New Results

Elucidation of novel miRNA candidates and their role in unraveling the pathology of Non-Alcoholic Fatty Liver Disease

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Abstract:

Non-Alcoholic Fatty Liver Disease (NAFLD) is a chronic liver disease which is observed in people who do not abuse alcohol. Main cause of NAFLD is Non-Alcoholic Steatohepatitis (NASH) where there is accumulation of fats such as triglyceride in liver and the disease progression ranges from simple steatosis to fibrosis and cirrhosis. MicroRNAs are critical players in post-transcriptional gene regulation of diseases with complex etiology. In this study, we have elucidated the role of microRNAs (miRNAs) in the pathophysiology of NAFLD/NASH and unravelled molecular markers for diagnosis of NAFLD. A subset of genes (n=10) responsible for NAFLD/NASH were selected and detailed *in silico* analysis carried out using multiple tools. miRDB and DIANA-microT were used to find putative miRNA binding sites followed by analysis using miRTarBase which is an experimentally validated database of miRNA-target interactions. The study elucidated a number of statistically significant predictions for both miRDB (scores >80) and DIANA -microT (values >0.90) and also strong experimental validation in miRTarBase. The analysis revealed that certain miRNAs like miR-7 & miR-548 family members are found in both the programmes, miRDB and DIANA-microT, targeting genes involved in liver function. They were also identified in the experimental validation database miRTarBase. These miRNAs probably play an important role in the pathophysiology of this disease. They can also be used as prognostic/diagnostic markers for assessment of NAFLD.

Introduction:

Non-alcoholic fatty liver disease or NAFLD is a chronic liver disease which leads to liver damage. This disease is seen in individuals who do not abuse alcoholic substances but still have the similar damage as an alcohol-induced damaged liver. Accumulation of different fats in liver cells is the reasons for NAFLD. This accumulation is caused due to metabolic syndrome and obesity.[1] As per population-based screening, at least 25% of US population suffers from NAFLD [2] and study conducted by Ankita *et.al,* in 2015, the prevalence of NAFLD in India is 9% in rural population and 32% in urban population.[3]

The individual suffering from NAFLD tends to develop NASH (a subset disease to NAFLD). There are no non-invasive tests to determine and differentiate between NAFLD and NASH which makes it more difficult to understand the exact incidence and prevalence of these diseases. However, use of serum aminotransferases as a surrogate marker for NASH, by a population-based study suggests that, about 6-8% of the adults suffering from NAFLD has NASH (that is; 25% of the population suffering from NAFLD).[4] Small liver biopsy study shows that 25% of patients with NASH develop cirrhosis as their condition progresses.[5] Considering these studies as representative to larger population, about 1-2.5% of the US population suffers from cirrhosis due to NAFLD/NASH. Cirrhosis is a leading factor for liver cancer in humans. However, growing number of liver cancer patients shows that liver cancer has been developing in individuals with NAFLD even though they don't suffer from cirrhosis. This shows that the risk factors for NAFLD like obesity and metabolic syndrome are also responsible for development of cancer in extra hepatic tissue.[6]

Patients with NAFLD are a higher risk of developing hepatocellular carcinoma (HCC). Out of all the HCC cases in Western countries, 4-22% of the cases are caused due to NAFLD. [7] NAFLD affecting 30% of the general population in North America and is closely associated with the growing obesity problem that people suffer from. Among this population, it is seen that 30-50% of the people suffer from NASH (Non-Alcoholic Steatohepatitis). Studies show that about 70% of the patients with NAFLD have normal liver enzymes.[8] However, alanine aminotransferases (ALT) appears to be seven-fold higher in obese people.[9] Some people with normal aminotransferase have been seen with NASH and indicative fibrosis. Thus, these surveys confirm, that the entire histopathological band of NAFLD could be seen in people with normal liver enzymes.[10] In Asia, where viral hepatitis is still an epidemic, 1-2% of the HCC cases are because of NAFLD.[11] Globally, cryptogenic cirrhosis is responsible for 15-30% of HCC cases and about half of these are believed to have risen due to NAFLD.[12]

