3. Bradshaw RA. Nerve growth factor. Annual Review of Biochemistry. 1978; 47: 191–216. https://doi.org/10.1146/annurev.bi.47.070178.001203 PMid:79332
4. Sofroniew MV, Charles LH, William CM. Nerve growth factor signaling, neuroprotection, and neural repair. Annual Review of Neuroscience. 2001; 24: 1217–81. https://doi.org/10.1146/annurev.neuro.24.1.1217 PMid:11520933
5. Shigeno T, et al. Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. Journal of Neuroscience. 1991; 11(9): 2914–9. https://doi.org/10.1523/JNEUROSCI.11-09-02914.1991 PMid:1880556
6. Pfister LA, et al. Nerve conduits and growth factor delivery in peripheral nerve repair. Journal of the Peripheral Nervous System. 2007; 12(2): 65–82. https://doi.org/10.1111/j.1529-8027.2007.00125.x PMid:17565531
7. Rich KM, et al. Nerve growth factor enhances regeneration through silicone chambers. Experimental Neurology. 1989; 105(2): 162–70. https://doi.org/10.1016/0014-4886(89)90115-5
8. Schwab ME. Repairing the injured spinal cord. Science. 2002; 295(5557): 1029–31. https://doi.org/10.1126/science.1067840 PMid:11834824
9. Fawcett JW, Richard AA. The glial scar and central nervous system repair. Brain Research Bulletin. 1999; 49: 377–91. https://doi.org/10.1016/S0361-9230(99)00072-6
10. Hefti F, William JW. Nerve growth factor and Alzheimer's disease. Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society. 1986; 20: 275–81. https://doi.org/10.1002/ana.410200302 PMid:3532929
11. Fahnestock M, et al. The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Molecular and Cellular Neuroscience. 2001; 18: 210–20. https://doi.org/10.1006/mcne.2001.1016 PMid:11520181
12. Siemann EH, Creasy LL. Concentration of the phytoalexin resveratrol in wine. American Journal of Enology and Viticulture. 1992; 43: 49–52.
13. Baur JA, David AS. Therapeutic potential of resveratrol: the in vivo evidence. Nature Reviews Drug discovery. 2006; 5: 493. https://doi.org/10.1038/nrd2060 PMid:16732220
14. Robb EL, Jeffrey AS. Trans-resveratrol as a neuroprotectant. Molecules. 2010; 15: 1196–212. https://doi.org/10.3390/molecules15031196 PMid:20335973 PMCid:PMC6257315
15. Granzotto A, Zatta P. Resveratrol and Alzheimer's disease: message in a bottle on red wine and cognition. Frontiers in Aging Neuroscience. 2014; 6: 95. https://doi.org/10.3389/fnagi.2014.00095 PMid:24860502 PMCid:PMC4030174
16. Ding X-Z, Thomas EA. Resveratrol inhibits proliferation and induces apoptosis in human pancreatic cancer cells. Pancreas. 2002; 25: 71–6. https://doi.org/10.1097/00006676-200211000-00024
17. Reddy NCS, Karnati PR. Protective effect of resveratrol against neuronal damage through oxidative stress in cerebral hemisphere of aluminum and fluoride treated rats. Interdisciplinary Toxicology. 2016; 9: 78–82. https://doi.org/10.1515/intox-2016-0009 PMid:28652849 PMCid:PMC5458107
18. Baxter RA. Anti-aging properties of resveratrol: review and report of a potent new antioxidant skin care formulation. Journal of Cosmetic dermatology. 2008; 7: 2–7. https://doi.org/10.1111/j.1473-2165.2008.00354.x PMid:18254804
19. Zhang L-N, et al. Neuroprotective effect of resveratrol against glutamate-induced excitotoxicity. Adv Clin Exp Med. 2015; 24: 161–5.https://doi.org/10.17219/acem/38144 PMid:25923101
20. Meftahi G, et al. Suppressive effects of resveratrol treatment on the intrinsic evoked excitability of CA1 pyramidal neurons. Cell Journal. 2015; 17: 532. PMid:26464825 PMCid:PMC4601874
21. Saleh, MC, et al. Co-administration of resveratrol and lipoic acid, or their synthetic combination, enhances neuroprotection in a rat model of ischemia/reperfusion. PloS One. 2014; 9: 87865.https://doi.org/10.1371/journal.pone.0087865 PMid:24498217 PMCid:PMC3909267
22. Frémont L. Biological effects of resveratrol. Life Sciences. 2000; 66: 663–73. https://doi.org/10.1016/S0024-3205(99)00410-5
23. Siemann EH, Creasy LL. Concentration of the phytoalexin resveratrol in wine. American Journal of Enology and Viticulture. 1992; 43: 49–52.
