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In Silico Identification of MicroRNA Predicted to Regulate Brain-Derived Neurotropic Factor Functions in Type 2 Diabetic Retinopathy

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Abstract: ***Diabetic Retinopathy Type 2 (T2DR) is the major visually impaired disease in patients with Type 2 diabetes mellitus and the prominent cause of blindness. MicroRNAs are small non-coding molecules of RNA that play a main role in regulating gene expression and certain biological procedures. Studies indicate that dysregulation of micro RNAs can lead to multiple illnesses, including T2DR, as they play a vital role in controlling gene expression and thus are likely to be helpful biomarkers for disease identification. Thus, it can be said that identifying biomarkers such as microRNAs could be considered as a better approach for the early and specific identification of T2DR and would have excellent potential for both diagnostic and therapeutic reasons. The primary focus of this research is to find out the connection between hsa-miR-206 and its prospective target gene Brain Derived Neurotrophic Factor (BDNF) with regard to the disease Diabetic Retinopathy and to validate the sort of regulation that hsa-miR-206 has on the target gene BDNF using Bioinformatics. Before analyzing BDNF expression regulation by hsa-miR-206 in retina of patients with type 2 diabetic retinopathy, an approach in silico is quite necessary. For hsa-miR-206 target genes prediction and binding site analysis, publicly accessible target prediction software was used. Gene network enrichment assessment and functional annotation of hsa-miR-206 targeted genes that play a part in T2DR anticipated the linkage, path and functional resemblance between BDNF and other genes using browser-based software namely HMDD, STRING, and DAVID. Neurotrophin signalling pathway and Mitogen activated protein kinase (MAPK) pathway enrichment analysis to predict the changes occurring in the pathway when BDNF gets dysregulated, using KEGG Pathway database for the pathogenesis of Type 2 Diabetic Retinopathy. Neurotrophin pathway signaling and Mitogen -activated protein kinase (MAPK) pathway enhancing assessment, using KEGG pathway database for the pathogenesis of type 2 diabetic retinopathy, predict the changes in pathways that occur when BDNF is dysregulated. Based on the findings of bioinformatics, this research anticipated BDNF expression regulation using hsa-miR-206, which is considered to be one of the most promising miRNA for biomarker as well as therapeutic agent in the prevention and tratment of T2DR. Future research therefore includes validation of this forecast using molecular methods such as Quantitative Real-Time PCR, BDNF-ELISA assay, and Western blots.***

Keywords: Type 2 Diabetic retinopathy, hsa-miR-206, Brain-Derived Neurotrophic Factor, MAPK signaling pathway, in silico analysis, miRNA-mRNA interactions.

I. INTRODUCTION

Type 2 Diabetic retinopathy (T2DR) is the foremost cause of blindness in adults troubling over 90% patients with 20 years of diabetes. It accounts for 4.8 percent of the global 37 million instances of eye disease correlated with blindness. With the incidence of diabetes rising at a surprising pace, the amount of individuals with diabetic retinopathy is projected to expand from 126.6 million in 2010 to 191.0 million by 2030, which is an alarming figure itself. (Congdon et al., 2012) Diabetic Retinopathy is intended to result from microvascular modifications in the retinal circulation. Microvascular occlusion and dilation happen in the original phase, which converted into Proliferative Retinopathy, leading in the growth of abnormal fresh blood vessels. With each passing phase, the incidence of diabetes rises gradually. (Tu et al., 2017) Hyperglycemia and genetic predisposition are associated with multiple pathophysiological occurrences recognized in the development of diabetic retinopathy. (Cai and Boulton 2002)

Early depictions show intraretinal hemorrhages, vascular sheathing and lipid exudates throughout the retina. These findings were confirmed with histopathological specimens, such as the work of Arthur Ballantyne, who, in 1945, showed that capillary wall changes contributed to the development of DR. (Wolfensberger & Hamilton 2001) To date, numerous major mechanisms are alleged to induce retinal stress in DR, comprising the polyol pathway, non-enzymatic glycation, activation of protein kinase C (PKC), etc. all of which have been implicated in the expansion of microvascular damage and retinopathy.(Lorenzi 2007; Safi et al.

2014) Anti-VEGF therapy for diabetic macular edema has been shown in multiple randomized clinical trials to be more effective at improving vision than laser, and several cost-effectiveness analyses have confirmed the value of these treatments to patients and society.(Brown et al. 2015; Haig et al., 2016) Although intravitreal injection is an effective means of supplying the retina with anti-VEGF medicines. This is however an invasive procedure associated, for example, with endophthalmitis or retinal detachment that can be important for long term serial therapy patients. Furthermore, although anti-VEGF drugs supplied within the vitreous , this could lead to higher blood pressure, proteinuria, enhanced cardiac events and impaired cleansing of wounds. (Simo and Hernandez, 2008)

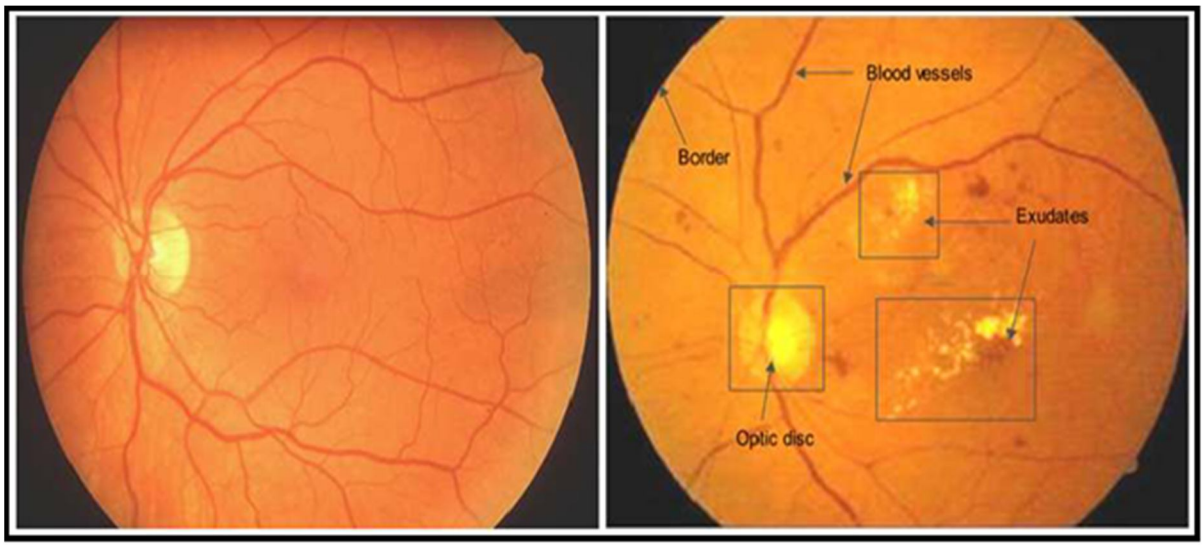

Figure.1: Sample retinal fundus images of Normal and Diabetic retinopathy subjects. This picture illustrates the difference between normal and T2DR where evidently the second eye displays signs of microaneurysms, lipid exudates and retinal degradation. (https://www.researchgate.net/publication/276528880)

