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Computational Identification of microRNA-15a predicted to regulate Brain Derived Neurotrophic Factor involved in Type 2 Diabetic Retinopathy

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Abstract: ***Type 2 Diabetic Retinopathy (T2DR), commonly classified as a microvascular complication of diabetes, is now recognized as a neurovascular complication resulting from disruption of the neurovascular unit. Neurodegenerative diseases are becoming an ever-increasing problem in aging populations. Low levels of brain-derived neurotrophic factor (BDNF) have previously been associated with the pathogenesis of numerous neurodegenerative diseases. Recently, microRNAs (miRNAs) have been proposed as potential novel therapeutic targets for treating various diseases of the central nervous system (CNS) and interestingly, few studies have reported several miRNAs that downregulate the expression levels of BDNF. The main focus of this investigation, therefore, was the early detection of T2DR by analysing microRNA-15a and its potential target gene BDNF associated with the onset and progression of T2DR. In this study we used homology-based analysis with available expressed sequence of miRNA-15a to predict conserved nature of miRNA, miR-15a targeting BDNF genes were identified. Functional Gene annotations revealed miR-15a were involved in growth and development and Encyclopaedia of Genes and Genomes (KEGG) pathway analyses showed miR-15a were involved in metabolic pathways. On the basis of the findings of in silico results, this study predicted the control of BDNF expression by microRNA-15a, which appeared to be one of the most promising miRNA for biomarker validation because of the role of the target genes associated with T2DR onset and progression.***
Keywords: Bioinformatics, Brain Derived Neurotrophic Factor, microRNA-15a,Type 2 Diabetic Retinopathy.

I. INTRODUCTION

Type 2 diabetes are expected to have some form of retinopathy by the first decade of incidence of diabetes (Klein et al., 1984). The risk of developing diabetic retinopathy can be reduced by early detection. The study of the mechanisms that lead to neurodegeneration will be essential to identify new therapeutic targets in the early stages of T2DR (Antonetti et al., 2006). The changes that have been described include the disruption of the blood-retinal barrier (BRB), vasoregression and impairment of neurovascular interaction. Glia and neurons closely interact with retinal vasculature to maintain the normal function of the retina. Diabetes disturbs the interaction between these cells by damaging neurons due to apoptosis and activating glial cells which is another feature of retinal neurodegeneration (Elena Vecino et al., 2006). Recent evidence suggests that neurotrophic factors play important roles in the interactions between neuronal and vascular cells, thereby regulating the survival, growth and functional maintenance of these cells. Neuronal retina produces a huge amount of neurotrophic factors such as *BDNF* which binds to its high affinity TrkB receptor and undergoes homodimerization, auto-phosphorylation and activation. It then recruits and activates several downstream effectors to regulate gene expression and protect neurons through mitogen activated protein kinase pathway (MAPK) pathway . Subsequent studies on *BDNF* have suggested that a marked decrease in the levels of this specific Neurotrophin is involved in the pathophysiology of numerous neurodegenerative diseases. However, the role of *BDNF* in diabetic retinopathy is not fully understood yet. Regulating the levels of *BDNF* may be a promising therapeutic target to protect neurons and prevent T2DR (Ola et al., 2013).

On the other hand, various miRNAs, that is, short non-coding RNA segments of approximately 22-nucleotides in length, on various levels ranging from transcription and processing to target site binding and miRNA stability, miRNA activity and abundance is also regulated. miRNAs have been linked with a variety of cellular processes and numerous signal transduction pathways (Hannon, 2004). There is a complex network when talking about miRNAs. While each miRNA could targets multiple genes, multiple miRNAs may regulate one gene in turn. Hence a unique opportunity is created by targeting one or a few miRNAs leading to

prevention of expression of multiple genes and for the development of RNA-based therapeutics. In human diseases, miR-15a family miRNAs have been involved, therefore miR-15a acts as a biomarker for the treatment of T2DR(Lagos et al., 2001). miR-15a was identified as key regulator of both pro-inflammatory and pro-angiogenic pathways (Wang et al., 2016). Angiogenesis, that is, the ailment characterized by the growth of new blood vessels originated from pre-existing ones, was shown to have a major role in the pathogenesis of T2DR (Tremolada et al., 2012). The area of bioinformatics or systems biology, which is the convergence of the disciplines of computational and biological science, was an important tool for organizing and analyzing the vast amount of biological data (Lewis, 2008). *BDNF*, which plays a significant role in T2DR, is one of miR-15a's target genes predicted using bioinformatics technology. Thus, with the aid of various online resources, bioinformatics tools helped predict changes occurring in T2DR pathways. No *in silico* and *in vitro* studies have been performed on miR-15a and its target gene *BDNF*, which play a very important role in T2DR and would be useful in preventing it. Therefore, in the current study, *BDNF* expression due to down regulated miR-15a in diabetic retina was predicted with the help of bioinformatics tools.

