

An In Silico Comparative and Pathway Analysis of Targeted Genes of Diabetic Retinopathy: A Prognostic Approach towards Diabetic Retinopathy

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ABSTRACT:Diabetic retinopathy is the leading cause of visual loss in working-age of adults and is characterized by retinal neurodegeneration and microvascular abnormalities in the eye. Without an early screening and effective diagnosis, patients with DR will develop serious complications. MicroRNAs are small non-coding RNA molecules which play a key role in controlling gene expression and certain biological processes. Studies show that dysregulation of microRNAs may lead to various diseases including DR, and thus, may be useful biomarkers for disease detection. Therefore, identifying biomarkers like microRNAs as a tool for the early and specific detection of DR have great potential for diagnostic purposes. The main focus of this investigation, therefore, is the early detection of DR by the identification and validation of novel biomarkers. Furthermore, based on previous studies, the aim of the investigation was to identify differentially expressed miRNAs as well as identify their potential target genes associated with the onset and progression of DR. An in-silico approach was used to identify miRNAs found to be differentially expressed in the serum/plasma of DR individuals. mirwalk which is publically available target prediction software was used for a compressive evaluation of target gene prediction of the identified miRNA. The target genes were subjected to functional analysis using web-based software, namely DAVID. Functions which were clustered with an enrichment score > 1.3 were considered significant. mirwalk was also used for pathway analysis of target gene and its appropriate miRNA.

KEYWORDS:Diabetic retinopathy, micro-RNA target prediction, novel biomarkers,

I. INTRODUCTION

Diabetic retinopathy (DR) is a common microvascular complication of diabetes and the leading cause of legal blindness among people with diabetes [1, 4, 49]. Due to the increased lifespan of diabetic patients, the prevalence of DR will continue to increase as a result of the longer duration of diabetes [5, 9]. Non-invasive diagnostic methods have been shown a lot of interest. Specifically, diagnostic methods which are quick, painless, cost-effective, and sensitive as well as being specific for a particular disease [3, 11]. Most of the current diagnostic tests for most diseases usually involve painful or uncomfortable methods for retrieving samples e.g. tissue samples from biopsies [7, 10, 51]. Particular interest is also focussed on diagnostic methods which can detect diseases in its early stages. If diabetic retinopathy were to be diagnosed in its early stages, the development of overt diabetic retinopathy, and its long-term complications, can be avoided. miRNAs have shown remarkable promise as potential biomarkers for the early (and specific) detection for DR, as well as for other diseases, due to its stability compared to other biomolecules such as mRNA and proteins. The