Brain-derived neurotrophic factor (BDNF) is a protein that belongs to a family of growth factors, called neurotrophins, whose other mammalian members include nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4).Neurotrophins are basically involved in regulating the development, survival and functioning of neurons.(Skaper 2012) Pro-BDNF has a high affinity to p75NTR, and it stimulates neuronal apoptosis .(Friedman 2000; Lee et al. 2001) Mature BDNF is considered as the biologically active form, which has a high affinity to the TrkB receptor. It promotes development and differentiation of neurons, cell survival, and synaptic plasticity(Cowansage et al., 2010; Hofer and Barde 1988) BDNF promotes survival in injured RGCs induced by axotomy or retinal ischemia, and also promotes regeneration of the nerve fiber(Mey and Thanos 1993; Peinado et al. 1996) In addition, BDNF promotes the survival of retinal interneurons and is important for establishing phenotypes and synaptic connections in the developing retina.(Pinzon et al. 2004) BDNF has been reported to inhibit neuro-retinal cell death under conditions of ischemia and hypoxia, and to inhibit apoptosis in rat RGCs at early stages of T2DR. (Seigel et al., 2000) Other studies have reported that activation of the ERK/MAPK pathway leads to cell death and PI3K/PKB is the main pathway involved in the protection of neurons induced by BDNF. (Klöcker et al., 2000)

miRNAs are short, non-coding RNA molecules predicted to interact with the transcripts of about 60 % of all mammalian protein-coding genes . miRNAs bind their target mRNAs through a fully complementary seed sequence of 7–8 nucleotides in their 5′ end and less complementary area in the 3′ end, inducing translational repression and/or mRNA degradation .(Bartel 2004; Filipowicz et al., 2008) In general, miRNA sites near the ends of the 3′UTR are more effective than sites in the center of the 3′UTR, partly because regions in the middle of the 3′UTR are more likely to be incorporated into hairpin structures, hindering access to miRNA. (Grimson et al. 2007) A study conducted by Lee et al., in 2012 showed a clear link between miR-206 and BDNF in which miR-206 targeted the 3'UTR of BDNF mRNA that decreased the BDNF level in AD transgenic mouse. Importantly, from a therapeutic standpoint, inhibition of miR-206 resulted in increased levels of brain BDNF and improved memory performance in AD mice Furthermore, miR-206 targeted the 3'UTR of BDNF mRNA and decreased the BDNF level in AD transgenic mouse neurons, which is also consistent with the report that miR-206 targets BDNF transcripts.(Lee et al. 2012)

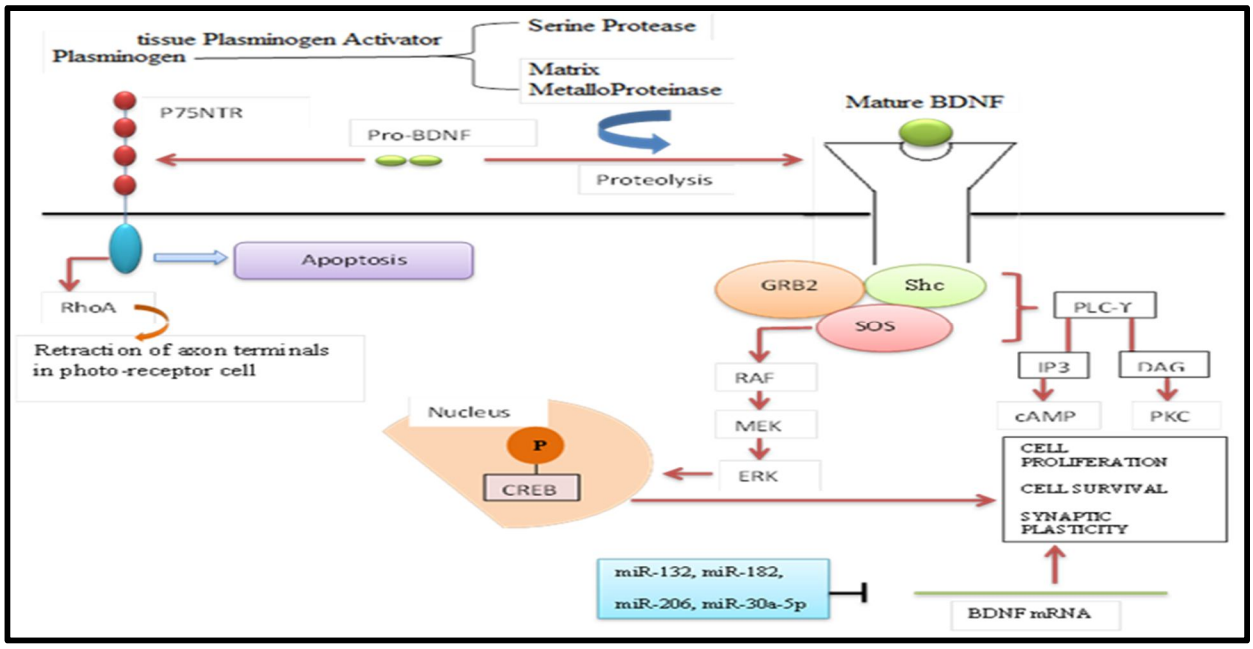

Figure. 2: Schematic representation of the various interaction involved in the regulation of brain-derived neurotrophic factor (BDNF) and miR-206, miR-132, miR-182, miR-30a-5p via TrkB/MAPK signaling pathway. Adapted from: https://link.springer.com/article/10.1007/s12017-016-8407-9

A key tool for organizing and analyzing the vast amount of data is the field of bioinformatics or system biology, that consists of combining the computer-based and the sciences of biology. (Lewis, 2008) In order to recognize significant trends and patterns that would eventually lead to a novel biomarker discovery both for diagnostic and therapeutic reasons, bioinformatics mainly aims to discover biological key information concealed among a mass of raw data. (Anthony, 2015) To date, no investigation has been carried out into the prospective impact of another miR-1/206 family member, miR-1, which differs from hsa-miR-206 by four nucleotides outside the seed region and has a separate pattern of speech from hsa-miR-206. (Varendi et al., 2014) There were not adequate information accessible about the impact of hsa-miR-206 on the target gene BDNF that could play an significant part in T2DR pathogenesis. Hence, before evaluating BDNF expression regulation for type 2 diabetic retinopathy patients, *in silico*-based approach is quite compulsory. Therefore, BDNF expression is anticipated in the present research with the assistance of bioinformatics software and databases owing to up-regulated hsa-miR-206. hsa-miR-206 target prediction was done using target prediction tools. Online bioinformatics instruments were used to analyze gene network enrichment, functional gene annotation and pathway evaluation. Thus, the regulation of BDNF expression by hsa-miR-206 for T2DR pathogenesis as a prognostic strategy was anticipated in the silico research.

II. MATERIALS AND METHODS

Computer with high speed internet access and various online bioinformatics tools were used.

A. BDNF sequence retrieval using NCBI

BDNF protein sequences in FASTA format of the organisms Homo sapiens, Gorilla gorilla, Pan troglodytes, Mus musculus, Rattus norvegicus, were retrieved from NCBI. A FASTA Formatted file was saved in notepad.

B. Target gene (BDNF) sequence homology using Clustal Omega

Clustal Omega was accessed using online link http://www.ebi.ac.uk/Tools/msa/clustalo/ .Home page of Clustal Omega appeared from the above link. The earlier saved Sequence file was then uploaded and used for multiple sequence alignment as input. To get the outcomes, the Submit button was pressed. Multiple sequence alignment and percentage identity index of BDNF protein sequences of distinct species was acquired to predict the resemblance of human BDNF protein sequence to other species. (Higgins and Seivers 2018)

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C. Literature Mining for Understanding the miRNA-disease Association using HMDD

HMDD V3.0 was accessed using online link http://www.cuilab.cn/hmdd. The miR-Target network option on the homepage of HMDD was then clicked. The search was made either by miRNA or disease. hsa-miR-206 was entered to find experimentally validated miRNA-Target gene network. Human microRNA Disease Database result presented more detailed annotations to the human miRNA-disease association data, including miRNA-disease association data from the evidence of miRNA-target interactions. (Cui et al., 2019)

D. miRNA sequence retrieval of hsa-miR-206 using miRBase.

MiRBase was accessed using home page link http://www.mirbase.org/index.shtml. On the rightmost side of miRBase home page, Search by miRNA name or keyword option was clicked. Mature sequence of hsa-miR-206 along with their Accession numbers and ID was retrieved by scrolling down the resulting page. (Jones et al., 2019)

E. miRNA Sequence Analysis Using NCBI-BLASTN.

BLAST page was opened by clicking BLAST on popular resources menu from National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). BLAST Nucleotide was selected to run from the Basic BLAST menu. hsa-miR-206 FASTA sequence was obtained from NCBI. The hsa-miR-206 FASTA formatted sequence was copied from the NCBI page and pasted to the wide text box underneath the Enter Query sequence in BLAST page. Other organisms such as Gorilla gorilla, Pan troglodytes were selected in the Organism choice to compare the sequences miR-206 with the sequence of human template. To submit the search, the BLAST button was pressed and the output was acquired. (Madden, 2003)

