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In Silico Analysis of BDNF Gene Regulation by miRNA-15a: A Prognostic Approach towards Type 2 Diabetic Retinopathy

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Abstract: ***Type 2 Diabetic retinopathy (T2DR) is a serious sight-threatening disease and the leading cause of blindness among people with diabetes. Micro RNAs are small non-coding RNA molecules which play a key role in controlling gene expression and certain biological processes. Studies show that dysregulation of micro RNAs may lead to various diseases including T2DR, and thus, may be useful biomarkers for disease detection. Therefore, identifying biomarkers like microRNAs as a tool for the early and specific detection of T2DR, have great potential for diagnostic purposes. The main focus of this investigation, therefore, was the early detection of T2DR by analysing microRNA-15a and its potential target gene Brain Derived Neurotrophic Factor (BDNF) associated with the onset and progression of T2DR. An in silico approach was used to analyse regulation of BDNF expression by microRNA-15a in retina of Type 2 Diabetic Retinopathy patients. Publically available target prediction software were used for miRNA-15a target genes prediction and binding site analysis. Gene network enrichment analysis and functional annotation of microRNA-15a target genes which play role in of T2DR predicted the linkage, pathway and functional similarity between BDNF and other genes, using a web-based software namely STRING, Enrich Net and DAVID. Mitogen-activated protein kinase (MAPK) pathway enrichment analysis to predict the changes occurring in the pathway when BDNF gets dysregulated, using Signa Link and KEGG Pathway database for the pathogenesis of Type 2 Diabetic Retinopathy. Based on the bioinformatics results, this study predicted the regulation of BDNF expression by microRNA-15a which seemed to be one of the most promising miRNA for biomarker validation, due to the function of the target genes being associated with T2DR onset and progression. Future work, therefore, include validation of this prediction, using molecular approaches such as Quantitative Real-Time PCR, luciferase assays and western blots.***

Keywords- Type 2 Diabetic retinopathy, MicroRNA-15a, Brain Derived Neurotrophic Factor, Mitogen-activated protein kinase, Bioinformatics

I. INTRODUCTION

Type 2 Diabetic retinopathy (T2DR) is a serious sight-threatening disease and the leading cause of blindness among people with diabetes. T2DR is widely recognized as a neuro-vascular disease as opposed to being previously considered solely as a vascular disease (Peng et al., 2009; Van et al., 2010).T2DR is the result of damage to the tiny blood vessels that nourish the retina. They outflow blood and other fluids that roots swelling of retinal tissue and clouding of vision. The condition generally affects both eyes. The lengthier a person has diabetes, the more likely they will develop T2DR. If left untreated, T2DR can cause blindness (http://www.bvoptometry.com/diabetic-retinopathy/). Symptoms of T2DR include: Seeing spots or floaters in your field of vision, blurry vision, having a dusky or empty spot in the centre of your vision, difficulty seeing fine at night.

Previous studies have largely focused on the mechanisms underlying microvascular changes. Therefore, better understanding of the molecular mechanisms that lead to neuro degeneration is needed (Barber, 2003). Neuronal and glial tissues that are sensitive to hyperglycemia, might be involved in the pathogenesis of T2DR. Therefore, understanding the pathological changes which correlate with T2DR and the mechanisms that protect neurons, are expected to be important aims of future studies. Studies in animal models have confirmed that at early stages of T2DR, changes occur in Brain-derived neurotrophic factor (BDNF) expression (Fernyhough et al., 2003). BDNF is a member of the neurotrophin family of growth factors and is important in the development, differentiation and maintenance of nervous system. BDNF has been reported to protect retinal cells under conditions of ischemia and hypoxia and thus it plays major role in T2DR (Seigel et al., 2000).Tropomyosin-related kinase B (TrkB) is a receptor protein involved in the

development and maturation of the central and peripheral nervous systems. Upon ligand-binding, TrkB undergoes homodimerization, auto phosphorylation and activation. It then recruits and triggers several downstream effectors to regulate gene expression and protect neurons.

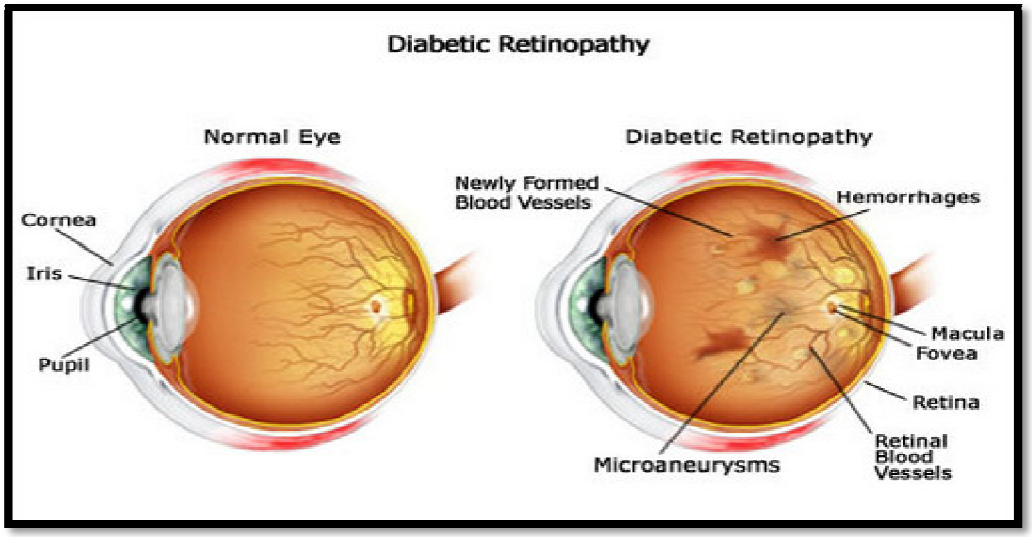

Figure.1: Difference between Normal eye and Type 2 Diabetic Retinopathy eye (http://www.bvoptometry.com/diabetic-retinopathy/)

In T2DR patient eye when compared to normal eye, microaneurysms or swelling, in the side of a blood vessel occurs. New abnormal blood vessels formation occurs which causes an escape of blood from a ruptured blood vessel i.e. haemorrhages.