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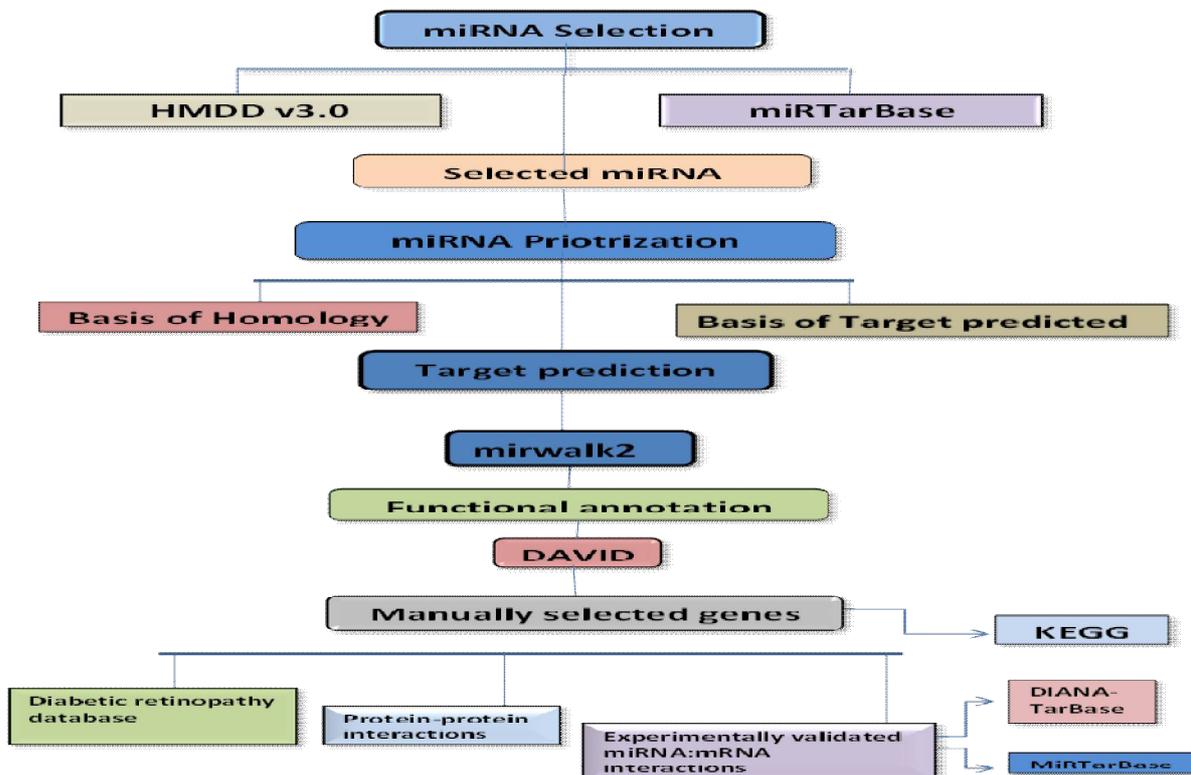
aim of this project, therefore, was to identify and analysis differentially expressed miRNAs in DR as well as to identify their potential target genes.

II. MATERIAL AND METHODS

Procedure	Database	Website
miRNA selection	HMDD v3.0	http://www.cuilab.cn/hmdd
	miRTarBase	http://mirtarbase.mbc.nctu.edu.tw/php/index.php
miRNA sequences	miRBase	http://www.mirbase.org/
Alignment of sequences	ClustalW2	http://www.ebi.ac.uk/Tools/msa/clustalw2/
Target prediction	mirwalk2	http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/generetsys-self.html
Functional annotation of target genes	DAVID v6.7	https://david.ncifcrf.gov/
Functional annotation of miRNAs	HMDD v3.0	http://www.cuilab.cn/hmdd
Network and pathway enrichment	STRING 10	http://string-db.org/
Experimental validation	DIANA-TarBase	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index
	miRTarBase	http://mirtarbase.mbc.nctu.edu.tw/php/index.php

Table 1.1 represented the list of methodology used in this investigation

Figure 1.1 Schematic representation of microRNA target prediction workflow.



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1. miRNA dataset selection

Experimentally validated microRNAs (miRNAs), which were shown to be differentially expressed in diabetic retinopathy, particularly in the blood/plasma/serum/tissue/cells, was selected from publically available databases i.e. the Human MicroRNA Disease Database v.2.0 (HMDD) (<http://202.38.126.151/hmdd/tools/hmdd2.html>) [34] and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/php/index.php>) [21,31].

2. miRNA prioritisation

A shortened list of miRNAs were obtained

(i) After target prediction and (ii) If human miRNAs were not 100 % homologous to rats.

miRNAs were eliminated if it did not generate a predicted target gene list, or if the target prediction software did not generate a statistically significant gene list. Lastly, 100 % homology of the mature human miRNA sequences to rats was only considered. Mature miRNA sequences of interest were obtained for both humans and rats from miRBase (<http://www.mirbase.org/>) [22,29]. The sequences of each specific miRNA from rats and humans were pairwise aligned using the ClustalW tool [21, 31]. Only sequences with a score of 100 % were considered for further analysis in this investigation. A list of miRNAs was generated after applying the above-mentioned criteria.

3. Target gene prediction

Target prediction was performed for each of the miRNAs obtained. The target prediction program used was mirwalk (<http://mirwalk.uni-hd.de/>) is a publicly available comprehensive resource. A comparative platform of miRNA-binding sites, resulting from ten different prediction datasets [27, 29, 50].