F. miRNA-target identification and binding site analysis using:

1) *TargetScan-7.2* : TargetScanHuman was accessed using online link http://www.targetscan.org/vert_72/ . The target gene BDNF was entered in the search box. Then, the option for selecting conserved miRNA families was clicked and predicted miRNA-gene interactions were obtained. (Varendi et al., 2014)
2) *miRDB:* miRDB database was accessed using online link http://mirdb.org/index.html . The search was made by entering the gene symbol i.e. BDNF . The database gave the predicted miRNAs with target score which could bind on target gene. (Wang and Liu 2019)
3) *miRabel:* miRabel miRNA target prediction tool was accessed using online link http://bioinfo.univ-rouen.fr/mirabel/. The search was made by entering Target gene BDNF and the results were obtained for both targeted pathways as well as miRNAs. Lastly the resultant scores were obtained. (Quillet et al., 2017)
4) *PicTar:* PicTar web interface was accessed using online link https://pictar.mdc-berlin.de/. The search boxes gave options to select the miRNA and target gene i.e. hsa-miR-206 and BDNF respectively. The results were obtained with respect to target score, binding free energy and probability score. (Krek et al., 2005 ; Faiza et al., 2017)

G. Pathway Enrichment Analysis of BDNF Gene Using KEGG:

KEGG pathway database was accessed using online link https://www.genome.jp/kegg/pathway.html. The selection of the organism was done by typing “hsa” identifier for Homo sapiens. The keyword BDNF was entered in the dialog box. The results were obtained giving map of pathways followed by BDNF gene. (Kaneshisa et al., 2017)

H. Network Gene Enrichment Analysis Using STRING

STRING was accessed using online link https://string-db.org/. The STRING visualizes protein networks and shows the biological relationships of the gene products. The mode was selected to be multiple proteins. The genes were retrieved from NCBI by searching specific disease i.e. Diabetic Retinopathy. It gave 272 genes which play their role in the progression of the disease. The 25 genes were selected manually on the basis of relevance and gene weightage. Hence, the gene list was uploaded. The network results were obtained and the network file data was downloaded. CytoScape 3.7.1 software was installed using online link https://cytoscape.org/. STRING gene network visualization was done using CytoScape App i.e NetworkAnalyzer. In the present research, protein interaction analysis was performed by using Cytoscape to understand network ($p<0.05$ and coefficient=0.7 were significant). (Szklarczyk et al., 2016)

I. Functional Gene Enrichment Using DAVID

DAVID was accessed using online link https://david.ncifcrf.gov/. To do functional annotation of genes, Start analysis option was clicked. Under the option Enter gene list, gene list was uploaded. The identifier ENSEMBL_GENE_ID was selected. Next step was to specify if it is a gene list or the background, Gene list was clicked and then submit list option was clicked. The organism 'Homo sapiens' was selected. Gene enrichment analysis was done. Annotation results summary was displayed and analyzed. (Huang et al., 2007)

III. RESULTS AND DISCUSSION

A. BDNF Sequence Retrieval Using NCBI

SPECIES NAME	GENE_ID	LOCATION	NCBI Reference Sequence ID
Homo sapiens	627	Chromosome 11, NC_000011.10 (27654893..27722030, complement)	CAA62632.1
Gorilla gorilla	101134399	Chromosome 11,NC_018435.2 (27636808..27706521, complement)	XP_018892897.1
Pan troglodytes	503511	Chromosome 11, NC_036890.1 (27358928..27359671, complement)	Q5IS78.1
Rattus norvegicus	24225	Chromosome 3, NC_005102.4 (100768637..100819216)	AAH87634.1
Mus musculus	12064	Chromosome 2, NC_000068.7 (109674700..109727043)	P21237.1

Table 1. This table shows the number of organisms from which the BDNF protein sequences were retrieved from NCBI.

B. Target Gene (BDNF) Sequence Homology Using Clustal Omega

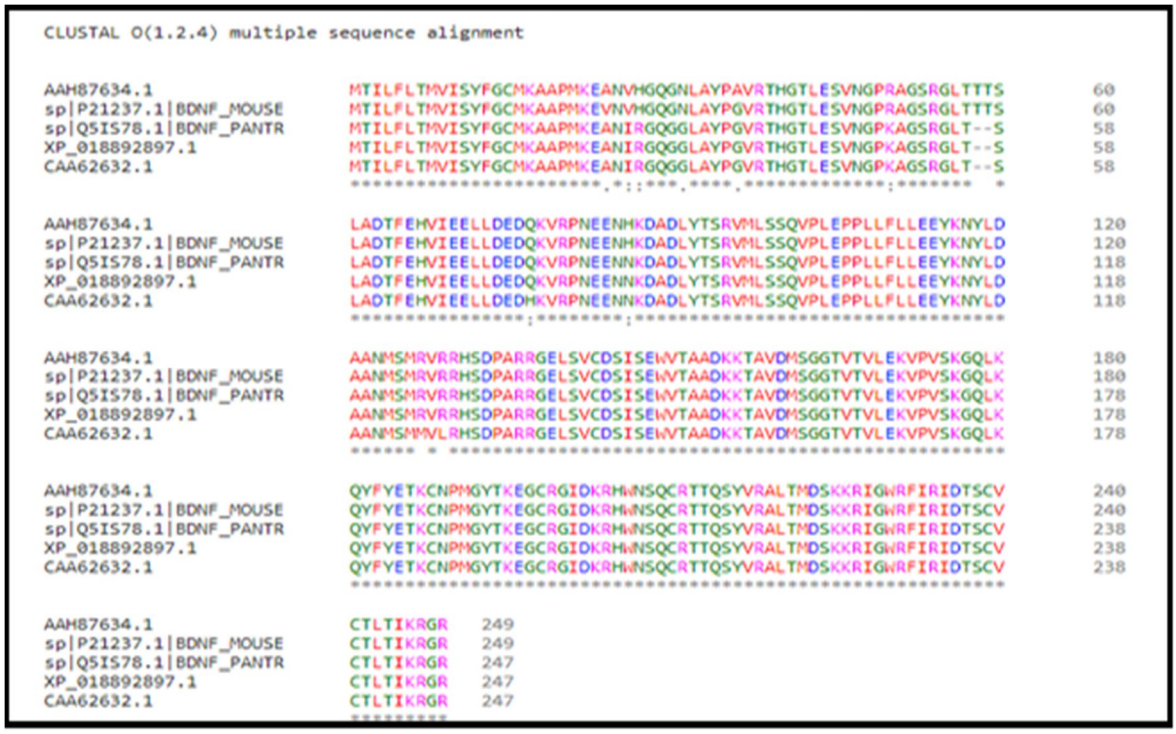

Figure 1: Clustal Omega Multiple sequence alignment showing gaps and dissimilar amino acid in selected organisms.

The Alignment scores are called the percent identity matrix (PIM). The percent identity matrix from the results shows that the human BDNF protein has abundant identity with Gorilla gorilla and Pan troglodytes i.e. 98.79 whereas Rattus norvegicus and Mus musculus attain the percent identity of 96.36.

C. Literature mining for understanding the miRNA-disease association using HMDD

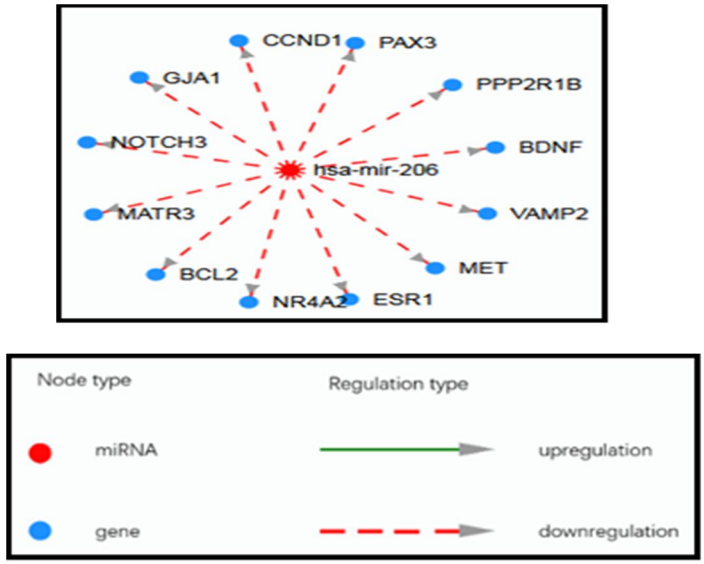

Figure 2(i): Human microRNA Disease Database (HMDD) showing downregulated genes by a specific miRNA (hsa-miR-206) in experimentally validated miRNA-Target gene network. Abbreviations: Brain-Derived Neurotropic Factor (BDNF), Cyclin D1 (CCND1), Paired box 3 (PAX3), Protein phosphatase 2 scaffold subunit A beta (PPP2R1B), Vesicle associated membrane protein 2 (VAMP2), MET proto-oncogene, receptor tyrosine kinase (MET), Estrogen receptor 1 (ESR1), Nuclear receptor subfamily 4 group A member 2 (NR4A2), BCL2 apoptosis regulator (BCL2), Matrin 3(MATR3), Notch receptor 3 (NOTCH3), Gap junction protein alpha 1 (GJA1).