The word “epigenetic” literally means “in addition to changes in genetic sequence”. MicroRNAs have avital role in epigenetic phenomenon (Chuang and Jones, 2007). MiRNAs are a large family of 20–22 nucleotide noncoding RNAs that are incorporated in numerous cellular processes (Jobe et al., 2012). Each miRNA has the target of multiple genes and multiple miRNAs may regulate one gene in turn. MiR-15a and miR-16 are clustered within 0.5 kilo bases at chromosome position 13q14, in humans. 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 field of bioinformatics, or systems biology, which is the merging of the computational and biological science disciplines, has been an important tool for the organisation and analysis of the vast amount of biological data (Lewis, 2008). The main aim of bioinformatics is to find key biological information hidden amongst a mass of raw data to identify important trends and patterns which would eventually lead to novel biomarker discovery for both diagnostic and therapeutic purposes (Anthony, 2015). BDNF, which plays major role in T2DR, is one of the target gene of miR-15a predicted with the help of bioinformatics software. Therefore, bioinformatics tools helped in analysing the interaction between miR-15a and BDNF, which in turn helped in predicting the changes occurring in the pathways of T2DR with the help of various online tools. In case of T2DR, number of studies were done on VEGF and anti-VEGF treatments provided the most fruitful T2DR therapy, but due to its side effects targeting on VEGF only was not the solution to prevent T2DR. Few studies have also shown that Anti-VEGF injections may not work for all (Keir et al., 2017). Therefore, there was a need for biomarkers which could be used as a tool for the early and specific detection of T2DR and to target more genes involved in T2DR. Previous studies showed that dysregulation of microRNAs may lead to various diseases including T2DR by targeting on its target genes thus leading to changes in expression level of those genes, and therefore, may be useful biomarkers for disease detection. To-date no in silico and in vitro study was done on miR-15a and its target gene BDNF which play very important role in T2DR and would be helpful in its prevention. Therefore, in the current study, BDNF expression due to down regulated miR-15a in diabetic retina was predicted with the help of bioinformatics tools. MiR-15a target prediction was done using target prediction tools. Gene network enrichment analysis, functional annotation of genes and pathway analysis was done using online bioinformatics tools. Thus, this in silico study predicted the regulation of BDNF expression by miR-15a for the pathogenesis of T2DR as a prognostic approach.

II. MATERIALS AND METHODS

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

A. Mirna-15a 5p And 3p Sequence Retrieval 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-15a-5p and hsa-miR-15a-3p along with their Accession numbers and ID was retrieved by scrolling down the particular page (Griffiths *et al.*, 2010).

B. Mirna-15a Sequence Analysis Using Blast

BLAST page was opened by clicking BLAST on popular resources menu from National Center for Biotechnology Information (NCBI, https://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 under Enter Query sequence in BLAST page. In the Organism option, other organisms such as *Rattus norvegicus*, *Mus musculus* were chosen to compare the miR-15a sequences with human query sequence. BLAST button was clicked to submit search and output was obtained (Madden, 2013).

C. Mirna-15a Target Identification And Binding Site Analysis

1) *miRDB:* miRDB was accessed using online link http://www.mirdb.org/. Web search page appeared where miRNA targets were searched by writing the particular miRNA for which targets are to be searched e.g. (hsa-mir-15a) or miRNAs could also be searched by searching gene symbol (e.g. BDNF) on the same page below the entry of search by miRNA name. Web page was appeared showing 2 Human miRNAs found for "hsa-mir-15a"- (i) hsa-miR-15a-5p targets (previously hsa-miR-15a); (ii) hsa-miR-15a-3p targets (previously hsa-miR-15a*). Click on one miRNA to retrieve its targets. Page showing number of target genes each of hsa-miR-15a-5p and hsa-miR-15a-3p was obtained. BDNF, VEGFA, FGF2, Smad3, MTHFR, G6PD, ITGA2, SLIT2, APLN, APLNR, CTGF, NAMPT were the obtained predicted targets of miR-15a. All of these miR-15a target genes play role in T2DR. Further, detailed analysis of miR-15a target BDNF gene was done for this study (Wong and Wang, 2014**).**
2) *DIANA Tool:* DIANA Tool was accessed using online link http://diana.imis.athena innovation.gr/Diana Tools index.php. MicroT-CDS was selected from microRNA target prediction DIANA Tools. MicroT-CDS software page was opened. BDNF gene symbol was entered in the search option to select its targeted miRNA. Result page appeared showing number of miRNAs along with hsa-miR-15a which confirmed that BDNF is the target of hsa-miR-15a.Clicked on hsa-miR-15a and the result was then interpreted (Paraskevopoulou *et al.*, 2013; Reczko *et al.*, 2012).
3) *MicroRNA.org:* MicroRNA.org tool was accessed using online link http://www.microrna.org/microrna/home.do. Home page of microRNA.org was appeared using the above link. MiRNA name was entered for which target gene was searched and clicked on Go. Result page of hsa-miR-15a target genes was obtained, one of the gene among them was BDNF. The result of hsa-miR-15a/BDNF alignment was interpreted (Betel *et al.*, 2008).
4) *PicTar:* Pic Tar was accessed by online link http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi. Home page appeared to enter microRNA ID. MicroRNA ID (hsa-miR-15a) was entered to search its target BDNF gene. Search for targets of a miRNA option was then clicked. Result page was obtained showing particular score values. Structure of predicted duplex was obtained from the result. Duplex structure was downloaded and results were interpreted (http://pictar.mdc-berlin.de/).