II. METHODOLOGY

A. microRNA-15a-5p sequence retrieval using miRbase

miRBase was accessed using home page link http;//www.mirbase.org/index.shtml.on the right most side of miRBase home page, Search by miRNA name or keyword option was clicked. miRNA name i.e. " hsa-miR-15a" was written in the search by miRNA name option and then clicked on Go. A particular page having all the information regarding hsa-miR-15a appeared e.g. Accession number, symbol, description, gene family, and stem loop structure. Mature sequence of hsa-miR-15a-5p along with their Accession numbers and ID was retrieved by scrolling down the particular page. **(**Griffiths et al., 2010).

B. Comparative Identification of Conserved miRNA-15a Between Homo Sapiens and other Mammalian Organism using BLAST

BLAST page was opened by clicking BLAST on popular resources menu from National Centre for Biotechnology Information (NCBI). Website link http://www.ncbi.nlm.nih.gov.Nucleotide BLAST program was chosen to run from the Basic BLAST menu. FASTA sequence of hsa-miR-15a was retrieved from NCBI. FASTA formatted sequence of hsa-miR-15a was copied from NCBI page and pasted into the large text box using Enter Query sequence in BLAST page. In the Organism option, other organisms such as *Macaca Mulatta , Rattus norvegicus, Mus musculus* were chosen to compare the miR-15a sequence with human query sequence. BLAST button was clicked to submit search and output was obtained. Some basic parameters were at the top of the page, along with graphical view and description below. Hits were sorted by total score from highest to the lowest and also from E-value from lowest to highest. Organism with lowest E-value was selected which showed the highest similarity with human miR-15a.The page was scrolled down further, alignments were obtained to check exact matches with human sequence and BLAST tree view constructed an implicit alignment between the database sequences (Madden,2013).

C. Target Prediction of miRNA-15a-5p miRmap

Target predictions for the miRNAs are based on the principle of nearly perfect complementation between the miRNA and target mRNAs. The first step is to select a species . The user may then 'Select' or input the 'Sequence' of a miRNA and/or a protein-coding gene. It is important to note that for browsing precomputed predictions, users may select a miRNA or a gene, or both, with an auto-complete capability to facilitate rapid selections. The gene selection and sequence input options may be combined such that the user may input a miRNA sequence and select a gene/transcript by name, or *vice versa*. When a gene name or identifier is entered, the canonical transcript of that gene is selected; it is also possible to select a specific transcript by directly entering the transcript identifier. When the form is valid, the user may then submit his/her query by clicking on the 'Get targets' button (Charles et al., 2012).

D. DAVID (The Database for Annotation, Visualization and Integrated Discovery)

List of all miR-15a target genes (verified by miRDB in this present study) which have role in T2DR was made with the help of National Centre for Biotechnology Information. By going to the link 'http: //www.ncbi.nlm.nih.gov/'.DAVID home page was opened with link https://david.ncifcrf.gov/.On the extreme left of DAVID home page shortcut to DAVID tools was present. To do functional annotation of genes, Functional Annotation option was clicked . Functional Annotation tool home page was displayed . Under the option Enter gene list, gene list was uploaded.

E. KEGG Pathway Analysis

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used to identify the significantly enriched pathways of miRNA target genes. KEGG Genome Net Web page was opened at http:// www.genome.jpand clicked on the KEGG2 link located in the navigation bar at the top of the page . KEGG table of contents was displaced which is the entry point to all of the KEGG databases . Clicked on KEGG PATHWAY in the Database column of the table near the top of the page . That brought up a list of KEGG's numerous Pathways organized in a Hierarchical manner Clicked the "6 Human diseases" link to display the KEGG metabolic pathway data . Number of pathway names appeared which are linked to the respective disease. Clicked on MAPK signalling pathway will be displayed showing *BDNF* role in MAPK pathway. (Aoki and Kanehisa , 2005)

III. RESULTS AND DISCUSSION

A. microRNA-15a-5p Sequence Retrieval using miRbase

hsa-miR-15a-5p sequence is of 22 nucleotides (fig.1). Accession number of this sequence 'MIMAT0000068' is unique identifier given to a sequence record to allow for lacking of different versions of that sequence record and the associated sequence over time in single data repository. All sequence information repositories implement the concept of "accession number" but might do so with subtle variations. Previous ID of this sequence was hsa-miR-15a but to make it more clear whether it is 5p or 3p sequence, it was named by new name as hsa-miR-15a-5p.