miRNA ▾ exact ▾ hsa-mir-206 Click to Search Reset all

download the result file

miRNA name	Evidence Code	Disease name	PMID	Description	Causality
hsa-mir-206	circulation_biomarker_diagnosis_ns	Lung Neoplasms	23337359	serum;Alteration of serum miR-206 and miR-133b is associated with lung carcinogenesis induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone	NO
hsa-mir-206	circulation_biomarker_diagnosis_ns	Muscular Dystrophy, Duchenne	24460924	Serum miR-206 and other muscle-specific microRNAs as non-invasive biomarkers for Duchenne muscular dystrophy.	NO
hsa-mir-206	circulation_biomarker_diagnosis_ns	Early-Stage Lung Carcinoma	24553369	4-(Methylnitrosamino)-1-(3-pyridyl) -1-butanone induces circulating microRNA deregulation in early lung carcinogenesis.	NO
hsa-mir-206	circulation_biomarker_diagnosis_ns	Amyotrophic Lateral Sclerosis	24586506	MicroRNA-206: a potential circulating biomarker candidate for amyotrophic lateral sclerosis.	NO
hsa-mir-206	circulation_biomarker_diagnosis_ns	Muscular Dystrophy, Duchenne	25150707	Taken together, our data demonstrate that levels of miR-1, miR-133a, and miR-206 in serum of BMD and miR-1 in sera of LGMD and FSHD patients showed no significant differences compared with those of controls by Bonferroni correction. However, the results might need increase in sample sizes to evaluate these three miRNAs as variable biomarkers.	NO
hsa-mir-206	circulation_biomarker_diagnosis_ns	Carcinoma, Hepatocellular	25391771	miR-30c-5p, miR-223-3p, miR-302c-3p and miR-17-9p could be used as novel non-invasive biomarkers of HCV-positive HCC in very early, even at cirrhosis stage of liver disease.	NO

Figure 2 (ii): HMDD- hsa-mir-206 based literature mining describes the role of its importance as biomarker and potential therapeutic target with respect to different diseases.

The network visualization of disease-based miRNA–target interaction results are based on the experimentally supported miRNA–target data from miRTarBase. The figure 2(i) shows that hsa-miR-206 specifically down-regulate genes, using the annotations from TarBase v8.

D. miRNA sequence retrieval of hsa-miR-206 using miRBase.

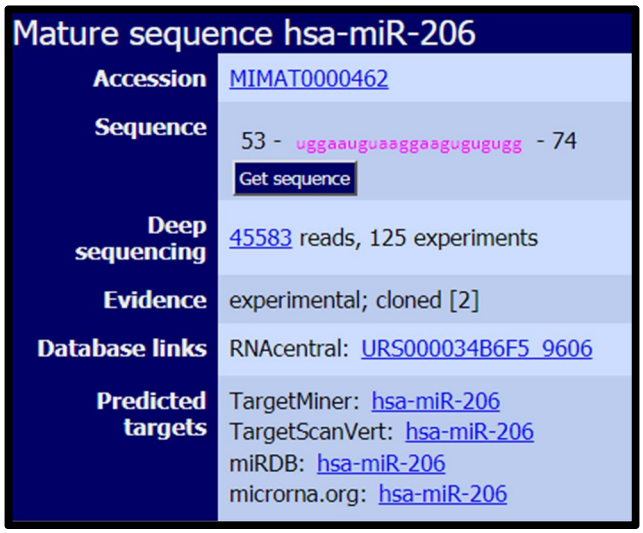

Figure 3: Sequence and structures of miRNA sequences were needed for understanding their function. Thus, stem-loop structure of hsa-mir-206 and mature sequence of hsa-miR-206 were retrieved from miRBase.

E. miRNA sequence analysis using NCBI-BLASTN.

Figure 4(i): The query sequence is represented at the top of the figure. Database hits are shown aligned to the query. The most similar are shown closest to the query (hsa-miR-206).

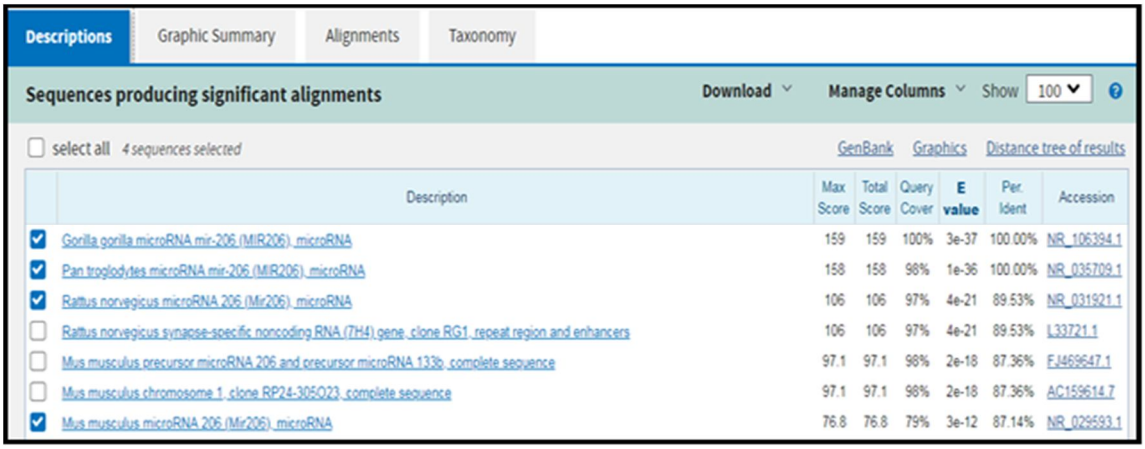

Figure 4(ii): The summary table given above shows all the sequences in the Refseq database that show significant sequence homology to our sequence By default, the results are sorted according to the Expect value (E-value) in ascending order.

Result demonstrated that the highest identity percentage and lowest E-value (3e-37) is of Gorilla gorilla miR-206 sequence (100% identity) when compared with query sequence of hsa-miR-206. Therefore, results clearly indicated the conserved nature of the miR-206 among various mammalian model organisms. It will help to infer the function of a sequence from similar sequence.