D. Bdnf Sequence Homology And Phylogenetic Analysis Using Clustal Omega

Clustal Omega was accessed using online link http://www.ebi.ac.uk/Tools/msa/clustalo/.Home page of Clustal Omega was appeared from the above link. BDNF protein sequences in FASTA format of the organisms Danio rerio, Mus musculus, Rattus norvegicus, Homo sapiens, Gorrila gorilla gorilla was retrieved from NCBI. Sequences file was then uploaded and used as input for the multiple sequence alignment. Submit button was clicked to obtain the results. Multiple sequence alignment and phylogenetic tree of the BDNF protein sequences of different organisms was obtained to predict the similarity of human protein sequence with other organisms (http://www.ebi.ac.uk/Tools/msa/clustalo/)**.**

E. Network Gene Enrichment Analysis Of Mirna-15a Target Genes In T2dr

1) *STRING:* STRING home page was opened by using link https://string-db.org/.Multiple proteins option was selected on the home page. Under list of names option, gene list was pasted. Clicked search. Then on the next page, *Homo sapiens* option was

clicked. Clicked continue. STRING result was displayed and interpreted. Clustering was done by clicked clusters option (K-means clustering). Clustering result was displayed and interpreted (Szklarczyk *et al.*, 2017).

2) *Enrich Net:* Enrich Net home page was opened by using link http://www.enrichnet.org/**.**Under the option Enter your gene/protein set of interest on the home page, upload the miR-15a target genes list. Identifier selected was HGNC symbol. Clicked on Go to next step. Next page displayed an option Choose an annotation database. KEGG as annotation database was selected. Result page was displayed showing annotation pathway process and results were interpreted. Graph computation was done by clicking on Compute graph visualization option and the results were interpreted (Glaab *et al.*, 2012).

F. Functional annotation of miRNA-15a target genes in T2DR using DAVID

List of all miR-15a target genes (verified by miRDB) which have role in T2DR was made with the help of National Center for Biotechnology Information by going to the link 'https://www.ncbi.nlm.nih.gov/'. DAVID home page was opened with the 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. Other option was to select Identifier. That button was clicked, from the drop down menu Official gene symbols was selected as gene symbols of gene names were used. Next step was to specify if its gene list or the background, Gene list was clicked and then submit list option was clicked. It gave all the species where these genes were identified. 'Homo sapiens' was selected. All the gene IDs were get recognized by DAVID. Clicked on select species button. Gene enrichment analysis was done. Annotation results page was displayed and the result was interpreted (Huang *et al.*, 2007).

G. Pathway enrichment analysis of BDNF gene

1) *Signa Link:* SignaLink home page was opened by home page link http://signalink.org/**.** Search option was clicked and BDNF was entered in the space provided under search option. Detailed information about BDNF was displayed. Clicked on the arrow for details of the interaction between pathway members of BDNF. Interaction between pathway members of BDNF result was displayed in the result**.** Post-transcriptional regulation result of miR-15a and BDNF was displayed and interpreted (Fazekas et al., 2013).

2) *KEGG:* KEGG Genome Net Web page was opened at *http://www.genome.jp/* and clicked on the. KEGG2 link located in the navigation bar at the top of the page. KEGG Table of Contents was displayed 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 "Human diseases" link to display the KEGG metabolic pathway data. Number of pathway names appeared which are linked to the respective diseases. Clicked on "04933 AGE-RAGE signaling pathway in diabetic complications". Clicked on MAPK signaling pathway in AGE-RAGE signaling pathway in diabetic complications. MAPK signaling pathway will be displayed showing BDNF role in MAPK pathway (Aoki and Kanehisa, 2005).

III. RESULTS AND DISCUSSION

A. Microrna-15a Sequence Retrieval Using Mirbase

Figure.2: Mature sequence of hsa-miR-15a-5p and hsa-miR-15a-3p

Sequence and structures of miRNA sequences were needed for understanding their function. Thus, stem-loop structure (83 nucleotide miRNA precursor) of hsa-mir-15a and mature sequence of hsa-miR-15a-5p and hsa-miR-15a-3p were retrieved from

miRBase. 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. According to past study (Molitoris and Molitoris, 2011), miRNA sequences should be of 20-25 nucleotides. In this study, miR-15a sequence obtained by miRBase is of 22 nucleotides. Thus, further analysis of sequence was done using other tools.

B. Mirna-15a Sequence Analysis Using Blast

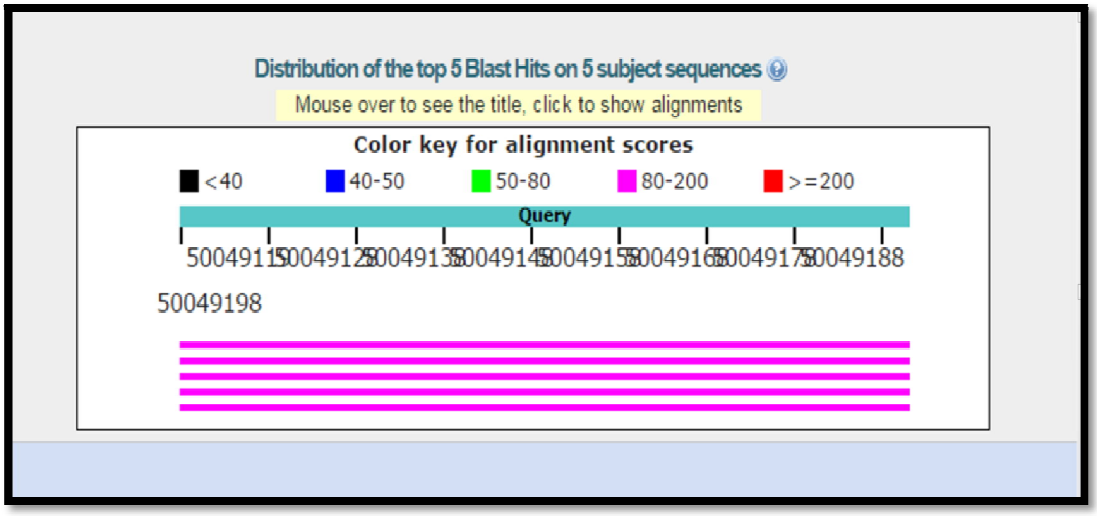

Figure.3: Graphical overview of BLAST result

The query sequence is represented by the numbered bar at the top of the figure 3. Database hits are shown aligned to the query, below the numbered bar. Of the aligned sequences, the most similar are shown closest to the query(human microRNA-15a).