The sequence retrieval was first and very important step for further analysis of sequences. Sequence retrieval helped in understanding the number of nucleotides present in the miRNA sequence and the stem loop structure of miR-15a. In many cases, mature miRNAs from both 5' and 3' arms of hairpin precursor are frequently identified, suggesting that both may be functional. Such miRNAs are given name of the form hsa-miR-15a-5p is retained in the miR set. According to past study (Molitoris , 2011), miRNA sequences are of 20-25 nucleotides. In this study, miR-15a sequence obtained by miRBase is of 22 nucleotides (uagcagcacauaaugguuugug). In the precursor miR-15a stem-loop structure, the 5p strand is present in the forward (5'-3') position and 3p strand (which will be almost complimentary to the 5p strand) is located in the reverse position. In terms of which strand is functional following Dicer cleavage of the stem loop to produce the two mature strands, either the 5p or 3p strand would be functional. The stability of the mature strand may influence its function and ability to enter the RISC complex to then bind to its target gene in general, the more stable strand will be functional, and less stable strand will be degraded. Thus, further analysis of sequence was done using other tools.

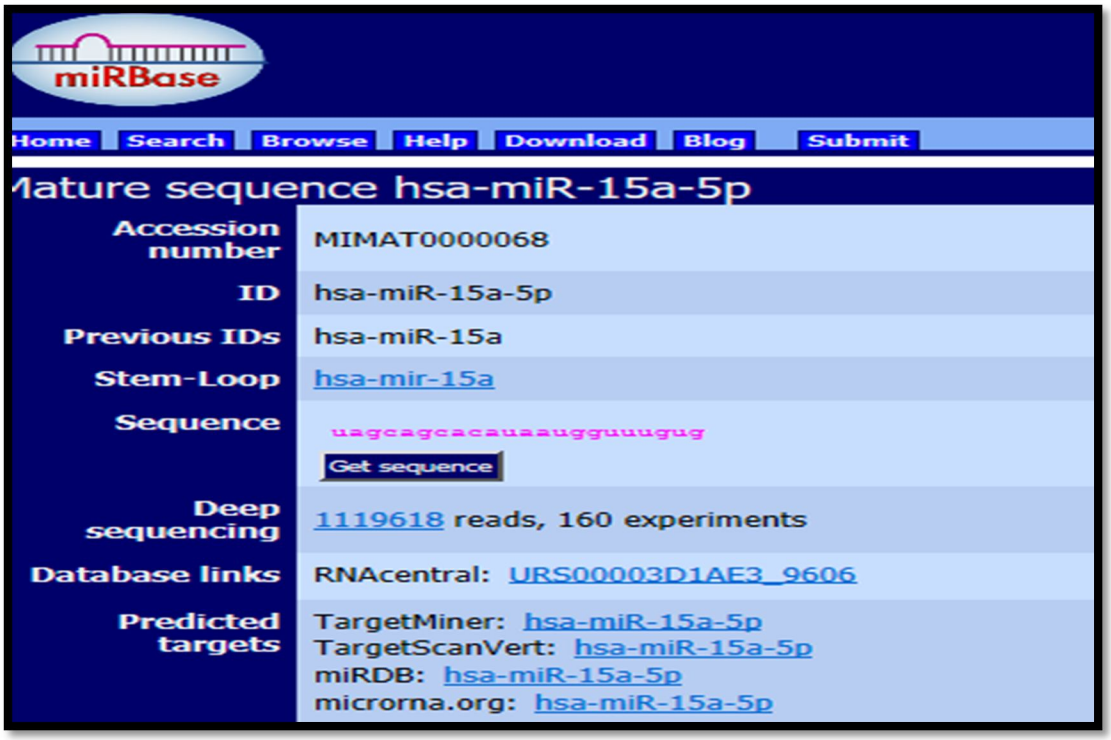

Figure:1 Mature sequence of hsa-miR-15a-5p.

B. miRNA-15a Sequence Analysis using BLAST

This BLAST result showed the numbered bar as query sequence in green. Other aligned sequence scoring 80- 200 (good score) are shown in pink. The query sequence is represented by the numbered bar at the top of the figure. Database hits are shown aligned to the query, below the numbered bar of the aligned sequences, the most similar are shown closet to the query. In this case, all seven are good scoring database matches that align to most of the query sequence (hsa-miR-15).