3.1 Target Prediction procedure

A total of 11,492 genes were obtained after a comprehensive evaluation of predicted targets of miRNA from mirwalk (<http://mirwalk.uni-hd.de/>) Before functional annotation, the selected genes were compared against 229 known genes involved in diabetic retinopathy, 102 genes were obtained from the Diaretinopathy database –A Gene database for diabetic retinopathy The database is freely available at <http://diaretinopathydatabase.com> and 166 genes were obtained from T2DiACoD: A Gene Atlas of Type 2 Diabetes Mellitus Associated Complex Disorders. The T2DiACoD is available at www.t2diacod.igib.res.in/. Two genes lists were obtained from these databases and combine, 39 duplicates genes were removed and retain 229 genes were obtained. To investigate whether the predicted target genes were previously linked to DR the VENNY tool v. 2.0 (<http://bioinfogp.cnb.csic.es/tools/venny/>) [40] was used to create Venn diagrams. The predicted targets genes list were compared with these 229 diabetic retinopathy genes a Venn diagram was generated and 109 common genes were found in predicted target gene list furthermore separately 7 Venn diagrams were also created from each micro RNA targeted genes list with known 229 diabetic retinopathy genes list.

4. Functional annotation of predicted target genes and miRNAs

Functional annotation was done separately for each gene list using the clustering tool available on DAVID (The Database for Annotation, Visualization and Integrated Discovery) (<http://david.abcc.ncifcrf.gov/>) [24]. Default parameters were used for functional annotation i.e. classification parameters were set to “Medium” and raw p-values were used [23]. Moreover, gene clusters with an enrichment score of > 1.3 [14]. By selecting the functional annotation summary and table, all data including biological processes, metabolic function and functional analysis of proteins were retrieved for all genes in the list. All genes mapped to the KEGG pathway database were selected. Genes potentially linked to DR were selected based on their regulatory role in metabolic pathways previously associated with the development of the disease. Thereafter, the roles of these genes in DR were confirmed using the genes list obtain from disease databases which provides information on candidate genes linked with DR.

MiRNA:gene interactions potentially linked to DR were further investigated using experimentally validated miRNA:mRNA target prediction databases such as DIANA-TarBase v7.0 (<http://www.microrna.gr/tarbase>) and miRTarbase v6.0 (<http://miRTarBase.mbc.nctu.edu.tw/thout>). For DIANA-TarBase, miRNAs of interest were inserted into the search box, and filters including, Homo sapien, normal and high throughput method types, target genes and interaction information was available by selecting the information links. For miRTarbase, standard default settings were used. a list of publications supporting each interaction, the experimental methods used for validation along with their outcomes, and links related to KEGG pathways and other external databases [14,15,29].

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5. Protein-protein interactions

To identify known and predicted protein-protein interactions of miRNA target gene/proteins, STRING v10.0 was used (<http://string-db.org/>). On the home page, the name of a predicted miRNA target gene/protein was inserted into the search box, and the species Homo sapien was selected to restrict the search. After identification of proteins, the protein network was inspected for interactive evidence, and the confidence score cut-off was set at 0.900 or greater to filter out false positives. Thereafter, a limit was set to show no more than 10 interacting proteins[21, 29].

6. Functional analysis of microRNAs

To determine the functional role of miRNAs in disease pathogenesis, the Human miRNA Disease Database (HMDD) v2.0 was used (<http://www.cuilab.cn/hmdd>) [34].

7. miRNA–target pathway interactions

For the miRNA–target pathway interactions, pathways can be interrogated by choosing a pathway (Biocarta or KEGG) from the drop-down menu of mirwalkdatabase. For the identification of miRNA–target pathway interactions, pathway interactions were performed by using mirwalk for, miRNA 21 and their targeted gene IL1B furthermore for the identification of their link with diabetic retinopathy KEGG disease pathway analysis were performed. KEGG database were available at <http://www.genome.jp/>. KEGG database interface shows numerous pathways on KEGG database “Human diseases” were selected. 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 role in MAPK pathway [2,26].