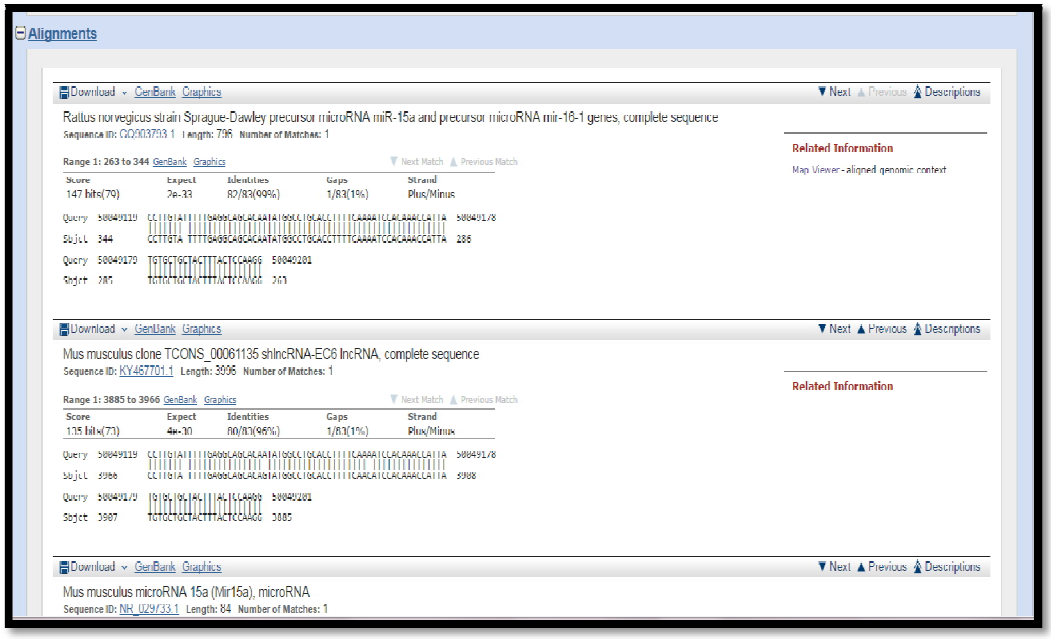

Figure.4: Pairwise sequence alignment from a BLAST report

Result demonstrated that the highest identity percentage and lowest E-value (2e-33) is of Rattus norvegicus microRNA-15a sequence (99% identity) when compared with query sequence. Therefore, results clearly indicated the conserved nature of the miR-15a among the various mammalian model organisms. It will help to infer the function of a sequence from similar sequence . Therefore, Rattus norvegicus can be used for better results for in vivo study of dysregulated miRNA-15a, which will be very helpful in identifying the changes occurring in BDNF gene in case of T2DR.

C. Mirna-15a target Identification And Binding site Analysis:

1) miRDB

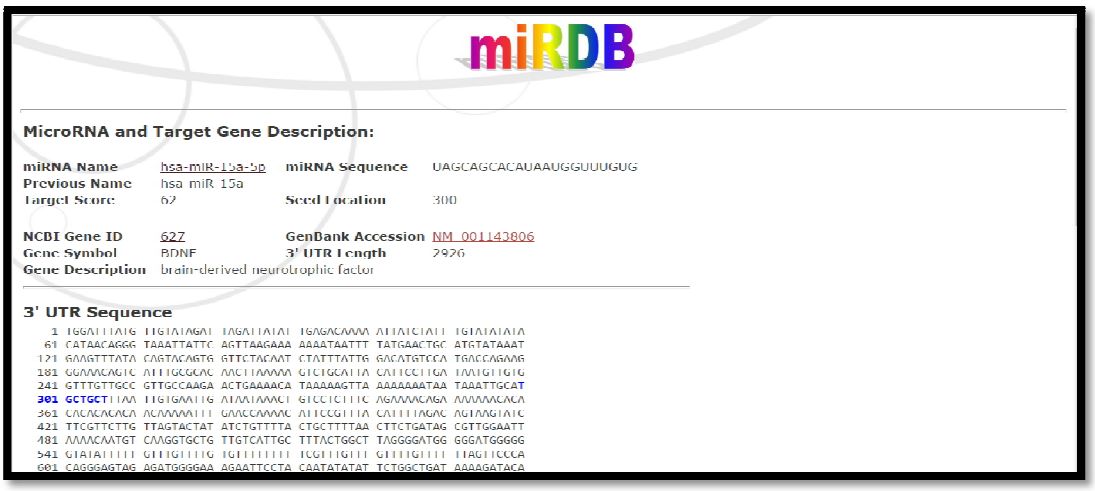

Figure.5: Target prediction data retrieved from miRDB

BDNF gene is the target of hsa-miR-15a-5p as predicted by miRDB. MiRNA seed location is at 300th nucleotide on 3'UTR sequence of BDNF. The target score is 62 which shows the high confidence in this prediction. As per the results obtained, BDNF is the target of hsa-miR-15a-5p. According to the past literature reviewed (Wong and Wang, 2014), the predicted targets should have scores between 50-100. If target score is more than 60, it comes under high confidence prediction. Therefore, the result obtained in this study has high confidence in prediction the target of miR-15a.