24. Mirnics K, et al. Analysis of complex brain disorders with gene expression microarrays: Schizophrenia as a disease of the synapse.Trends in Neurosciences. 2001; 24: 479–86. https://doi.org/10.1016/S0166-2236(00)01862-2
25. Mirnics K, Pevsner J. Progress in the use of microarray technology to study the neurobiology of disease. Nature Neuroscience. 2004; 7: 434. https://doi.org/10.1038/nn1230 PMid:15114354
26. Boccuto L, et al. Decreased tryptophan metabolism in patients with autism spectrum disorders. Molecular Autism. 2013; 4: 16. https://doi.org/10.1186/2040-2392-4-16 PMid:23731516 PMCid:PMC3680090
27. Brazma A, et al. Array Express- a public repository for microarray gene expression data at the EBI. Nucleic Acids Research. 2003; 31: 68–71. https://doi.org/10.1093/nar/gkg091 PMid:12519949 PMCid:PMC165538
28. Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Research. 2002; 30: 207–10.

https://doi.org/10.1093/nar/30.1.207PMid:11752295 PMCid:PMC99122
29. Chahrour M, et al. MeCP2, a key contributor to neurological disease, activates and represses transcription. Science. 2008; 320: 1224–9. https://doi.org/10.1126/science.1153252 PMid:18511691 PMCid:PMC2443785
30. Ishigaki S, et al. Differentially expressed genes in sporadic amyotrophic lateral sclerosis spinal cords–screening by molecular indexing and subsequent cDNA microarray analysis. FEBS Letters. 2002; 531: 354–8. https://doi.org/10.1016/S0014-5793(02)03546-9
31. Mullighan CG. The molecular genetic makeup of acute lymphoblastic leukemia. ASH Education Program Book. 2012; 2012: 389–96.
32. Freeman T. High throughput gene expression screening: its emerging role in drug discovery. Medicinal Research Reviews. 2000; 20: 197–202. https://doi.org/10.1002/(SICI)1098-1128(200005)20:3<197::AID-MED3>3.0.CO;2-1
33. Yadav R, Srivastava P. Clustering, pathway enrichment, and protein-protein interaction analysis of gene expression in neurodevelopmental disorders. Advances in Pharmacological Sciences. 2018; 2018.
34. Smyth GK, Yang YH, Speed T. Statistical issues in cDNA microarray data analysis. Functional Genomics. 2003; 224: 111036. https://doi.org/10.1385/1-59259-364-X:111 PMid:12710670
35. Durinck S, et al. Bio Mart and Bioconductor: A powerful link between biological databases and microarray data analysis. Bioinformatics. 2005; 21: 3439–40. https://doi.org/10.1093/bioinformatics/bti525 PMid:16082012
36. Saeed AI, et al. TM4 microarray software suite. Methods in Enzymology. 2006; 411: 134–93. https://doi.org/10.1016/S0076-6879(06)11009-5
37. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009; 37: 13. https://doi.org/10.1093/nar/gkn923PMid:19033363 PMCid:PMC2615629
38. Baldi P, Long AD. A Bayesian framework for the analysis of microarray expression data: Regularized t-test and statistical inferences of gene changes. Bioinformatics. 2001; 17: 509–19.https://doi.org/10.1093/bioinformatics/17.6.509 PMid:11395427
39. Dinu I, et al. Improving gene set analysis of microarray data by SAM-GS. BMC Bioinformatics. 2007; 8: 242. https://doi.org/10.1186/1471-2105-8-242 PMid:17612399 PMCid:PMC1931607
40. Cheadle C, et al. Analysis of microarray data using Z score transformation. The Journal of Molecular Diagnostics. 2003; 5: 73–81. https://doi.org/10.1016/S1525-1578(10)60455-2
41. Cahan P, et al. Meta-analysis of microarray results: challenges, opportunities, and recommendations for standardization. Gene. 2007; 401: 12–8. https://doi.org/10.1016/j.gene.2007.06.016 PMid:17651921 PMCid:PMC2111172
42. Butte A. The use and analysis of microarray data. Nature Reviews Drug discovery. 2002; 1: 951. https://doi.org/10.1038/nrd961 PMid:12461517
43. Feng J, et al. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. Nature Neuroscience. 2010; 13: 423. https://doi.org/10.1038/nn.2514 PMid:20228804 PMCid:PMC306077244. Liu L, et al. DNA methylation impacts on learning and memory in aging. Neurobiology of Aging. 2009; 30: 549–60. https://doi.org/10.1016/j.neurobiolaging.2007.07.020 PMid:17850924 PMCid:PMC2656583
45. Novarino G, et al. Exome sequencing links corticospinal motor neuron disease to common neurodegenerative disorders. Science. 2014; 343: 506–11. https://doi.org/10.1126/science.1247363 PMid:24482476 PMCid:PMC4157572
46. Benitez-King G, et al. The neuronal cytoskeleton as a potential therapeutical target in neurodegenerative diseases and schizophrenia. Current Drug Targets-CNS and Neurological Disorders. 2004; 3: 515–33. https://doi.org/10.2174/1568007043336761 PMid:15581421
47. Fujiki Y, Yagita Y, Matsuzaki T. Peroxisome biogenesis disorders: Molecular basis for impaired peroxisomal membrane assembly: in metabolic functions and biogenesis of peroxisomes in health and disease. Biochimicaet Biophysica Acta (BBA)-Molecular Basis of Disease. 2012; 1822: 42.
48. Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nature Medicine. 1997; 3: 614. https://doi.org/10.1038/nm0897-934 PMid:9176486
49. Myrie KA, et al. Mutation and expression analysis of human BUB1 and BUB1B in aneuploid breast cancer cell lines. Cancer Letters. 2000; 152: 193–9. https://doi.org/10.1016/S0304-3835(00)00340-2
50. Meech R, et al. A novel function for UDP Glycosyltransferase 8 (UGT8): Galactosidation of bile acids. Molecular Pharmacology. 2015; 87(3): 442–50. https://doi.org/10.1124/mol.114.093823 PMid:25519837
51. Cuevas R, et al. FGF-2 disrupts mitotic stability in prostate cancer through the intracellular trafficking protein CEP57. Cancer Research. 2013 Feb 15; 73(4): 1400–10. https://doi.org/10.1158/0008-5472.CAN-12-1857 PMid:23243019
52. Moosmang S, et al. Cellular expression and functional characterization of four hyperpolarization-activated pacemaker channels in cardiac and neuronal tissues. European Journal of Biochemistry. 2001; 268: 1646–52. https://doi.org/10.1046/j.1432-1327.2001.02036.x PMid:11248683
53. Hoffmann K et al. Mutations in the gene encoding the lamin B receptor produces an altered nuclear morphology in gran-

ulocytes (Pelger–Huet anomaly). Nature Genetics. 2002; 31: 410. https://doi.org/10.1038/ng925PMid:12118250
54. Marion V, et al. Exome sequencing identifies mutations in LZTFL1, a BBSome and smoothened trafficking regulator, in a family with Bardet–Biedl syndrome with situs inversus and insertional polydactyly. Journal of Medical Genetics. 2012; 49: 317–21. https://doi.org/10.1136/jmedgenet-2012-100737 PMid:22510444
55. Liu N-P, et al. Mutations in corneal carbohydrate sulfotransferase 6 gene (CHST6) cause macular corneal dystrophy in Iceland. 2000 Dec 13; 6: 261–4.
56. Flück CE, et al. Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. Nature Genetics. 2004 Mar; 36(3): 228–30. https://doi.org/10.1038/ng1300PMid:14758361
57. Tanaka K, et al. Analysis of a human DNA excision repair gene involved in group Axeroderma pigmentosum and containing a zinc-finger domain. Nature 1990; 348: 73. https://doi.org/10.1038/348073a0PMid:2234061
58. Hallstrand TS, et al. Secreted phospholipase A2 group X overexpression in asthma and bronchial hyperresponsiveness. American Journal of Respiratory and Critical Care Medicine. 2007; 176: 1072–8. https://doi.org/10.1164/rccm.200707-1088OC PMid:17901411 PMCid:PMC2176098
59. Zoncu R, et al. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H+-ATPase. Science. 2011; 334: 678–83. https://doi.org/10.1126/science.1207056 PMid:22053050 PMCid:PMC3211112
60. Yu S, et al. Identification of tripartite motif-containing 22 (TRIM22) as a novel NF-κB activator. Biochemical and Biophysical Research Communications. 2011; 410: 247–51. https://doi.org/10.1016/j.bbrc.2011.05.124 PMid:21651891
61. Okamoto Y, et al. Molecular characterization of a phospholipase D generating an andamide and its congeners. Journal of Biological Chemistry. 2004; 279: 5298–305. https://doi.org/10.1074/jbc.M306642200 PMid:14634025
62. Pendergast AM, et al. BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. Cell. 1993; 75: 175–85. https://doi.org/10.1016/S0092-8674(05)80094-7
63. Wei Q, et al. Tumor-suppressive functions of leucine zipper transcription factor–like 1. Cancer Research. 2010; 0008: 5472. https://doi.org/10.1158/0008-5472.CAN-09-3826
64. Bajorek, M, et al. Structural basis for ESCRT-III protein autoinhibition. Nature Structural and Molecular Biology. 2009; 16: 754.https://doi.org/10.1038/nsmb.1621PMid:19525971 PMCid:PMC2712734
65. Chen S, Wang J, Siegelbaum SA. Properties of hyperpolarization-activated pacemaker current defined by coassembly of HCN1 and HCN2 subunits and basal modulation by cyclic nucleotide. The Journal of General Physiology. 2001; 117: 491–504. https://doi.org/10.1085/jgp.117.5.491 PMid:11331358 PMCid:PMC2233656
66. Wu K, et al. Expression of neuronal protein synuclein gamma gene as a novel marker for breast cancer prognosis. Breast Cancer Research and Treatment. 2007; 101:259–67. https://doi.org/10.1007/s10549-006-9296-7 PMid:16821081