miRNAs or microRNAs, as the name suggests are small, non-coding RNA molecules which are involved in regulating gene expression at post-transcriptional level. The miRNAs are around 19-23 nucleotides long. MicroRNA was discovered by Ambrose and colleagues in 1993.[13] They regulate the gene expression by interfering in translation process.[14,15] From multiple studies, it is evident now that microRNA plays an important role in pathophysiology of complex multi-factorial disorders including NAFLD/NASH.[14,16,17] Studies have shown the role of miR-122 in progression of severe liver injury such as fibrosis.[18] Liver-specific knockout animal models have shown development and fibrosis, and Steatohepatitis.[18] miR-34a is involved in lipid metabolism, apoptosis, cell cycle control and studies in mice have shown that overexpression of miR-34a increases hepatic Triglyceride (TG) level. [19] Studies have shown that elevated miR-103 cause's stimulation of ectopic lipid accumulation in liver causing NAFLD. [20,21,22] Studies on NAFLD patients have also that down regulation of miR-155 enhances LXRα/SREBP-1c signal which lead to accumulation of lipids in liver.[23] Animal studies have shown that inhibition miR-29a causes enhancement of lipoprotein lipase (LPL) expression thereby causing lipid accumulation in liver. [24] Studies on humans showed that, up regulation of miR-192 have co-relation with liver disease.[14] However, we still don't know the involvement of various miRNAs and their role in the pathophysiology of NAFLD/NASH. Hence, a subset of genes responsible for NAFLD/NASH was selected from the study of Alessandra *et. al.,* and a detailed *in silico* work was carried out in this present study.[16] It is hoped that this information would serve as the basis for further studies in elucidating the role of microRNAs in the pathogenesis of NAFLD/NASH as well as provide diagnosis / prognostic markers for NAFLD/NASH.

Methodology:

The input gene dataset for this study included candidate genes from a spectrum of genetic factors implicated in the complex etiology of NAFLD. This present list of genes and their role and importance in NAFLD has been discussed in detail in the paper of Alessandra *et. al.* [16]. In all, 10 genes different physiological categories were selected for identifying the various miRNAs targeting these genes. The list of these 10 candidate genes and their gene description are presented in Table 1 of this study.

miRDB analysis-

For the task of identifying the various miRNAs targeting these different genes involved in NAFLD pathophysiology, the miRDB database was used.[25] miRDB is a database for miRNA target prediction and annotation. The various genes selected in this study were analysed by a tool called Mir Target v4 integrated in miRDB and which functions by analysing thousands of genes regulated by miRNAs with a Support Vector Machines learning framework. Common features involved in

miRNA target binding have been identified and incorporated in this tool to predict miRNA targets in species like human, rat, mouse, dog and chicken. The Mir Target v4 prediction algorithm has been validated by independent experimental data for predicting a large number of miRNA down-regulated gene targets. In this study, the selected genes were searched individually in miRDB by entering the appropriate 'Gene Symbol' in the search box and using 'Human' as the option for organism. Now for each gene of interest and if predicted to be targeted by mature miRNAs, this results in a table where the multiple mature miRNAs targeting that gene are ranked according to a score assigned by the target prediction algorithm. Other details like miRNA sequence, 3'UTR sequence and gene description are also given. According to the scoring scheme in miRDB, the prediction scores for predicted targets range between 50-100. However, higher the score, the more is the confidence in the prediction and scores >80 are considered to be most likely to be real. Based on this, Table 2, was prepared having only those hits which have scores above or equal to 80 for the various genes predicted to be targeted by miRNAs.

DIANA-microT analysis -

Continuing with our miRNA elucidation process, further analysis of the candidate genes was carried out with DIANA (DNA Intelligent Analysis) Lab miRNA target prediction tool DIANA-microT 5.0. [26] This algorithm works on parameters that are calculated individually for each miRNA and for each miRNA recognition element or MRE and takes into account binding characteristics and conservation levels. The miRNA: target interaction is primarily judged on the basis of the total score or the miRNA targeted gene (miTG) score and this predicted score is the sum of the conserved and non-conserved MREs of a particular gene. Along-with this, a signal-to-noise ratio or SNR and a precision score for each interaction is also provided for estimating the confidence of the predicted result. The DIANA microT web server allows one to search miRNA targets on a specific gene and using this feature the candidate genes were searched one by one for prospective miRNAs targeting them using a score threshold of 7.0. This web server apart from the main parameters mentioned above also gives information about the binding type, UTR position, conservation and online linkages to bibliographical and biological resources for further analysis. A list of the DIANA microT 5.0 analysis of the candidate genes with significance values (above 0.90) and their miRNAs are given in Table 3 of this study.