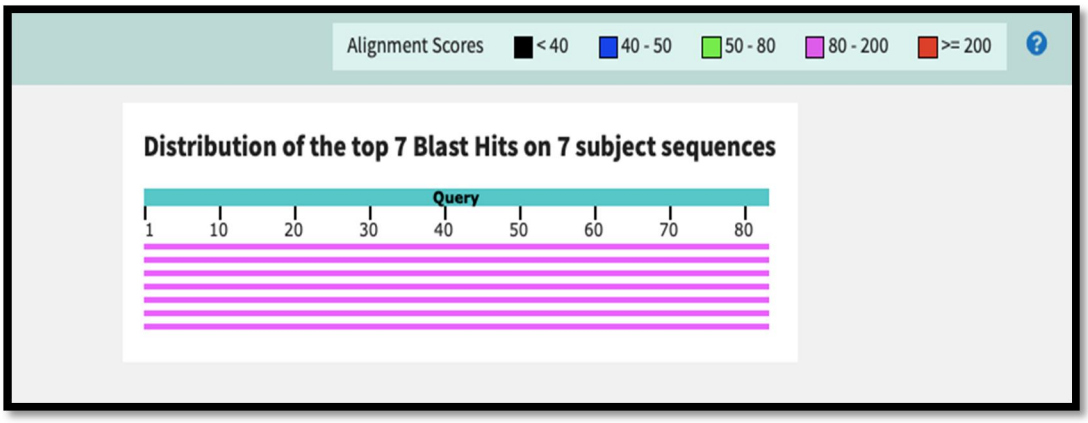

Figure: 2 Graphical overview of BLAST result

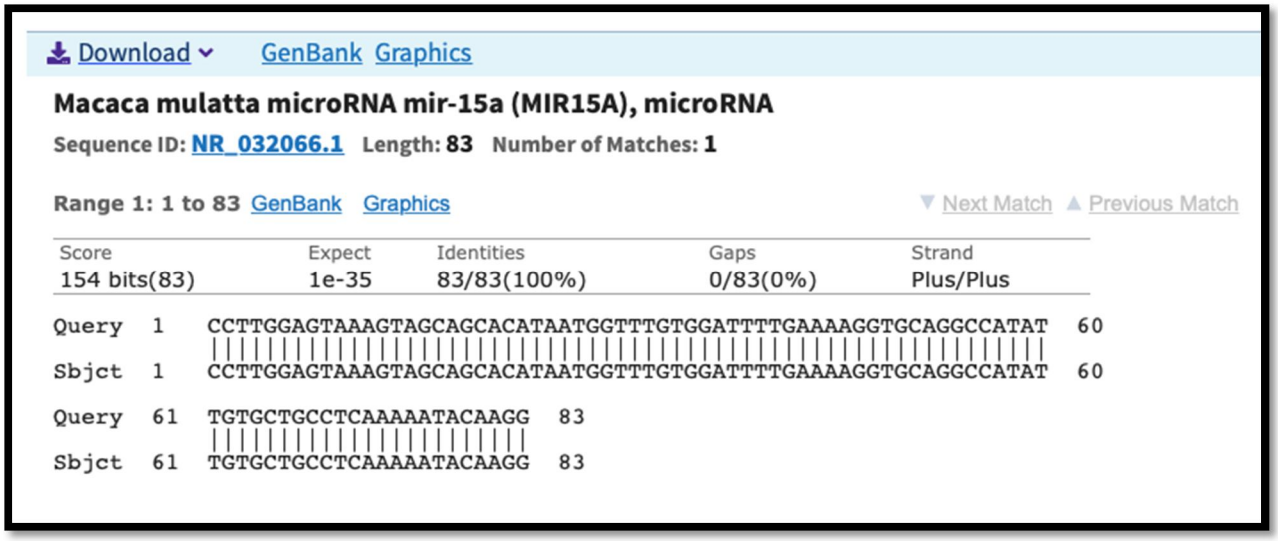

Figure: 3 Pair wise sequence alignment from a BLAST report.

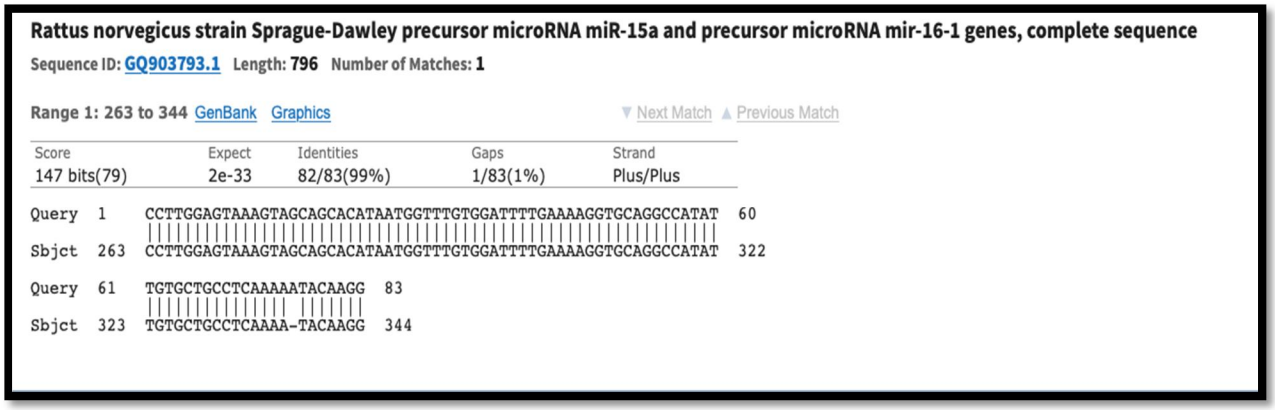

Figure: 4 Pair wise sequence alignment from a BLAST report.