F. miRNA-target identification and binding site analysis using:

1) TargetScan

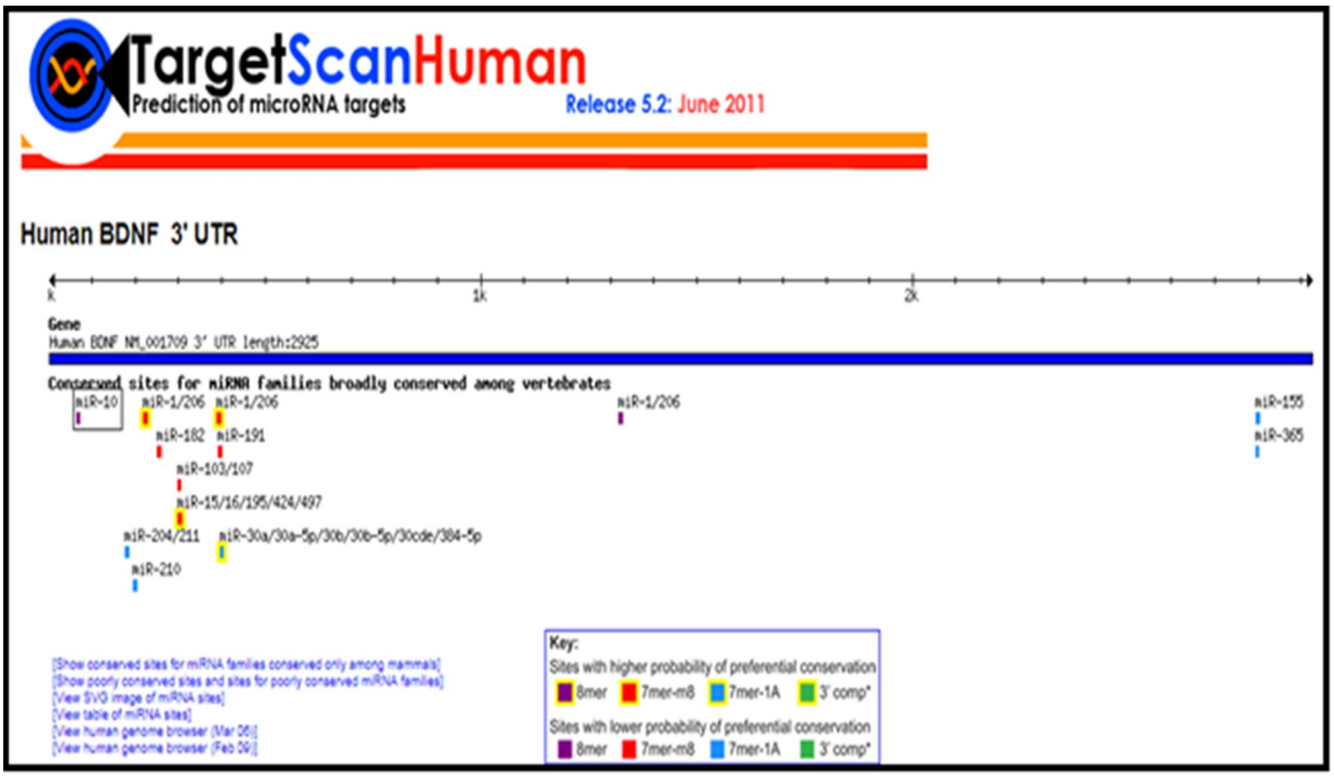

Figure 5(i): TargetScanHuman showing three binding site of microRNA-206 on Human BDNF 3' UTR seed location 220-226, 390-396, 1322-1329 respectively.

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 220-226 of BDNF 3' UTR hsa-miR-613	5' ...UAAAAAGUCUGCAUUACAUUCCU... 3' CCGUUUCUUCCU---UGUAAGGA	7mer-m8	-0.38	97	-0.37	6.475	0.83
Position 220-226 of BDNF 3' UTR hsa-miR-206	5' ...UAAAAAGUCUGCAUUACAUUCCU... 3' GGUGUGUGAAGGAAUGUAAGGU	7mer-m8	-0.37	97	-0.36	6.475	0.83
Position 220-226 of BDNF 3' UTR hsa-miR-1-3p	5' ...UAAAAAGUCUGCAUUACAUUCCU... 3' UAUGUAUGAAGAAAUGUAAGGU	7mer-m8	-0.37	97	-0.36	6.475	0.83
Position 390-396 of BDNF 3' UTR hsa-miR-1-3p	5' ...AAAAUUUGAACCAAAACAUUCCG... 3' UAUGUAUGAAGAAAUGUAAGGU	7mer-m8	-0.35	97	-0.31	6.565	0.83
Position 390-396 of BDNF 3' UTR hsa-miR-613	5' ...AAAAUUUGAACCAAAACAUUCCG... 3' CCGUUUCUUCCUUGUAAGGA	7mer-m8	-0.35	97	-0.31	6.565	0.83
Position 390-396 of BDNF 3' UTR hsa-miR-206	5' ...AAAAUUUGAACCAAAACAUUCCG... 3' GGUGUGUGAAGGAAUGUAAGGU	7mer-m8	-0.35	97	-0.31	6.565	0.83
Position 1322-1329 of BDNF 3' UTR hsa-miR-613	5' ...GGCAUGGUAUUUGAGACAUUCCA... 3' CCGUUUCUUCCUUGUAAGGA	8mer	-0.24	92	-0.02	2.686	0.40
Position 1322-1329 of BDNF 3' UTR hsa-miR-1-3p	5' ...GGCAUGGUAUUUGAGACAUUCCA... 3' UAUGUAUGAAGAAAUGUAAGGU	8mer	-0.23	92	-0.02	2.686	0.40
Position 1322-1329 of BDNF 3' UTR hsa-miR-206	5' ...GGCAUGGUAUUUGAGACAUUCCA... 3' GGUGUGUGAAGGAAUGUAAGGU	8mer	-0.23	92	-0.02	2.686	0.40

Figure 5(ii): TargetScanHuman v 7.0 Database showing type of binding sites with Context score and Probability of conserved Targeting (P_{CT}).

It estimates the cumulative weighted context++ score (CWCS) for each miRNA. The CWCS score ranks based upon the predicted repression or P_{CT} (probability of conserved targeting) aggregated score of the longest 3'-UTR isoform. First, the 6mer, 7mer-A1, 7mer-m8, and 8mer are first filtered to remove overlapping locations for each miRNA family, then the CWCS is calculated for each member of the miRNA family, and the member representing the largest expected score of repression is selected to represent that family and the 3'-UTR reference with the most 3p-seq tags represents the gene. (Faiza et al., 2017)

2) miRDB

Figure 6(i): miRDB database showing hsa-miR-206 and target gene prediction. This result describes the predicted miRDB Target score and seed location of hsa-miR-206-BDNF interaction.

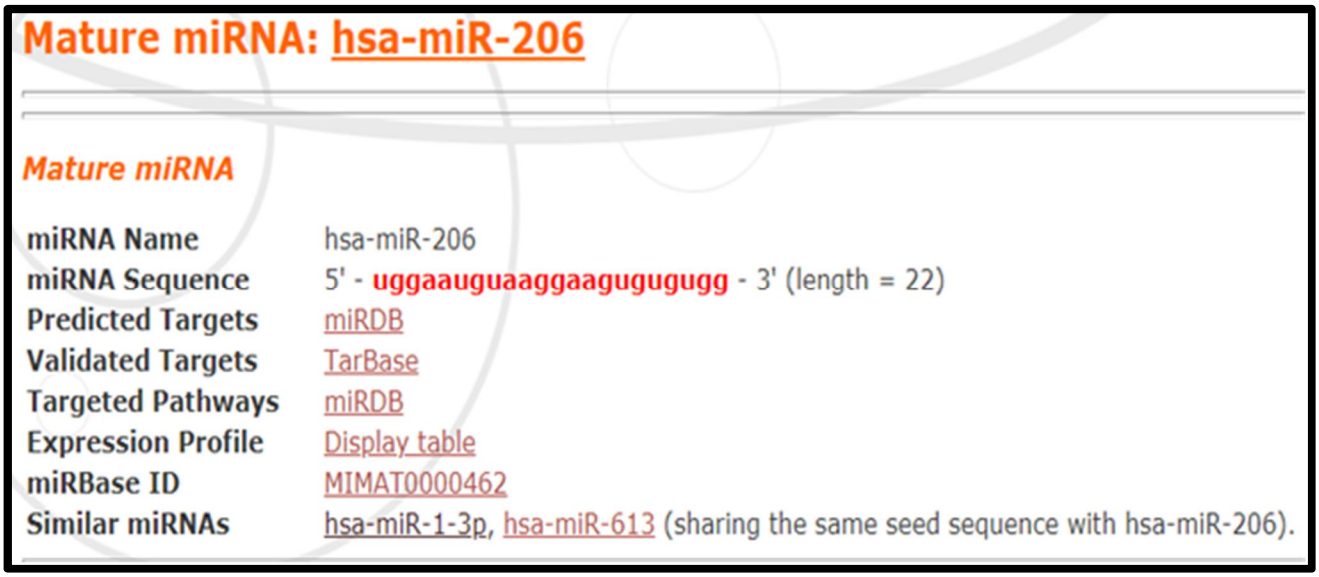

Figure 6(ii): miRDB Database shows that functional miRNA hsa-miR-206 has similar seed sequence as hsa-miR-1-3p and hsa-miR-613.

BDNF gene is found to be the target of hsa-miR-206 as predicted by miRDB. There are 3 has-miR-206 seed locations i.e. 220, 389 and 1321th nucleotide on 3'UTR sequence of BDNF. The target score is 97 which build the high confidence in this prediction. As per the results obtained, BDNF is the target of hsa-miR-206. If target score is more than 60, it is predicted to be highly confident. (Wong & Wang, 2014) miRDB database also describes that there are 2 more miRNA (hsa-miR-1, hsa-miR-613) which shares the same seed sequence with hsa-miR-206.