2) DIANA TOOL (microT-CDS)

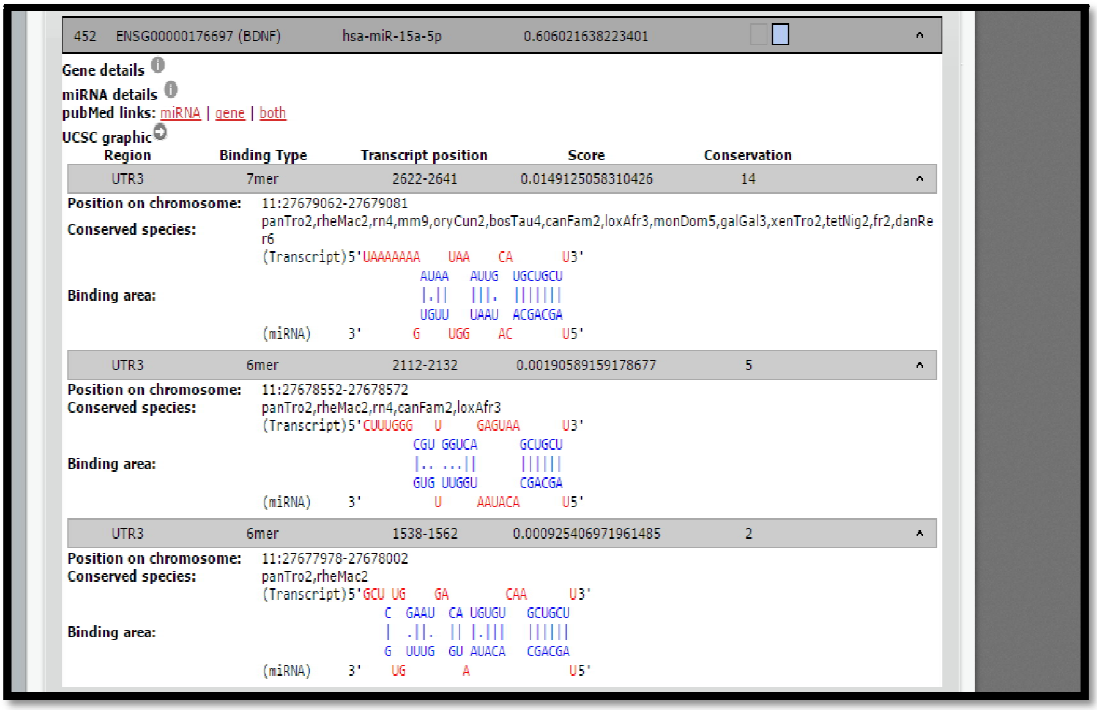

Figure.6: The figure showing information regarding the specified predicted miRNA15a–BDNF mRNA interaction

The miTG score of miRNA and target gene binding is 0.606021638223401. According to past literature reviewed (Paraskevopoulou et al., 2013; Reczko et al., 2012), miRNA target gene (miTG) score should be higher than the threshold value '0.5' which

corresponds to higher possibility of correct prediction. The miTG score obtained in this study is higher than the threshold value. Thus, binding interactions predicted with the help of this tool is correct.

3) MicroRNA.org

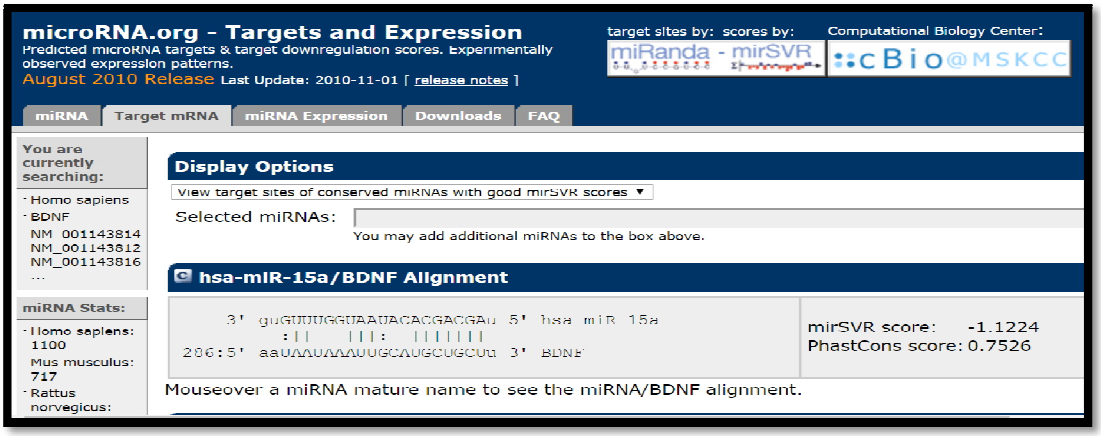

Figure.7: The figure showing hsa-miR-15a/BDNF alignment mirSVR score and PhastCons score

Alignment result shows that mirSVR score value is "-1.1224". PhastCons score value is "0.7526" which tells the conservation of nucleotide positions across multiple vertebrates. According to past study (Siepel *et al.*, 2005), the score value should be between 0-1. Thus, score value obtained in this result was 0.7526, which is a good score.

4) Pic Tar

Table.1: Pic Tar score, probability and free energy of miR15a-BDNF interaction

Organism	MicroRNA	Target gene	PicTar final score	PicTar cut-off value	Probability	Free energy
Homo sapiens(hsa)	MicroRNA-15a	BDNF	2.45	2.00	0.87	-18.1