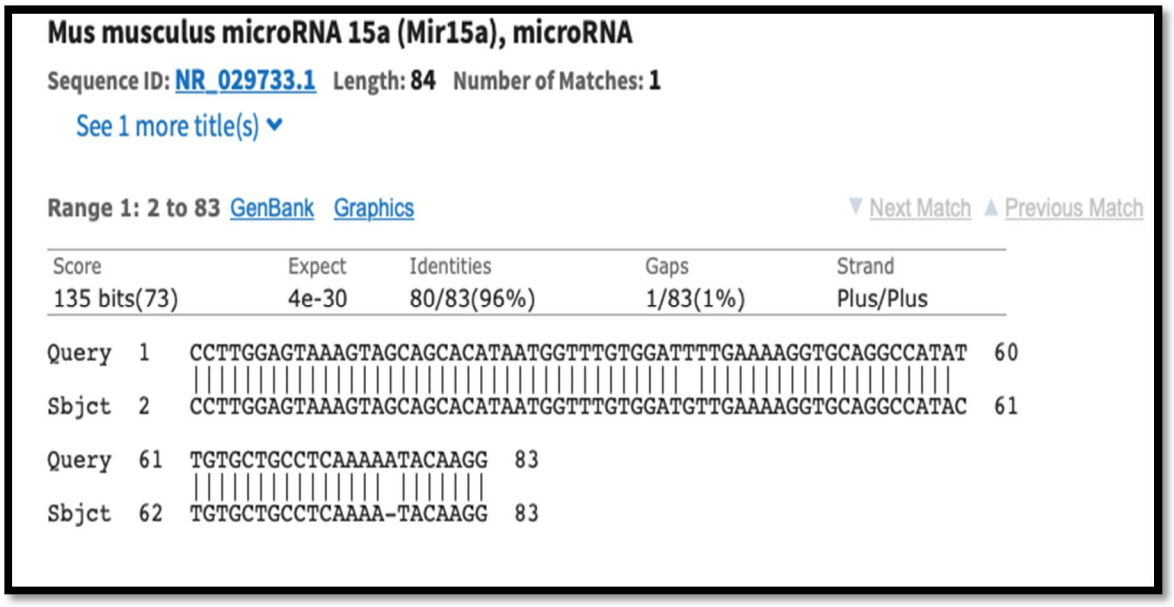

Figure: 5 Pairwise sequence alignment from a BLAST report.

In this case (fig.3, 4, 5), human microRNA-15a sequence is query sequence. Result demonstrated that the highest identity percentage and lowest E-value (1e-35) is of Macaca mulatta microRNA-15a sequence (100% identity); 99% identity with Rattus norvegicus; 96% identity with Mus musculus when compared with Homo sapiens with Macaca mulatta and Rattus norvegicus will also be almost similar as their sequences are almost conserved. Therefore, results (Fig.2) clearly predicted the conserved nature of the miR-15a among the various mammalian model organisms.

C. Target Prediction Tool miRmap

Raw miRmap scores for each feature, e.g. ΔG duplex' in kcal/mol and 'P. over exact' as a probability, can be converted to percentiles to simplify and standardize a scale from strong to weak re- pression. This is achieved by ranking targets for each species from the weakest to the strongest predicted repression for each individual feature and assigning scores to percentiles from 0 to 100%, with 100 representing the strongest repression. Users therefore do not have to be familiar with the ranges of different feature values, nor their directions, i.e. whether larger or smaller values correspond to higher or lower repression.