miRTarBase analysis for experimentally validated miRNAs -

In order to validate our search strategy, the same gene dataset was used for identification of miRNAs using a database called miRTarBase.[27] miTarBase is a comprehensive database containing collections of experimentally validated miRNA-Target interactions or MTIs. These

MTIs are collected manually from relevant literature reporting functional miRNA studies. The experimental validation of these miRNAs is mostly done using reporter assays, western blots or microarray experiments with over-expression or knockdown of miRNAs. miRTarBase contains more than 422517 experimentally validated MTIs involving 4076 miRNAs and 23,054 target genes. Using this database, we investigated whether for any of our candidate gene(s), there is experimental evidence of miRNA-Target Interactions from literature using the validation methods. For this purpose, all the candidate genes were searched in miRTarBase by using the 'Gene Symbol' option and 'Human' in the species option. One can also browse through the gene list provided under a specific species like Human and check whether one's gene of interest is listed in the database. When a specific hit for a target gene is found, information about both the pre- and mature miRNA including the secondary structure as well as the mature sequence is provided along-with information about the target gene, the validation methods used and the relevant literature report. Using miRTarBase, all the 10 genes were selected in the database according to the given protocol and the result of this study is given in Table 4.

Based on this *in silico* elucidation analysis, a meta-list of candidate genes and their elucidated cognate miRNAs common to both miRDB and DIANA were created and listed in Table 5. The entries here were searched to identify putative hits having experimental validation in miRTarBase database also and only those hits with strong experimental evidence selected for further discussion.

Results and Discussion:

The gene list (Table 1) was obtained from the study of Alessandra *et. al.,* and the genes were put through the *in silico* miRNA elucidation pipeline. In this study, miRDB and T-CDS were used to identify putative miRNA binding sites. This was followed by analysis using miRTarBase which is an experimentally validated database of miRNA-target interactions. In miRDB study, a target score of greater than 80 is selected as a statistically significant value according to the miRDB program itself. A list of these candidate genes with various miRNA's targeting them is given in Table 2. This list contains all the genes with miRNAs having a minimum target score of 80 and above only. In T-CDS study, a target score of greater than 0.90 is selected as a statistically significant value which is according to the T-CDS program. A list of these candidate genes with various miRNA's targeting them is given in Table 3 and this list contains all the genes having a minimum target score of 0.90 and above. miRTarBase is a database containing a collection of experimentally validated miRNA-Target interactions. This database has been used to check miRNA-Target interactions and Table 4 contains the candidate miRNAs which are validated according to miRTarBase criteria. Table 5 of this study is comparative data of candidate genes having significant miRNA hits which have been found to be common in both miRDB and T-

CDS programs. The common miRNA hits of miRDB and DIANA T-CDS were also searched in miRTarBase database.

NAFLD with its abnormal fat accumulation is the most common cause of chronic liver disease especially in the western world.[28] This disorder can manifest in multiple ways including steatohepatitis, fibrosis and cirrhosis.[29] Additionally, NAFLD is also strongly associated with metabolic syndrome and cardiovascular disease and a subset of such patients may end up with hepatocellular carcinoma (HCC). The hallmark of NAFLD is the accumulation of triglycerides within hepatocytes with increased *de novo* lipogenesis, high adipose tissue lipolysis, obesity and diabetes.[29] The progression of NAFLD to fibrosis is mediated by a complex web of factors including inflammatory mediators, lipotoxicity, fatty acids, dietary factors, genetic factors and epigenetics.[30] However, despite understanding the factors responsible for NAFLD and its progression, little is known about the regulation of these factors at both transcriptional or post-transcriptional levels as well as the identity of novel biomarkers that can help in predicting clinical outcome. Therefore, in this study, we have attempted to elucidate the novel regulatory miRNA candidates involved in post-transcriptional regulation of cognate genes implicated in the pathophysiology of NAFLD and its progression using an *in silico* approach.