8. A comparative miRNA target prediction

comparative view of miRNA-binding sites identified by mirwalk and other eight established programs within the mRNA 3'-UTR region and Predicted miRNA-binding sites within the promoter region. has-miR-21 and their targeted gene IL1B, their comparative miRNA target prediction was performed by using mirwalk comparative miRNA target search[29].

III. RESULTS AND DISSCUTIONS

1. miRNA dataset selection

Lists of 11 miRNAs from HMDD and, miRTarBase respectively, were obtained The two lists were combined and duplicate miRNAs were eliminated, which resulted in a final list of 10 miRNAs in total Thereafter, this list was shortened even further if the mature miRNA sequences were not 100 % homologous to those in rats. A total of seven miRNAs was identified as significant for further analysis in this investigation.

Table 1.1summary of miRNAprioritisation

Procedure	Number of miRNAs eliminated	Total miRNAs
miRNA collection from databases	0	11
Elimination of duplicates	1	10
Target prediction	10	10
Homology	3	7*

*MicroRNAs of interest

2. Target gene prediction

A total of 11,492 genes were obtained after a comprehensive evaluation of predicted targets of miRNA from mirwalk. In a predicted 11,492 genes, 6392 duplicates genes were removed and the remaining 7289 genes were only retained bringing the putative target gene list. In putative target gene list 109 genes were found common in combine diabetic retinopathy genes list for the separated investigation for each miRNA putative target gene list common genes for disease respectively find (table.1.2)

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Table 1.2 lists for the separated investigation for each miRNA putative target gene

miRNA	Target genes	DR genes	Common with DR genes
hsa-mir-181c	2190	109	34
hsa-mir-21	1147	109	14
hsa-mir-200b	2099	109	30
hsa-mir-152	1893	109	40
hsa-mir-195	2387	109	35
hsa-mir-27b	2462	109	31
hsa-mir-126	370	109	10

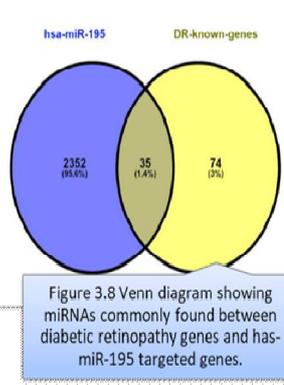
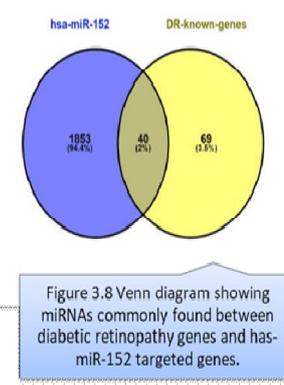
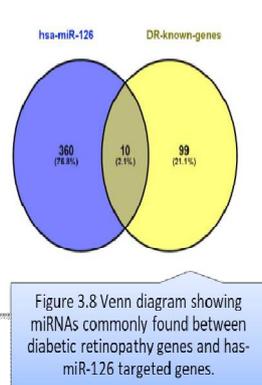
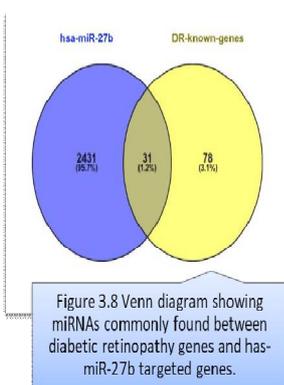
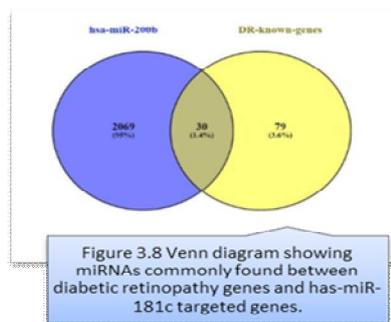
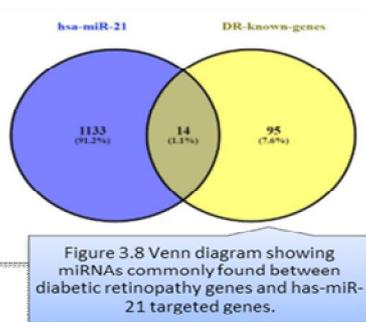
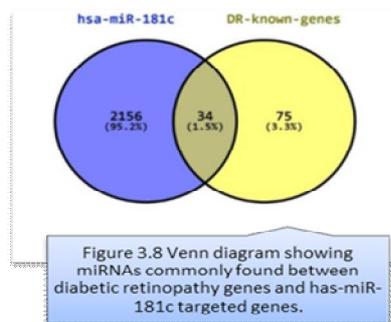