3) miRabel

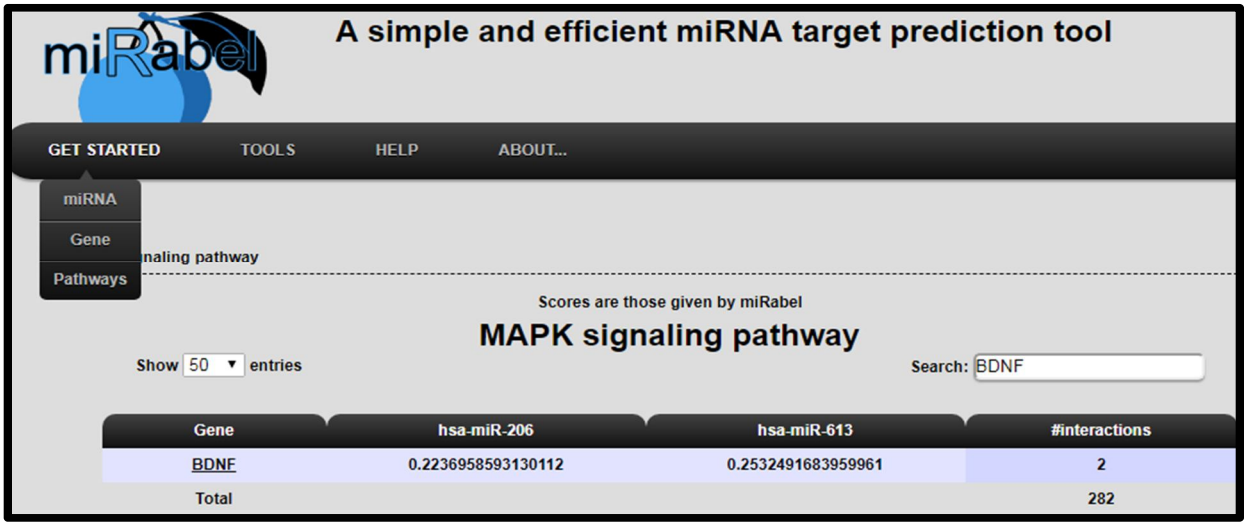

Figure7: miRabel, a target prediction tool which also compute the potential impact of miR-206 and BDNF in metabolic pathway i.e. MAPK signalling by predicting the target prediction score between hsa-miR-206 and BDNF i.e. 0.2236.

4) PicTar

Org	PicTar score	PicTar score per species	microRNA	Probabilities	Nuclei mapped to alignments	Nuclei mapped to sequence	Free Energies kcal/mol
hs	8.42	8.26	hsa-miR-206	0.94 0.94 0.94	355 621 1706	220 390 1323	-24.4 -22.9 -20.0
pt	8.42	8.30	hsa-miR-206	0.94 0.94 0.94	355 621 1706	220 389 1316	-24.4 -22.9 -20.0
mm	8.42	8.54	hsa-miR-206	0.94 0.94 0.94	355 621 1705	213 407 1306	-25.0 -17.7 -17.4
rn	8.42	8.47	hsa-miR-206	0.94 0.94 0.94	355 621 1705	214 392 1295	-25.0 -17.7 -17.4
cf	8.42	8.48	hsa-miR-206	0.94 0.94 0.94	355 621 1698	215 402 1383	-24.9 -18.2 -18.6
gg	8.42	6.58	hsa-miR-206	0.96 0.96	355 621	281 430	-20.2 -17.2
fr	8.42	8.31	hsa-miR-206	0.98 0.98	355 621	249 453	-17.6 -15.0
dr	8.42	7.95	hsa-miR-206	0.98 0.98	355 621	248 437	-19.4 -18.2

Figure 8(i): PicTar result summary table showing target prediction score, free energies and three nuclei sites for hsa-miR-206 with respect to BDNF gene.

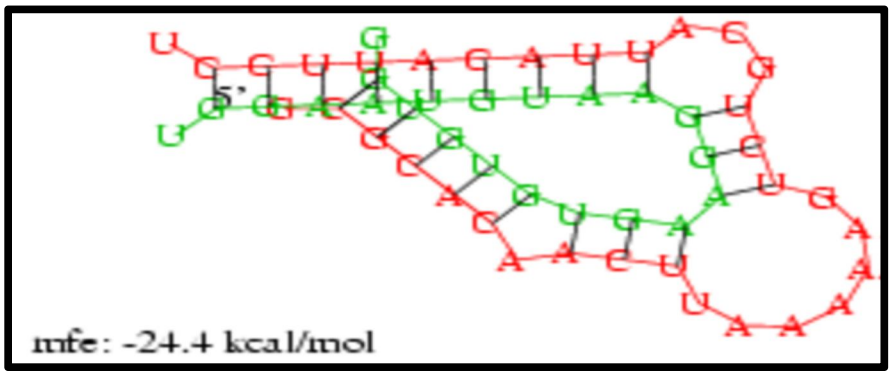

Figure 8(ii): Structure of predicted duplex of hsa-miR-206 and BDNF gene showing required free energy.

The summary table of outcomes demonstrates that there are three nuclei locations in hsa-miR-206 and all three sites have distinct optimum free energy. Each nuclei's optimum free energy is anticipated to narrow down to lower objectives. The extremely likely nuclei with optimum free energy falling into overlapping positions are called anchors in the alignment of the species being regarded. If there are enough anchors in the 3'-UTR alignment, each UTR in the alignment is then subjected to the main PicTar highest probability operation, after which all the results of the orthological transcripts are combined. (Krek et al., 2005)

SERIAL NO.	LIST OF MICRORNAs	PICTAR SCORE	MiRDB SCORE
1	hsa-miR-1	8.42	97
2	hsa-miR-206	8.42	97
3	hsa-miR-22	2.41	---
4	hsa-miR-368	1.94	---
5	hsa-miR-382	1.89	---
6	hsa-miR-15a	1.85	68
7	hsa-miR-15b	1.85	68
8	hsa-miR-107	1.85	59
9	hsa-miR-103	1.85	59
10	hsa-miR-195	1.85	68
11	hsa-miR-16	1.85	68
12	hsa-miR-10b	1.69	89
13	hsa-miR-10a	1.69	89
14	hsa-miR-182	1.55	---
15	hsa-miR-30e	1.11	69
16	hsa-miR-369	0.46	---

Table 2: This table comprises the list of microRNAs which were predicted to target BDNF gene.

The Table 2 displayed above also shows the predicted target score from 2 major databases i.e. PicTar and miRDB. These target scores clearly describes that hsa-miR-206 and hsa-miR-1 has the highest prediction data in both of the databases individually. Therefore, the result obtained in this study has high confidence in prediction the target of hsa-miR-206.

G. Pathway Enrichment Analysis of BDNF Gene Using KEGG:

Tropomyosin-related kinase B (TrkB) is a receptor protein that contributes to central and peripheral nervous systems development and maturation. BDNF has a strong affinity for TrkB and p75 improves BDNF-TrkB interaction. TrkB undergoes homodimerisation, autophosphorylation and activation after ligand-binding. In order to control gene expression and defend neurons, it then recruits and activates several downstream effectors. Members of the TrkB downstream signaling cascade, including ERK/MAPK and PI3K/PKB, have been reported to be responsive to BDNF. Several studies have hypothesized that BDNF largely activates the ERK/MAPK pathway. (Reichardt, 2006)