miRNAs as endogenous single-stranded RNAs have emerged as very important component of gene expression regulation through post-transcriptional mechanisms in wide variety of pathological conditions.[31] The stability of miRNA in circulatory system makes them an attractive candidate for biomarker discovery.[32] The input genes in our study were taken from the review of Alessandra *et. al.,* and in that they were classified under genetic factors category involved in NAFLD/NASH pathophysiology. The various genes belong to diverse physiological domains and all have been found to be associated significantly in genetic studies in NAFLD cases in multiple population types. The functional categories in this subset of genes include those involved in retinol metabolism, synthesis of fatty acids, lipid transport, oxidative stress, glucose metabolism and fibrosis.

miRNA have a very important role in metabolic homeostasis in an individual. In the liver, miR-112 influences the genes responsible for the metabolism of hepatic cholesterol and lipids. Using antisense methods, obstruction of miR-112 resulted in the reduction of plasma cholesterol levels in mice and chimpanzees.[33,34] A separate study showed that in a mice model where miR-122 encoding gene was deleted, development of steatohepatitis, HCC and fibrosis was observed.[35]

For normal liver homeostasis, miR-122 is essential and the decreased levels of miR-122 have damaging effects on the liver. [35] Lipid and cholesterol regulatory genes are regulated by miRNA such as miR-33, miR-34, miR-103, miR-104 and miR-370.[36]

The mitochondrial enzyme, carnitine palmitoyl transferase that is involved in the movement of long-chain fatty acids across the membrane is affected by miR-370.[37] At many levels miRNAs are responsible for the regulation of liver fibrosis. Upon fibrogenic injury of the liver, the Hepatic Stellate Cells (HSCs), a primary type of cells responsible for liver fibrosis, experience growth and differentiation into myofibroblast-like cells. Many varieties of miRNA are identified in regulating the HSC stimulation.[14] Increased serum levels of miR-571 are considered as a potential biomarker for liver fibrosis.[38] In bile-duct-ligated rats, the decrease in miRNA-150 and miRNA-194 has been identified. This miRNA-150 and miRNA-194 targeted c-Myc and Rac-1 respectively, thus restraining the HSC activation.[39]

Apart from the above abnormalities, increased levels of miR-705, miR-1224, miR-486, miR-320 and decreased levels of some other miRNAs like miR-192, miR-183, miR-199a, miR27b, miR-214 was observed in the livers of the mice subjected to chronic feeding of alcohol.[40] In alcoholic steatohepatitis and NASH, the Kuffer cell activation is common. Up-regulation of miRNA-155 occurs in Kuffer cell in ALD, so we can theorize that it is the same for NASH as well. In patients with NASH, altered hepatic expression has been found.[41]

In Alcoholic Steatohepatitis and NASH, the Kupffer cell activation is common. Up-regulation of miRNA-155 occurs in Kupffer cells in ALD, so we can theorize that it is the same for NASH as well. In patients with NASH, altered hepatic expression has been found.[41] It has been seen that, in patients with NASH miRNA-122 decreases as their disease progresses. These decreased levels of miRNA-122 contribute to the metabolism of altered hepatic lipids.[36]

In our *in silico* study, Table 5 shows the comparison of DIANA-microT, miRDB and miRTarBase analysis and in this table miR-7-5p (miR-7 family) is present and this have been found in literature to be an anti-oncogenic miRNA and its repression by a complex mechanism causes manifestation of HCC which can be observed is almost 22% of NAFLD cases in western countries.[7,42] In the same table, miR-548p and 548-3p (miR-548 family) are also present in all three above mentioned tools. miR-548 is a large, poorly conserved primate-specific miRNA family and it has been implicated in multiple pathological processes like cancer and signalling pathways.[43] miR-548a-5p been reported to negatively regulate the tumour inhibitor gene Tg737

and promote tumorigenesis *in vitro* and *in vivo* especially with respect to HCC cell proliferation and apoptosis. [44]

From the above discussion, it is evident that certain miRNA's like miR-7-5p and miR548p/548-3p elucidated in our study are involved in pathophysiology of NAFLD/NASH. Such miRNA candidates can also serve as prognostic markers for accurate assessment of NAFLD/NASH early on. Our analysis however has its own limitation as far as the providing functional significance and information is concerned. This analysis gives an idea of specific miRNA targeting gene implicated in liver disease. Our study also does not exclude the involvement of other miRNA(s) targeting such pathways. This analysis is putative and functional aspects of this post-transcriptional regulation needs to be validated experimentally. Some techniques by which such validation in clinical sample can be achieved includes Reporter Assay, qPCR, and pSILAC etc.