Figure 1.2 Venn diagrams to investigate whether the predicted target genes were previously linked to DR.

3. Functional analysis of predicted targets

Commonly predicted mRNA target gene lists for the seven miRNAs (Table 1.2) were copied into DAVID for pathway analysis. Results of the individual miRNAs and their predicted miRNA targets are indicated. Predicted miRNA target genes were mapped to KEGG pathways separately each. Of these, predicted target genes were selected based on their potential role in DR disease progression. Thereafter, the role of these targets in DR was confirmed using the diseased databases.

For miR-181c, 675 of the 2190 mRNA targets predicted by mirwalk mapped to KEGG pathways. Of these, three genes; SP1, THBS2 and IL1B, which play a role in the TGF-beta signalling and MAPK signalling pathway respectively were selected as genes linked to DR.

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For miR-21, 456 of the 1147 mRNA targets predicted by mirwalk mapped to KEGG pathways. Of these, eight genes; INSR, AKT3, IL1B, PRKCD, KITLG, ITGA2, PTGS2 and STAT3, which play a role in the Insulin signalling pathway, MAPK signalling, TGF-beta signalling and neurotrophin signalling pathway respectively were selected as genes linked to DR.

For miR-200b, 607 of the 2099 mRNA targets predicted by mirwalk mapped to KEGG pathways. Of these, eight genes; AKT3, VEGFA, ROCK2, SP1, CRK, IRS1, MAPK7 and PPARGC1A, which play a role in the Insulin signalling pathway, MAPK signalling, TGF-beta signalling and neurotrophin signalling pathway respectively were selected as genes linked to DR.

For miR-152, 903 of the 1893 mRNA targets predicted by mirwalk mapped to KEGG pathways. Of these, five genes; INSR, IRS1, PPARGC1A, SOCS3 and SP1 which play a role in the Insulin signalling pathway, T2DM, TGF-beta signalling and neurotrophin signalling pathway respectively were selected as genes linked to DR.

For miR-195, 1123 of the 2387 mRNA targets predicted by mirwalk mapped to KEGG pathways. Of these, seven genes; GRB2, MAPK3, INSR, IRS1, AKT3, RAF1 and VEGFA which play a role in the Insulin signalling pathway, T2DM, TGF-beta signalling and VEGF signalling pathway respectively were selected as genes linked to DR.

For miR-27b, 465 of the 2462 mRNA targets predicted by mirwalk mapped to KEGG pathways. Of these, seven genes; SP1, GRB2, INSR, IRS1, AKT3, MAPK7 and PPARGC1A which play a role in the Insulin signalling pathway, T2DM, MAPK signalling, TGF-beta signalling and neurotrophin signalling pathway respectively were selected as genes linked to DR.

For miR-126, 103 of the 370 mRNA targets predicted by mirwalk mapped to KEGG pathways. Of these, three genes; IRS1, CRK, and PPARGC1A, which play a role in the Insulin signalling pathway, T2DM and neurotrophin signalling pathway respectively were selected as genes linked to DR.