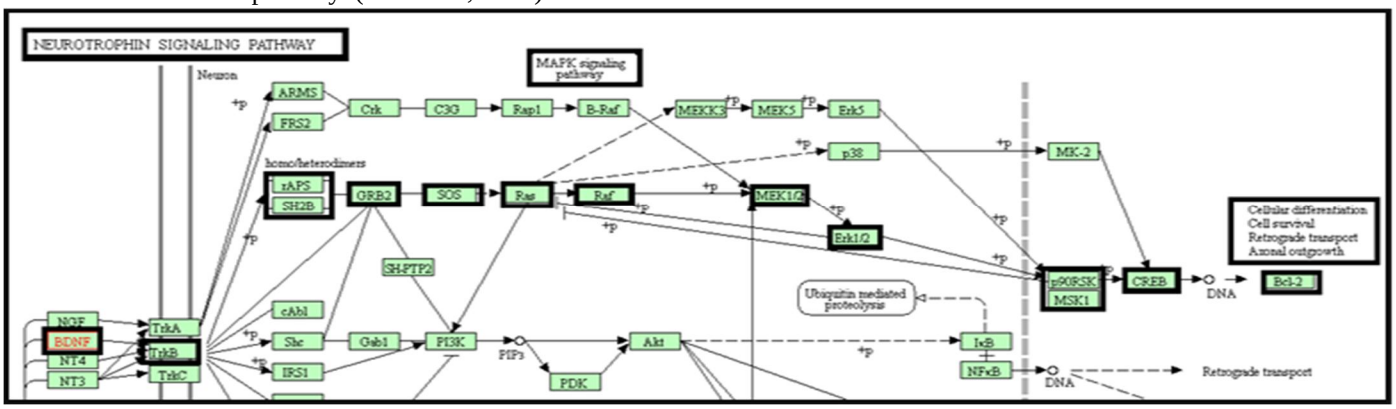

Figure 9: Neurotrophin signalling pathway map 04722 visualization using Kegg pathway database.

The Kegg pathway database describes the map 04722 known as Neurotrophin signalling pathway. It is found in increasing number of studies that the pathogenesis of T2DR correlates with neurodegeneration of the retina. (Barber, 2003) Like studies showed earlier that BDNF follows ERK/MAPK Pathway though binding its receptor TrkB. Here, in Kegg pathway it is shown that after ligand binding to TrkB, receptor dimerizes, autophosphorylates and binds to Grb2 through SH2 domain and similarly effector protein SOS binds to Grb2. This activates SOS which recruits RAS which has GTPase action which further activates RAF. The RAF phosphorylates MEK further phosphorylating ERK. ERK goes into the nucleus to regulate gene expression and cause cellular differentiation and survival.

H. String

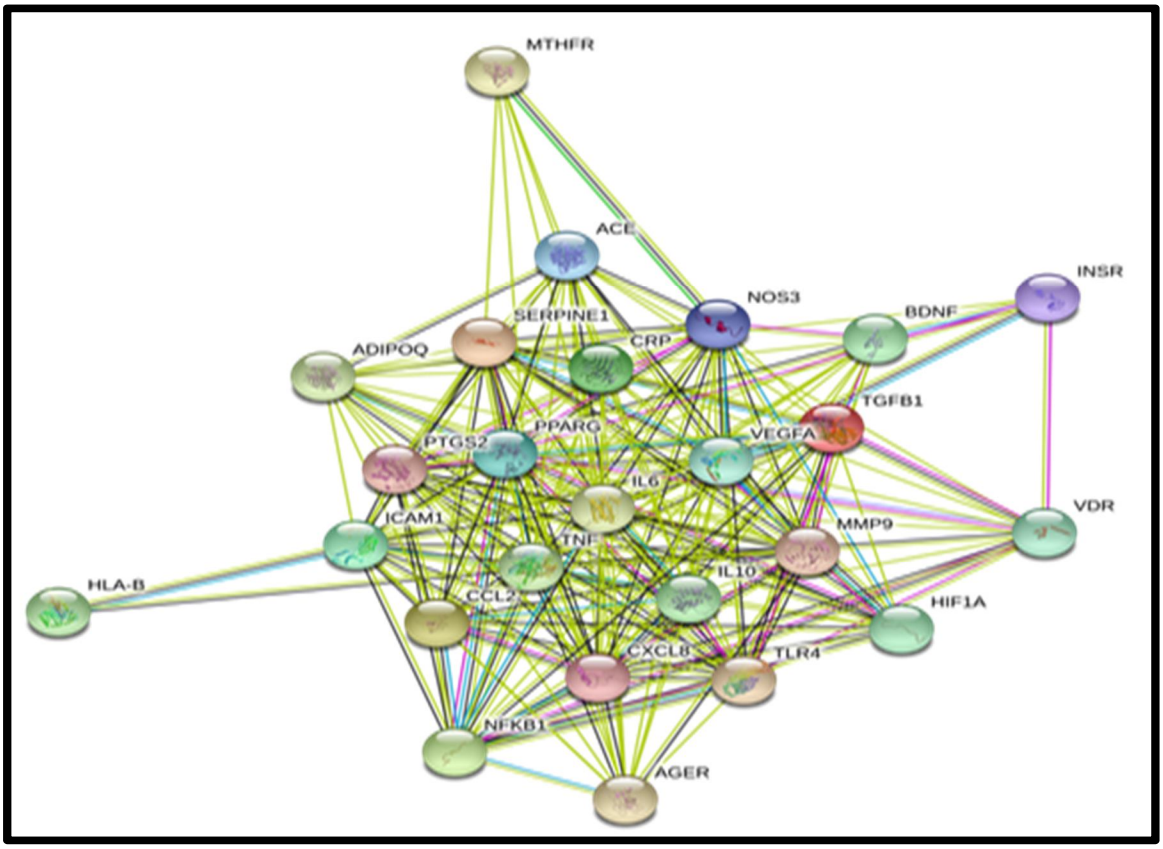

Figure 10(i): String Database displaying protein-protein network interaction with statistics summary. Colored lines between the proteins indicate the various types of interaction evidence.

String database showed the protein-protein interaction within 25 genes, which are relevant to the disease Type 2 diabetic retinopathy. The result data shows that protein-protein interaction network is significantly enriched more than expected. A small PPI enrichment P-value <1.0e-16 indicates that the nodes are not random and number of edges are significant. The average local clustering coefficient is 0.857 which shows how connected is the node in this network.