This study represents an advance in biomedical science because it provides a novel and unique approach to understand the complex regulation of genes involved in NAFLD/NASH as well as provide prognostic /diagnostic markers for this disease.

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Gene name	Gene Description
APOB	apolipoprotein B
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1
GCKR	glucokinase (hexokinase 4) regulator
HFE	hemochromatosis
PNPLA3	phospholipase domain containing 3
MBOAT7	membrane bound O-acyltransferase domain containing 7
IRS1	insulin receptor substrate 1
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1
TM6SF2	transmembrane 6 superfamily member 2
TMC4	transmembrane channel-like 4

Table 1: The list of 10 candidate genes implicated in the complex etiology of NAFLD and used as input for miRNA elucidation pipeline.

Gene symbol	Target score	miRNA name
APOB	94	hsa-miR-5008-3p
APOB	94	hsa-miR-7157-3p
APOB	94	hsa-miR-6737-3p
APOB	92	hsa-miR-6124
APOB	91	hsa-miR-449c-5p
APOB	91	hsa-miR-34b-5p
APOB	88	hsa-miR-548p
APOB	87	hsa-miR-2682-5p
APOB	87	hsa-miR-10522-5p
APOB	86	hsa-miR-1237-3p
APOB	84	hsa-miR-1468-3p
APOB	80	hsa-miR-7161-5p
ENPP1	100	hsa-miR-3613-3p
ENPP1	100	hsa-miR-3163
ENPP1	98	hsa-miR-4424
ENPP1	98	hsa-miR-371b-5p
ENPP1	98	hsa-miR-616-5p
ENPP1	98	hsa-miR-373-5p
ENPP1	95	hsa-miR-548c-3p
ENPP1	93	hsa-miR-607
ENPP1	93	hsa-miR-4319
ENPP1	92	hsa-miR-7110-3p
ENPP1	92	hsa-miR-7854-3p
ENPP1	91	hsa-miR-4307
ENPP1	90	hsa-miR-3120-3p
ENPP1	90	hsa-miR-6873-3p
ENPP1	90	hsa-miR-125a-5p
ENPP1	90	hsa-miR-125b-5p
ENPP1	89	hsa-miR-1294
ENPP1	89	hsa-miR-9986
ENPP1	88	hsa-miR-7843-3p
ENPP1	87	hsa-miR-202-5p

ENPP1	86	hsa-miR-5586-5p
ENPP1	85	hsa-miR-3169
ENPP1	83	hsa-miR-556-3p
ENPP1	83	hsa-miR-10523-5p
ENPP1	83	hsa-miR-4316
ENPP1	82	hsa-miR-6867-3p
ENPP1	82	hsa-miR-6734-5p
ENPP1	82	hsa-miR-4740-5p
ENPP1	82	hsa-miR-4768-5p
ENPP1	82	hsa-miR-338-5p
ENPP1	82	hsa-miR-6857-5p
ENPP1	82	hsa-miR-6833-3p
ENPP1	80	hsa-miR-2117
ENPP1	80	hsa-miR-12125
ENPP1	80	hsa-miR-3200-5p
GCKR	83	hsa-miR-4306
GCKR	80	hsa-miR-4656
HFE	99	hsa-miR-5093
HFE	91	hsa-miR-5696
HFE	88	hsa-miR-361-5p
HFE	88	hsa-miR-1245b-3p
HFE	87	hsa-miR-7159-3p
HFE	86	hsa-miR-5700
HFE	85	hsa-miR-3915
HFE	83	hsa-miR-12124
HFE	82	hsa-miR-324-5p
HFE	82	hsa-miR-3662
PNPLA3	90	hsa-miR-6800-5p
PNPLA3	83	hsa-miR-3171
PNPLA3	82	hsa-miR-8064
PNPLA3	81	hsa-miR-1304-3p