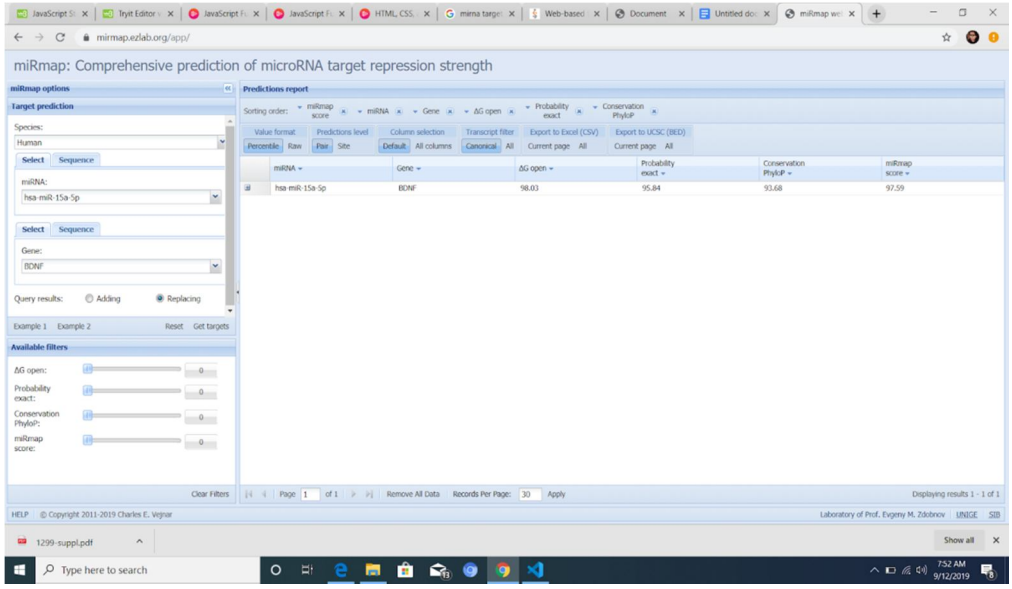

Figure: 6 Target prediction between miR-15a-5p and BDNF using miRmap target prediction tool.

miRmap is used to identify the target as *BDNF* for the selected miRNA hsa-miR-15a-5p and it showed a free energy about 98.03, probability about 95.84 and 97.59 miRmap score.

These outputs represents the suitable chances to target the selected gene. The novel feature based on PhyloP , which evaluates the significance of negative selection, is the best performing predictor in the evolutionary category. The higher values of the output represent the strongest repression.

D. DAVID

Functional annotation was done for 12 miR-15a targets having role in Retinopathy (searched with help of NCBI) using the clustering tool Database for Annotation, Visualization and integrated Discovery (DAVID), David provides a comprehensive set of functional annotation tool for investigators to understand biological meaning behind target list of genes, P-value for gene enrichment analysis I obtained from the functional annotation chart report by DAVID for gene list (Huang et al., 2014).

Table:1 Functional annotation cluster 1 of miRNA-15a predicted target genes for biological process characterization

Annotation cluster 1			
Category	Term	Count	P-value
GOTERM_BP_DIRECT	GO.0001666-RESPONSE TO hypoxia	4 (MTHFR, VEGF-A, *BDNF*, SMAD3)	0.19

Overlapping genes MTHFR, VEGF-A, *BDNF*, SMAD3 are linked biologically as they all are responsive to hypoxia and therefore are considered as significant, P-value of annotation cluster 1 is 0.19 respectively, which signifies cluster 1 to be considered strongly enriched in the annotation category.

Table:2 Functional annotation cluster 2 of miRNA-15a predicted target gene for biological process characterisation

Annotation cluster 2			
Category	Term	Count	P-value
GOTERM_BP_DIRECT	O:0002042=cell migration involved in sprouting angiogenesis	3 (VEGF-A, FGF2, *BDNF*)	0.14
GOTERM_MF_DIRECT	GO:0008083-growth factor activity	4 (*BDNF*, CTGF, VEGF-A, FGF2)	0.19

Overlapping genes of category GOTERM_BP_DIRECT-VEGF-A, FGF2, *BDNF* are linked biologically as they all are involved in same biological process i.e. angiogenesis. Overlapping genes of category GOTERM_MF_DIRECT-*BDNF*, CTGF, VEGF-A, FGF2 having same molecular function of growth factor activity. Therefore, genes of both categories are considered as significant. P-value of category GOTERM_BP_DIRECT and GOTERM_MF_DIRECT is a 0.14 and 0.19 respectively, which signifies cluster 2 to be considered strongly enriched in the annotation category. Functional annotation was done for gene list using the clustering tool available on Database for Annotation, Visualization and integrated Discovery (DAVID) . The clustering tool grouped genes that may be linked biologically i.e. similar genes were grouped in the same cluster. The threshold of EASE Score, a modified Fisher Exact P-value, for gene-enrichment analysis ranges from 0 to 1. Fisher Exact P-Value=0 represents perfect enrichment. Usually P-value is equal or smaller than 0.50 to be considered strongly enriched in the annotation categories. The genes that overlapped were especially considered as significant and will be validated for future studies. According to past studies, in diabetic retina, down regulated miRNA-15a up regulate VEGF-A and FGF2. Thus, STRING, and David result helped in predicting that VEGF-A, *BDNF* and FGF2 may be linked together and share some common pathways which includes PI3-K/Akt pathway that are required for cell proliferation and survival in many cell types.