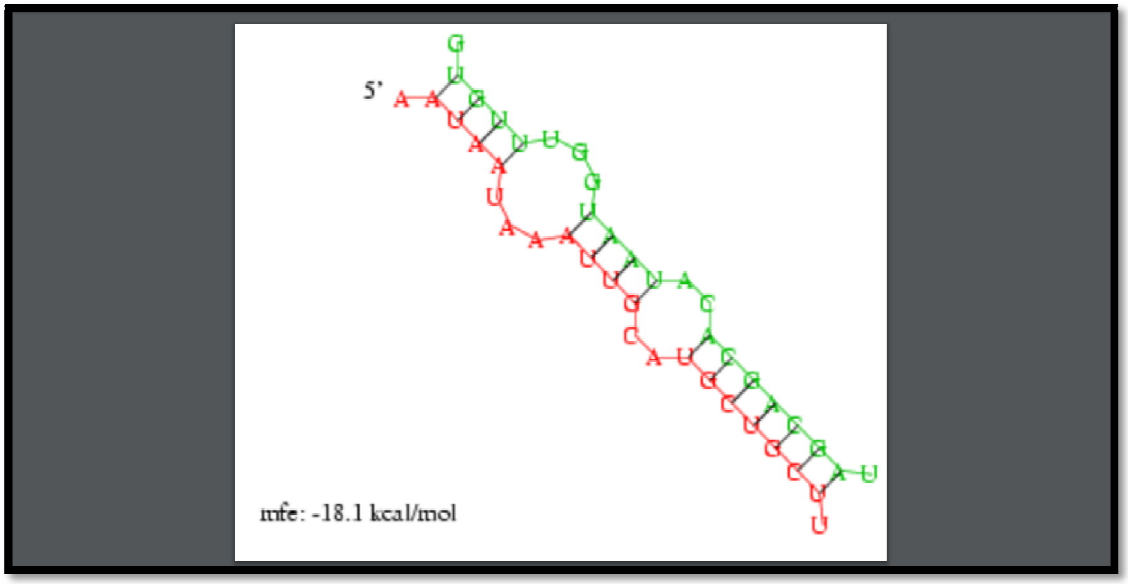

Figure.8: Structure of predicted duplex (miRNA-15a: mRNA BDNF) by PicTar

PicTar computed score is 2.00 reflecting the likelihood that 3'UTR of BDNF mRNA is targeted by miRNA-15a based on a hidden Markov model. Probability of binding is 0.87 and the free energy calculated is "-18.1" showing perfect matching of UTR with the miRNA seed region. The result indicated the good conservation and perfect binding of duplex.

D. Target gene (BDNF) sequence homology and phylogenetic analysis using Clustal Omega

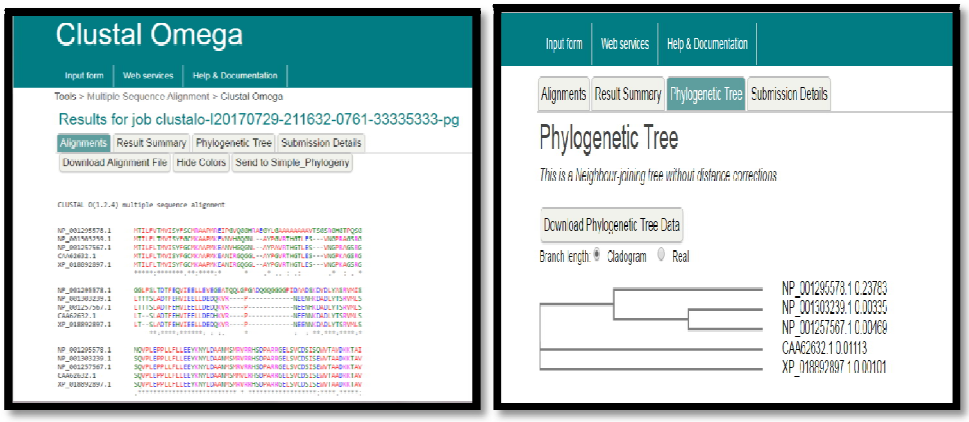

Figure.9: Multiple sequence alignment and Phylogenetic Treeby Clustal Omega

The multiple sequence alignment and Phylogenetic Tree of BDNF protein sequence between Homo sapiens (query sequence) and other species was done using Clustal Omega. Rattus norvegicus and Gorilla gorilla gorilla shows highly conserved nature with Homo sapiens, as obtained in the result. Thus, the changes occurring in BDNF sequence can be analysed by in vivo study in any of these organisms for the pathogenesis of T2DR. Therefore, with BLAST and Clustal Omega, this comparative analysis result (between other organism and human) of miRNA-15a sequences and BDNF gene sequences would be helpful in identifying the changes occurring at a particular stage in T2DR.

Table.2: BDNF protein NCBI Reference Sequence of 5 different species for Clustal Omega

S.No.	NCBI Reference Sequence (BDNF Protein)	Species
1.	NP_001295578.1	Danio rerio
2.	NP_001303239.1	Mus musculus
3.	NP_001257567.1	Rattus norvegicus
4.	CAA62632.1	Homo sapiens
5.	XP_018892897.1	Gorrila gorilla gorilla

E. Gene network enrichment analysis and Functional annotation of microRNA-15a target genes in case of Type 2 Diabetic Retinopathy

1) String

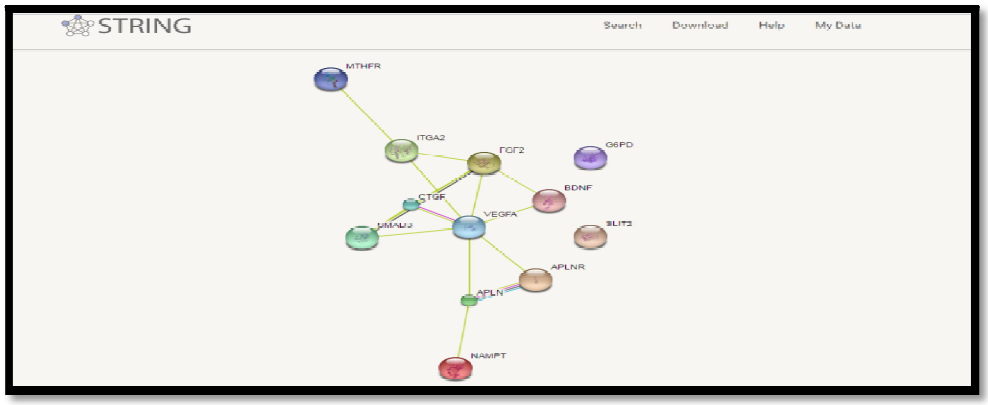

Figure.10: Protein-Protein interaction network visualized by STRING

According to results obtained, FGF2, VEGFA and BDNF shows functional and pathways similarity. Thus, alteration in FGF2 and VEGF will majorly affect the expression of BDNF in vascular endothelial cell through autocrine and paracrine mechanisms which could enhance neoangiogenes is in patients suffering from T2DR.