Table 1.3 miRNAs and their mRNA targets. Genes listed were identified to play a potential role in DR-related pathways, identified using the DAVID/KEGG pathway database.

miRNA	No. of predicted mRNA targets	No. of linked to KEGG pathway	pathways linked to DR*	Genes linked to DR*	Role of genes in DR related metabolic pathways
has-mir-181c	2190	675	4	INSR	Insulin signalling
				AKT3	Insulin signalling,
					MAPK signalling,
					TGF-beta signalling
				IL1B	Neurotrophin
				PRKCD	MAPK
KITLG ITGA2 PTGS2 STAT3	TGF-beta signalling				
has-mir-21	1147	456	2	SP1	TGF-beta signalling
				THBS2	
				IL1B	
has-mir-200b	2099	607	4	AKT3	MAPK signalling
					insulin signalling
					neurotrophin signalling
				VEGFA	MAPK
				ROCK2	TGF-beta signalling
				SP1	TGF-beta
CRK	Neurotrophin signalling				
	insulin signalling				

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				IRS1	Neurotrophin and insulin signalling
				MAPK7	Neurotrophin
				PPARGC1A	insulin signalling
hsa-mir-152	1893	903	4	INSR	Insulin signalling T2DM
				IRS1	Insulin and T2DM , Nerotrophin
				PPARGC1A	Insulin signalling
				SOCS3	Insulin signalling
				SP1	TGF BETA signalling
hsa-mir-195	2387	1123	4	GRB2	Insulin signalling
				MAPK3	TGF-beta Signalling insulin signalling VEGF signalling, T2DM
				INSR	Insulin signalling T2DM
				IRS1	Insulin signalling T2DM
				AKT3	Insulin signalling VEGF signalling
				RAF1	Insulin signalling VEGF signalling
				VEGFA	VEGF signalling
hsa-mir-27b	2462	465	5	SP1	TGF-BETA Signalling
				GRB2	MAPK Signalling, insulin signalling nurotrophin signalling
				INSR	Insulin signalling T2DM
				IRS1	Insulin signalling T2DM
				AKT3	MAPK signalling insulin signalling nurotrophinsignaaliing
				MAPK7	MAPK signalling nurotrophin signalling
				PPKCD	Neurotrophin
hsa-mir-126	370	103	3	IRS1	T2DM Neurotrophin insulin signalling
				CRK	Neurotrophin insulin signalling
				PPARGC1A	Insulin signalling pathway

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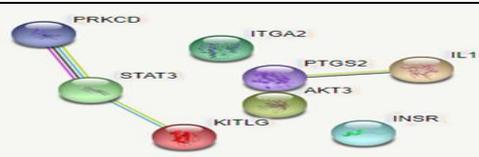
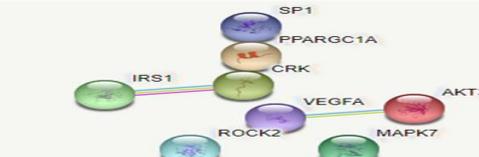
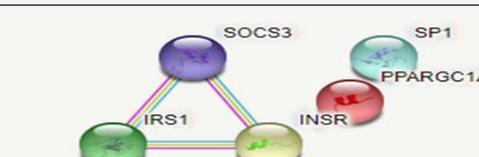
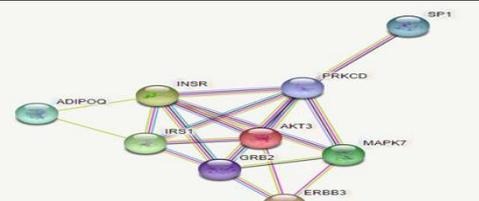
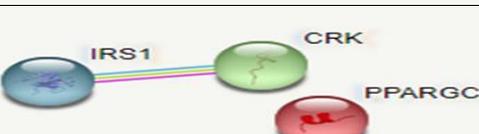
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4. Protein-protein interactions regulated by targeted microRNAs

Protein-protein interactions possibly regulated by the targeted miRNAs were investigated using the protein interaction database called STRING. The estimated likelihood that the predicted proteins interact in the same metabolic map in the KEGG database was indicated by a 95% confidence score generated by the active prediction methods in the STRING.