E. KEGG

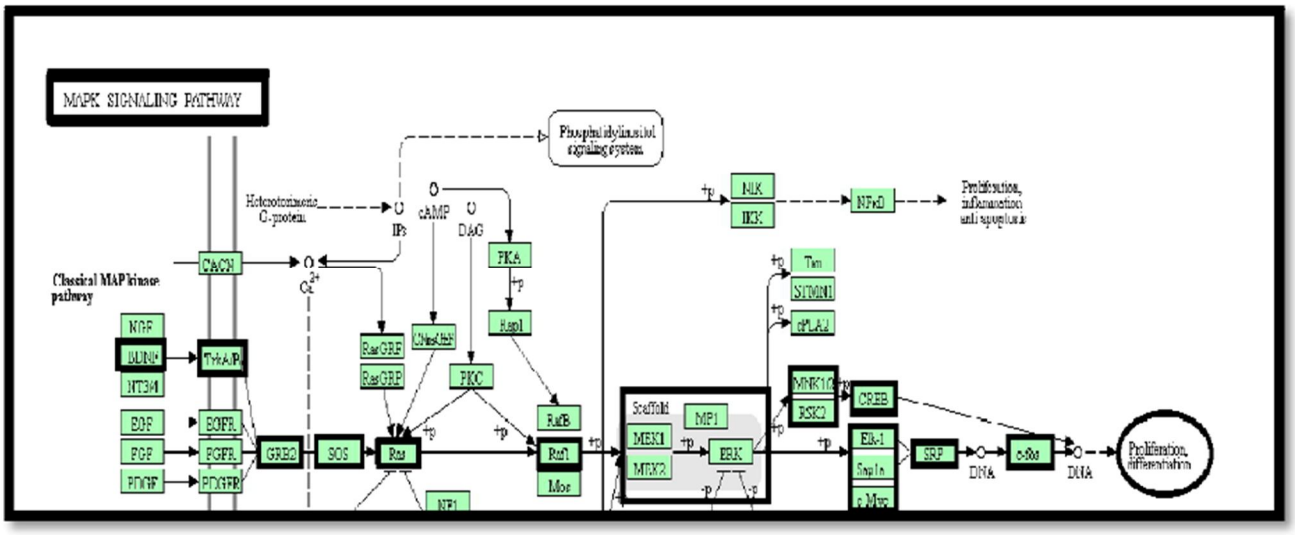

Figure: 7 *BDNF*-MAPK signalling pathway obtained by KEGG for T2DR

BDNF binds to its receptor protein TrkB which undergoes homodimerization, autophosphorylation and activation. It then recruits and activates several downstream effectors to regulate gene expression and cell survival/proliferation. KEGG database resource was used for Mitogen-activated protein kinases (MAPK) pathway enrichment analysis to predict the changes in the MAPK pathway when *BDNF* get upregulated in Type 2 Diabetic Retinopathy. When *BDNF* (cell growth factor) gets up regulated or over expressed, then as up regulated *BDNF* keeps on binding to its receptor TrkB , it leads to mutation of Ras which may have dramatic consequences for the cell. Mutated Ras protein is activated just like normal protein. Mutated active Ras-GTP is also able to bind and to activate the kinase Raf. A major difference between mutated and normal Ras protein is mutated protein loses capability to be inactivated by the GTPase activating protein (GAP). GAP can still bind to mutated Ras in its GTP bound form. However, GAP is not able to provide the domain that is crucial for activation of Ras-GTPase. GTP is not hydrolysed to GDP, consequently, mutated Ras-GTP stays active. The kinase Raf Stays bound to Ras-GTP and remains active. Raf phosphorylation and activates the increasing number of MEK1/2 proteins. In turn MEK1/2 activate ERK1/2 which activates *fun* and *fos.* Thus, kinase cascade is not turned off. A permanently activated MAPK pathway results in continuously activated cell proliferation which is important feature of mutated cell. Cells will lose the control over cell division and abnormal cells will keep on growing (Duman & Voleti, 2012). In T2DR patients, due to hyperglycaemia those continuously growing cell will be deprived of oxygen (hypoxia) and *BDNF* is responsive to hypoxia thus retina will not get nourishment by those cells, due to which retina will send signal to brain for more blood vessels to provide nourishment which leads to neovascularization/neoangiogenesis. Due to *BDNF* upregulation, highly activation of TrkB receptor expressed on a sub-population of retinal cells may contribute to neoangiogenesis. New vessel formation in T2DR causes visual loss with vitreous hemorrhage, retinal detachment, and neovascular glaucoma which are the complication of T2DR.