2) Enrich Net

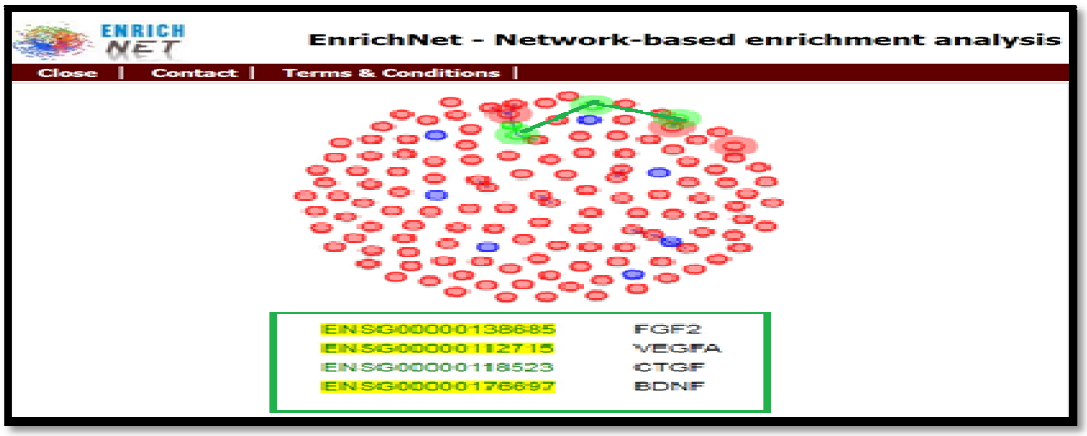

Figure.11: Interactive sub-network visualization of FGF2, VEGFA, BDNF (green in colour) by EnrichNet

The result of gene enrichment analysis using information from molecular interaction networks/process interpret that FGF2, VEGFA, BDNF share some of the common pathways and are linked together. Thus, if any change occur in one gene, it will affect other linked genes simultaneously.

F. Functional Annotation Of Mirna-15a Target Genes In T2dr Using David

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 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 VEGFA and FGF2 in case of T2DR. Thus, STRING, Enrich Net and DAVID result helped in identifying that VEGFA, BDNF and FGF2 are linked together and share some common pathways. Therefore, based on the results, it was predicted that BDNF will also get up regulated due to down regulated miRNA-15a in case of T2DR.

G. Pathway Enrichment Analysis Of Mirna-15a Target Gene Bdnf In T2dr

1) Signa link

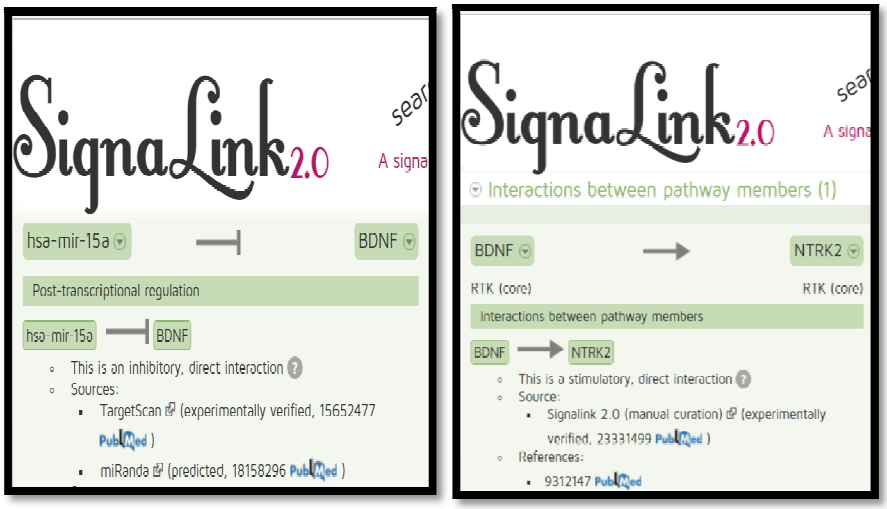

Figure.12: Results of Post-transcriptional BDNF gene expression regulation by hsa-mir-15a and Interaction between BDNF and NTRK2/TrkB in RTK/MAPK pathway

The post transcriptional regulation process showing the inhibitory interaction between hsa-miR-15a and BDNF. This clearly indicate that if due to several factors hsa-mir-15a is downregulated then BDNF expression may get upregulated or vice versa.The GO semantic similarity confidence score generated by the interaction between BDNF and NTRK2/TrkB is 0.538474. The direct interaction of BDNF and its receptor NTRK2/TrkB (located at cellular membrane e.g. at cellular membrane of retinal cells) generate impulse that travels through cells to the brain. This interaction score indicate that BDNF is negatively regulated by hsa-miR-15a which means that when hsa-miR-15a is downregulated then BDNF level will get upregulated.