PNPLA3	81	hsa-miR-8075
MBOAT7	96	hsa-miR-4700-5p
MBOAT7	94	hsa-miR-24-3p
MBOAT7	92	hsa-miR-23a-3p
MBOAT7	92	hsa-miR-23b-3p
MBOAT7	92	hsa-miR-23c
MBOAT7	91	hsa-miR-6739-5p
MBOAT7	91	hsa-miR-8089
MBOAT7	91	hsa-miR-6733-5p
MBOAT7	91	hsa-miR-4667-5p
MBOAT7	89	hsa-miR-450a-1-3p
MBOAT7	89	hsa-miR-3153
MBOAT7	84	hsa-miR-4725-3p
MBOAT7	82	hsa-miR-4731-5p
MBOAT7	81	hsa-miR-610
IRS1	100	hsa-miR-5011-5p
IRS1	100	hsa-miR-3148
IRS1	100	hsa-miR-8485
IRS1	100	hsa-miR-190a-3p
IRS1	99	hsa-miR-5696
IRS1	98	hsa-miR-922
IRS1	97	hsa-miR-3646
IRS1	97	hsa-miR-660-5p
IRS1	97	hsa-miR-6071
IRS1	97	hsa-miR-651-3p
IRS1	97	hsa-miR-544b
IRS1	97	hsa-miR-4291
IRS1	96	hsa-miR-3619-5p
IRS1	96	hsa-miR-128-3p
IRS1	96	hsa-miR-3919
IRS1	96	hsa-miR-1284

IRS1	95	hsa-miR-216a-3p
IRS1	95	hsa-miR-761
IRS1	95	hsa-miR-7-5p
IRS1	95	hsa-miR-214-3p
IRS1	94	hsa-miR-4503
IRS1	94	hsa-miR-4324
IRS1	94	hsa-miR-4789-3p
IRS1	93	hsa-miR-570-3p
IRS1	93	hsa-miR-3681-3p
IRS1	92	hsa-miR-12122
IRS1	92	hsa-miR-7160-5p
IRS1	92	hsa-miR-5692a
IRS1	91	hsa-miR-4799-5p
IRS1	90	hsa-miR-6828-3p
IRS1	90	hsa-miR-3941
IRS1	90	hsa-miR-4670-3p
IRS1	89	hsa-miR-654-5p
IRS1	89	hsa-miR-3149
IRS1	89	hsa-miR-541-3p
IRS1	89	hsa-miR-96-5p
IRS1	88	hsa-miR-6739-3p
IRS1	88	hsa-miR-1250-3p
IRS1	88	hsa-miR-181c-3p
IRS1	88	hsa-miR-3180-5p
IRS1	88	hsa-miR-5093
IRS1	87	hsa-miR-30b-5p
IRS1	87	hsa-miR-30e-5p
IRS1	87	hsa-miR-30c-5p
IRS1	87	hsa-miR-466
IRS1	87	hsa-miR-30a-5p
IRS1	87	hsa-miR-30d-5p

IRS1	86	hsa-miR-3617-3p
IRS1	86	hsa-miR-1271-5p
IRS1	86	hsa-miR-4328
IRS1	86	hsa-miR-12133
IRS1	86	hsa-miR-4729
IRS1	85	hsa-miR-1245b-3p
IRS1	85	hsa-miR-6124
IRS1	85	hsa-miR-12120
IRS1	85	hsa-miR-3613-3p
IRS1	85	hsa-miR-3928-3p
IRS1	85	hsa-miR-1323
IRS1	85	hsa-miR-183-5p
IRS1	85	hsa-miR-1200
IRS1	84	hsa-miR-4666a-3p
IRS1	84	hsa-miR-7152-5p
IRS1	84	hsa-miR-4680-3p
IRS1	84	hsa-miR-550a-3p
IRS1	82	hsa-miR-548o-3p
IRS1	82	hsa-miR-7162-3p
IRS1	81	hsa-miR-302b-5p
IRS1	81	hsa-miR-32-3p
IRS1	81	hsa-miR-6759-3p
IRS1	81	hsa-miR-3133
IRS1	81	hsa-miR-2115-5p
IRS1	81	hsa-miR-302d-5p
IRS1	80	hsa-miR-7856-5p
IRS1	80	hsa-miR-559
IRS1	80	hsa-miR-890
IRS1	80	hsa-miR-1261
SLC2A1	96	hsa-miR-5011-5p
SLC2A1	93	hsa-miR-4427