Table 1.4 Functional protein-protein interactions determined by STRING

MicroRNA target gene/protein	Functional protein-protein interaction network
Ligand for the receptor-type protein-tyrosine kinase KIT	
Interleukin 1 beta (IL-1 β)	
AKT3	
PPARGC1A	
RAF1	
AKT3 RAC-gamma serine/threonine-protein kinase	
PPARGC1A	

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5. Functional analysis of microRNAs

In addition to functional analysis of mRNA target genes, biological activity of the differentially expressed miRNAs was also explored. Using HMDD, the biofluid and disease association of the differentially expressed miRNAs were investigated using the miRNA database.

Table 1.5 Functional analysis of miRNAs using the HMDD (14).

miRNA ID		Bio-fluid /tissue/celldetection					Disease association			
miRNA	Target gene	Prediction program		Validation method					Predictive score	Refer-enc
		DIANA-Tarbase	miRTar Base	Reporter assay	Western blot	qRT-PCR	Microarrays	Other		
miR-195	GRB2	Yes	Yes					✓	0.603	2
miR-152	IRS1	Yes	Yes	✓	✓				0.666	1
	FGF2	Yes	Yes	✓	✓	✓			0.630	1
miR-21	IL1B	Yes	Yes	✓	✓		✓		0.668	1
hsa-miR-181c		Serum					Proliferative Diabetic Retinopathy			
hsa-miR-21		Serum					Proliferative Diabetic Retinopathy			
hsa-miR-200b		retinas of streptozotocin-induced diabetic rats					Diabetic Retinopathy			
hsa-miR-152		hRECs and rat retinal tissues under HG conditions.					Diabetic Retinopathy			
hsa-miR-195		human retinal and dermal microvascular ECs (HRECs, HMECs)					Diabetic Retinopathy			
hsa-miR-27b		Serum					Diabetic Retinopathy			
hsa-miR-126		Retina					Diabetic Retinopathy			

6. Experimental validation of microRNA:mRNA target interactions

The databases DIANA-TarBase and miRTarBase were used to investigate whether the interactions between differentially expressed miRNAs and their predicted mRNA targets were experimentally validated. The predictive scores ranged from 0 - 1, with 1 representing the highest likelihood of an interaction, and 0 representing the lowest likelihood of a true interaction (Table 1.4). Both DIANA-TarBase and miRTarBase gave high predictive scores for the interaction between miR-21 and IL1B, miR-152 and IRS1, and between MiR-195 and GRB2.

Table 1.4 Experimental validation of miRNA:mRNA target interactions.

7. miRNA-target pathway interactions

miRNA – target pathway interactions were performed for miRNA 21 and their targeted gene IL1B by using mirwalk database, their interaction was found in apoptosis and MAPK signalling pathway which are previously link with DR. furthermore for the identification of their link with diabetic retinopathy KEGG disease pathway analysis was performed. Which shows MAPK signalling and apoptosis both pathways are affected in case of diabetic retinopathy.

8. A comparative miRNA target prediction

has-miR-21 and their targeted gene IL1B, their comparative miRNA target prediction was performed by using mirwalk comparative miRNA target search. out of 10 databases 6 target prediction programs were conform that miRNA 21 targets the gene IL1B. DIANA-T, miRanda, mirwalk, PICTAR5 and Targetscan were conform that has-miR-21 targets the gene IL1B.

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MicroRNA	Gene	DIANaT	miRanda	miRDB	miRWalk	RNAhybrid	PICTAR4	PICTAR5	PITA	RNA22	TargetsScan	SUM
hsa-miR-21	IL1B	1	1	1	1	0	0	1	0	0	1	6

Figure 1.3 miRWalk result Represented that has-miR-21 targets IL1B gene were conform 6 target prediction databases.

IV. CONCLUSION

There is a need for identification and validation of good biomarkers Particular interest is also focussed on diagnostic methods which can detect diseases in its early stages. If diabetic retinopathy were to be diagnosed in its early stages, the development of overt diabetic retinopathy, and its long-term complications, can be avoided. miRNAs have shown remarkable promise as potential biomarkers for the early (and specific) detection for DR, as well as for other diseases, due to its stability compared to other biomolecules such as mRNA and proteins. The aim of this project, therefore, was to identify and analysis differentially expressed miRNAs in DR as well as to identify their potential target genes.

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