2) KEGG

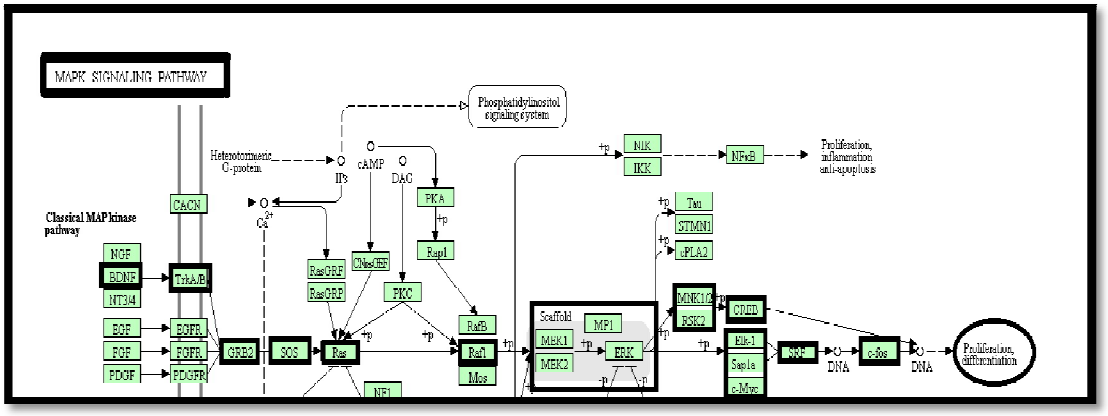

Figure.13: BDNF-MAPK signaling pathway obtained by KEGG for T2DR

BDNF binds to its receptor protein TrkB which undergoes homodimerization, auto phosphorylation and activation. It then recruits and activates several downstream effectors to regulate gene expression and cell survival/proliferation. In T2DR patients, due to hyperglycaemia those continuously growing cells 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 neovascularisation neoangiogenesis. Due to BDNF upregulation, highly activation of TrkB receptor expressed on a sub-population of endothelial cells (ECs)/ retinal cells contribute to neoangiogenesis. New vessel formation in T2DR causes visual loss with vitreous hemorrhage, retinal detachment, and neovascular glaucoma which are the complications of T2DR.

IV. CONCLUSIONS

hsa-miR-15a-5p and hsa-miR-15a-3p sequences retrieval was first and very important step for further analysis of sequences**.** Sequence retrieval helped in understanding the number of nucleotides present, target genes and their interaction pattern and functionality of the miR-15a sequence by using software. Interaction pattern between miR-15a and BDNF gene was very important to further predict the changes occurring in the pathways related to T2DR. From the pathways results predicted by various bioinformatics tools, it was finally concluded that as hsa-miR-15a level gets decreased in diabetic retina/retinal endothelial cells, BDNF protein levels may get increase which in turn will activate further downstream protein targets and will contribute to neoangiogenes is leading to T2DR. Thus, controlling the miR-15a levels may help in preventing this abnormal phenomenon which will help to prevent T2DR.

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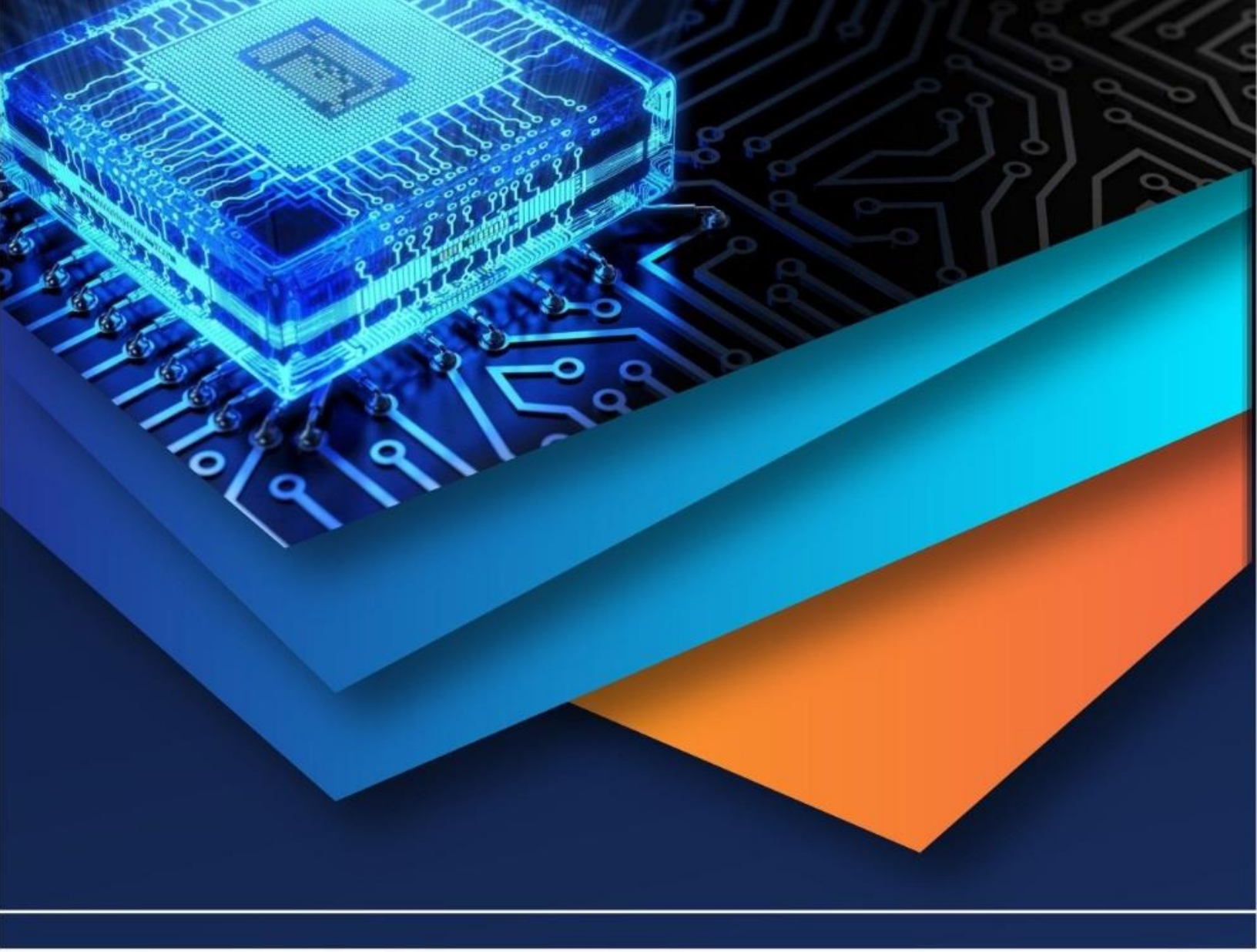

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