IV. CONCLUSION

It was concluded that the sequence retrieval of hsa-miR-15a-5p was the first and most important step for further sequence analysis. In miR-15a annotation, sequence retrieval helped identify target genes, their pattern of interaction using software. The pattern of interaction between hsa-miR-15a and the BDNF gene was very significant in predicting further changes in the T2DR-related pathways. From the results of the pathways predicted by different bioinformatics methods, it was finally concluded as a prediction that when hsa-miR-15a levels in my diabetic retina decrease, BDNF protein levels that increase which may cause more downstream protein targets and lead to T2DR neoangiogenesis. Therefore, the silico research predicted that hsa-miR-15a negatively controlled the expression of BDNF genes, and the results obtained from bioinformatics tools could be useful as a prognostic approach for T2DR.

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2) Conflict of Interests: There are no conflicts of interest.

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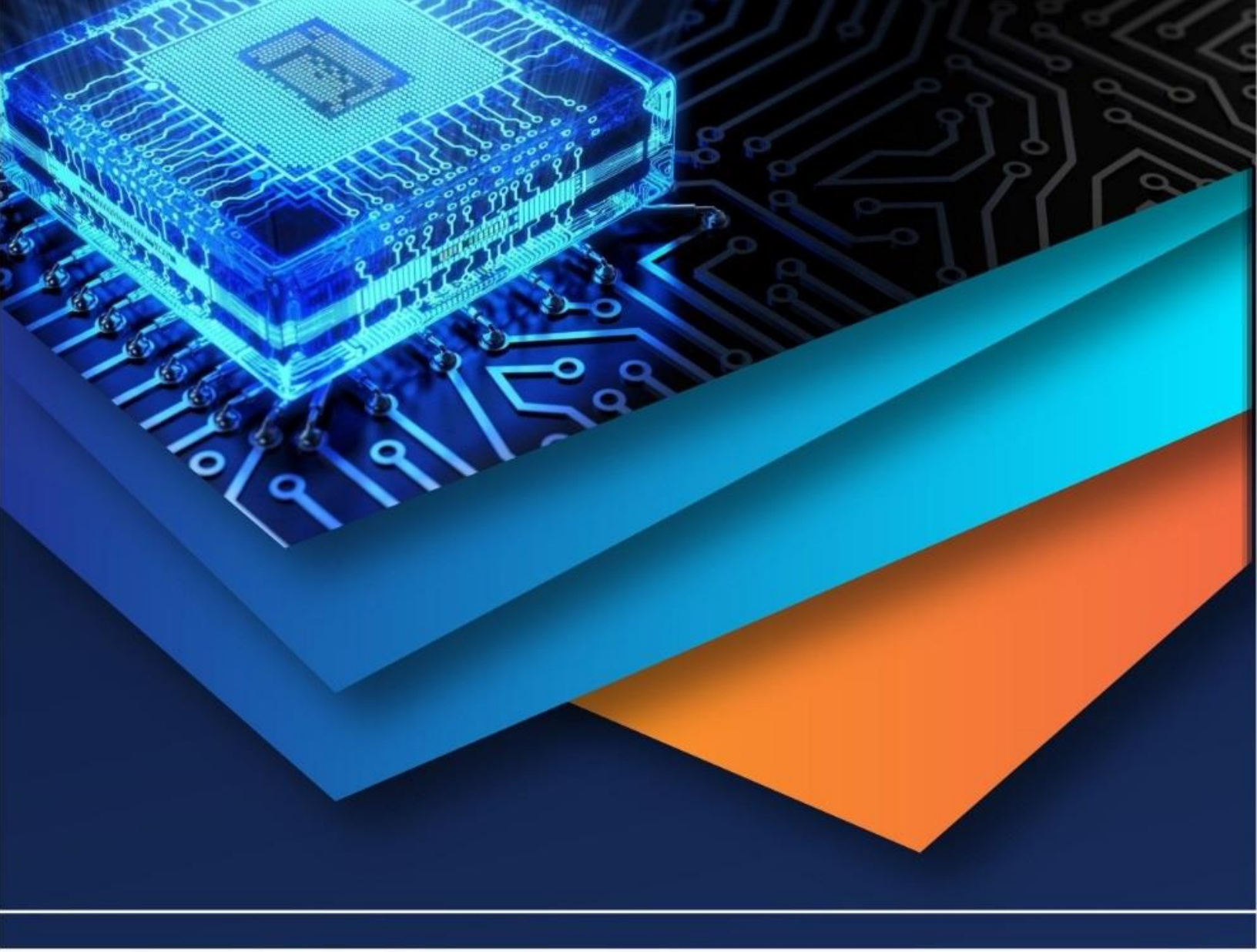

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