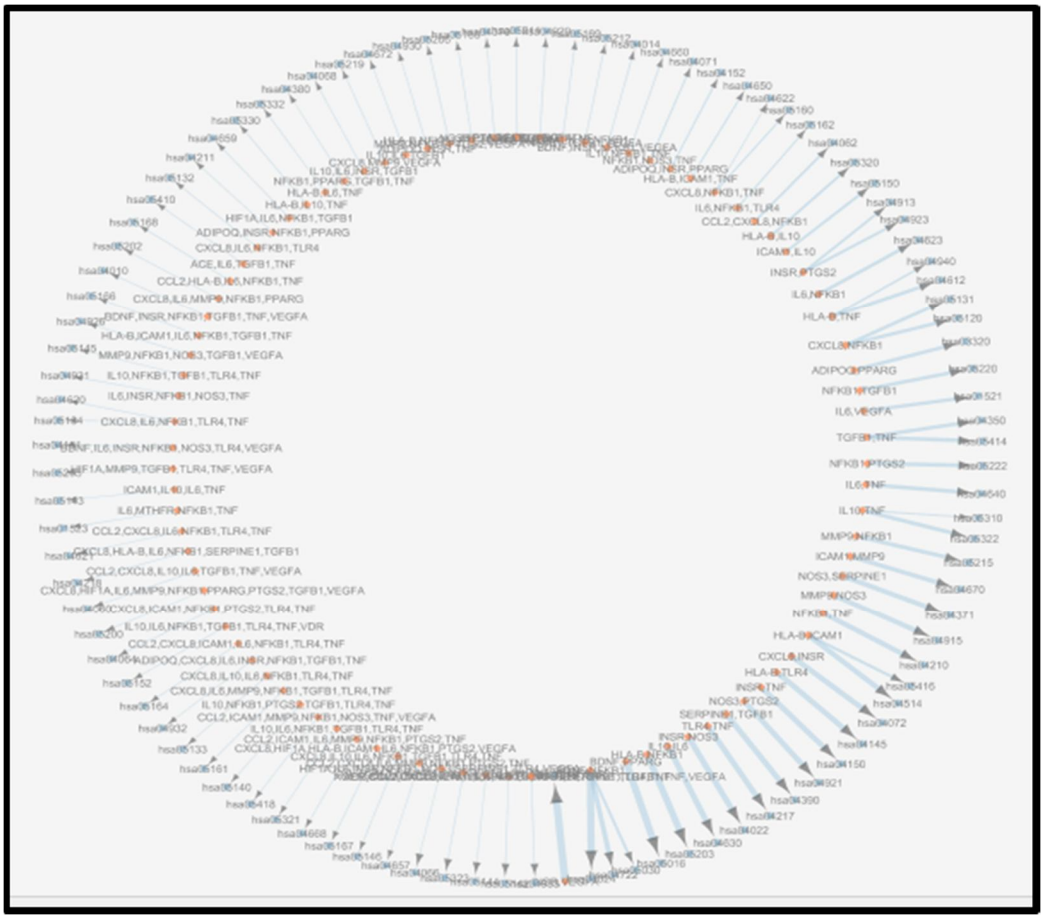

Figure 10(ii): String PPI network visualization using CytoScape Network Analyzer

I. Functional Gene Enrichment using DAVID

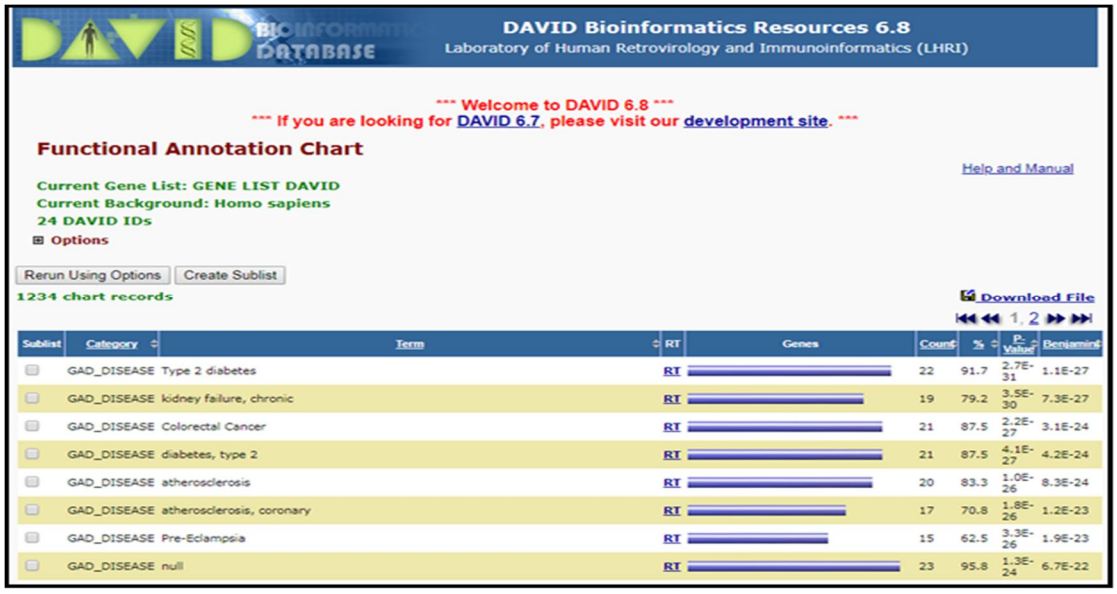

Figure 11: The representative biology terms associated with the top 8 diseases showing a much clearer and non-redundant view of the functional annotations associated with the study.

DAVID changes functional annotation analysis from gene centric to biological module centric. This method takes into account the redundant and network nature of biological annotation contents in order to concentrate on the larger biological picture rather than an individual terms or genes. In the result summary table it clearly shows that 22 out of 25 genes are involve in the Diabetes mellitus with least E-value of 2.7e-31. It also gives the idea that the genes that overlapped and showed multiple interactions in multiple biological functions could be considered significant and should be validated for future studies.

IV. CONCLUSIONS

Diabetic Retinopathy is age-related severe eye-threating disease which is caused due to hyperglycemia. There are various therapies which are currently used for the prevention of T2DR. Central laser photocoagulation, vitrectomy and anti-VEGF agents are most currently used in the treatment of this disease. Type 2 Diabetic retinopathy has proliferative and progressive nature, it is mandatory to find a way of its prevention in its earlier stage. BDNF is one of the most relevant genes whose dysregulation plays a crucial role in initiation of retinal degeneration. Therefore, targeting the expression of BDNF gene through miRNAs could play a positive role. The current *in silico* study has resulted that hsa-miR-206 has a better chances of targeting BDNF due to the target prediction scores, site accessibility, structure stability and optimal free energy which were found with the help of different tools and databases. The literature mining also gave experimentally validated down-regulated genes targeted by hsa-miR-206. Therefore, hsa-miR-206 could be considered as a therapeutic agent in the prevention & treatment of T2DR. For future aspects, the *in vitro* analysis of hsa-miR-206 and BDNF interaction should be studied in T2DR patients.

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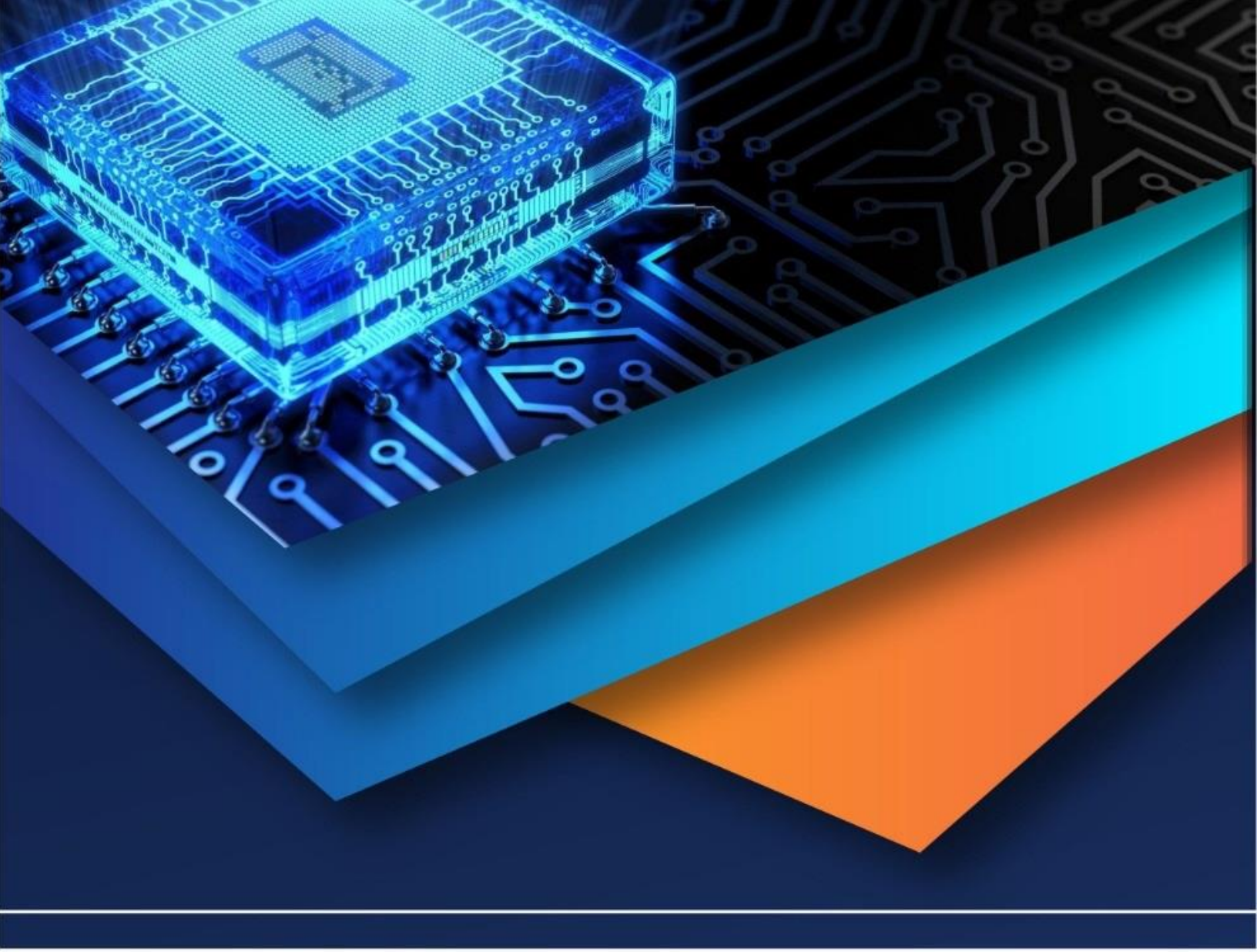

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