SLC2A1	92	hsa-miR-4758-5p
SLC2A1	92	hsa-miR-1238-5p
SLC2A1	91	hsa-miR-92a-1-5p
SLC2A1	90	hsa-miR-6077
SLC2A1	88	hsa-miR-152-3p
SLC2A1	88	hsa-miR-148a-3p
SLC2A1	88	hsa-miR-6081
SLC2A1	88	hsa-miR-148b-3p
SLC2A1	87	hsa-miR-4653-3p
SLC2A1	86	hsa-miR-4742-3p
SLC2A1	86	hsa-miR-8055
SLC2A1	86	hsa-miR-3664-3p
SLC2A1	84	hsa-miR-1263
SLC2A1	84	hsa-miR-223-5p
SLC2A1	81	hsa-miR-1266-5p
SLC2A1	81	hsa-miR-1284
SLC2A1	81	hsa-miR-6773-5p
SLC2A1	81	hsa-miR-4518
SLC2A1	80	hsa-miR-3163
SLC2A1	80	hsa-miR-8063
SLC2A1	80	hsa-miR-3140-3p
SLC2A1	80	hsa-miR-548t-3p
SLC2A1	80	hsa-miR-548aa
SLC2A1	80	hsa-miR-548ap-3p
SLC2A1	80	hsa-miR-140-5p
SLC2A1	80	hsa-miR-1277-5p
TM6SF2	87	hsa-miR-6778-5p
TM6SF2	87	hsa-miR-1233-5p
TM6SF2	85	hsa-miR-488-3p
TM6SF2	85	hsa-miR-5681a
TMC4	91	hsa-miR-11400

Table 2: List of candidate genes with various miRNAs targeting them and with cut-off score ≥ 80 according to miRDB analysis (Mir Target v4).

Gene Id(name)	Transcript Id	miRNA Name	miTG score
ENSG00000084674 (APOB)	ENST00000399256	hsa-miR-6816-3p	0.989967521
ENSG00000084674 (APOB)	ENST00000399256	hsa-miR-659-5p	0.948997332
ENSG00000084674 (APOB)	ENST00000399256	hsa-miR-5585-3p	0.921929567
ENSG00000084674 (APOB)	ENST00000399256	hsa-miR-548c-3p	0.912467713
ENSG00000084674 (APOB)	ENST00000399256	hsa-miR-183-3p	0.912447313
ENSG00000084674 (APOB)	ENST00000399256	hsa-miR-155-5p	0.905450717
ENSG00000197594(ENPP1)	ENST00000360971	hsa-miR-125a-5p	0.99998178
ENSG00000197594(ENPP1)	ENST00000360971	hsa-miR-125b-5p	0.9999598
ENSG00000197594(ENPP1)	ENST00000360971	hsa-miR-4319	0.99943457
ENSG00000197594(ENPP1)	ENST00000360971	hsa-miR-3163	0.99651834
ENSG00000197594(ENPP1)	ENST00000360971	hsa-miR-670-5p	0.98776546
ENSG00000197594(ENPP1)	ENST00000360971	hsa-miR-6873-3p	0.97890705
ENSG00000197594(ENPP1)	ENST00000360971	hsa-miR-1273g-3p	0.96987193
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-4282	0.9698025
ENSG00000197594 (ENPP1)	ENST00000360971	has-miR-548c-3p	0.96847277
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-3613-3p	0.96115681
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-6734-5p	0.95897725
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-5690	0.95819912
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-4324	0.95443301
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-6833-3p	0.95004084
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-607	0.94175328
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-5695	0.93258301
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-7110-3p	0.93171827
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-6817-3p	0.93120509
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-3678-5p	0.93000864
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-3148	0.92928568
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-3909	0.92916088
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-105-5p	0.92458385
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-942-5p	0.92404109
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-3926	0.92120792
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-6809-3p	0.92047367
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-590-3p	0.91909087
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-7702	0.9054111
ENSG00000197594 (ENPP1)	ENST00000360971	hsa-miR-6835-3p	0.90232333
ENSG00000084734 (GCKR)	ENST00000424318	hsa-miR-2115-3p	0.934023234
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-5093	0.998296651
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-4450	0.99733991
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-6857-5p	0.989243863

ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-1237-3p	0.976014323
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-4282	0.96832407
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-3915	0.964237738
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-335-3p	0.96392008
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-361-5p	0.962235864
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-4490	0.961079043
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-3670	0.953763237
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-1236-3p	0.940866582
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-3163	0.937822762
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-2110	0.936225184
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-5700	0.935967631
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-3662	0.931178207
ENSG00000010704 (HFE)	ENST00000357618	hsa-let-7i-5p	0.927500375
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-7159-3p	0.922299852
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-607	0.919034704
ENSG00000010704 (HFE)	ENST00000357618	hsa-let-7b-5p	0.91623146
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-589-3p	0.91440342
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-133a-5p	0.910355816
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-6813-5p	0.908833774
ENSG00000010704 (HFE)	ENST00000357618	hsa-miR-651-3p	0.908488478
ENSG00000010704 (HFE)	ENST00000357618	hsa-let-7g-5p	0.902552165
ENSG00000100344(PNPLA3)	ENST00000216180	hsa-miR-5702	0.954747123
ENSG00000100344(PNPLA3)	ENST00000216180	hsa-miR-7162-5p	0.952451962
ENSG00000100344(PNPLA3)	ENST00000216180	hsa-miR-516b-3p	0.950287063
ENSG00000100344(PNPLA3)	ENST00000216180	hsa-miR-516a-3p	0.950287063
ENSG00000100344(PNPLA3)	ENST00000216180	hsa-miR-5682	0.904732946
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-6739-5p	0.998490394
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-6733-5p	0.997028138
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-662	0.967740166
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-4725-3p	0.950021997
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-5589-3p	0.945604077
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-7162-5p	0.940505903
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-3153	0.926373081
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-6780b-5p	0.923085009
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-450a-1-3p	0.919470489
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-24-3p	0.916453614
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-516b-3p	0.914665976
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-516a-3p	0.914665976
ENSG00000125505(MBOAT7)	ENST00000437868	hsa-miR-770-5p	0.914472144
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-7160-5p	0.999995859

ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-5011-5p	0.999908943
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-216a-3p	0.999343799
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-128-3p	0.999337078
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-7-5p	0.99622645
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-548au-3p	0.993246198
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-1277-5p	0.991928479
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3681-3p	0.991154647
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-559	0.987208851
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-183-5p	0.986272451
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-27a-3p	0.985359885
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-466	0.984662639
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-27b-3p	0.984439868
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4291	0.982757361
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-1271-5p	0.982698658
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4503	0.982266463
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-660-5p	0.980524168
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-190a-3p	0.980115168
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-495-3p	0.979116743
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-5696	0.978722101
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-5580-3p	0.97722777
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-548c-3p	0.976486428
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4672	0.975124686
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4775	0.975081911
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3941	0.970245974
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4789-3p	0.969589024
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-5007-5p	0.965283204
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-1185-1-3p	0.96282615
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4680-3p	0.962534826
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-1185-2-3p	0.962422907
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4328	0.961524128
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-664a-3p	0.961390409
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3148	0.960285047
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-203a-3p	0.957995548
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-664b-3p	0.950877947
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4729	0.95074604
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-545-3p	0.949029505
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-541-3p	0.947749673
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-548az-5p	0.946946362
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-5692a	0.946840355
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-548t-5p	0.946834738

ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-7152-5p	0.946394186
ENSG00000169047 (IRS1)	ENST00000305123	hsa-let-7f-2-3p	0.945190779
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-654-5p	0.944376625
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-890	0.942962739
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-1324	0.942358279
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-5000-5p	0.941386506
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-545-5p	0.940482229
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-7978	0.940118526
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-369-3p	0.939703826
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-1273h-3p	0.93910484
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4666a-5p	0.93656348
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3149	0.936238754
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-96-5p	0.933520332
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-579-3p	0.93313342
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3144-3p	0.929398729
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3646	0.927390299
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-7856-5p	0.925517605
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-641	0.924253406
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4471	0.924041411
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-548aq-5p	0.923926882
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4694-3p	0.923229436
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-624-5p	0.921746264
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-2052	0.92118378
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-1272	0.920893017
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-544b	0.920132674
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-2115-5p	0.9191133
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-548k	0.918657182
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-6885-3p	0.918463385
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-922	0.917740713
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-548av-5p	0.916369563
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-877-3p	0.915804596
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4789-5p	0.912567152
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-576-3p	0.912337044
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3606-3p	0.910296365
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-30d-5p	0.905398415
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-424-5p	0.905260446
ENSG00000169047 (IRS1)	ENST00000305123	hsa-let-7b-3p	0.905097984
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3617-5p	0.905048975
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3133	0.903479524
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4496	0.903174768

ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-1261	0.902881689
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-30e-5p	0.902179054
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-30a-5p	0.901892426
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-497-5p	0.901444509
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-4703-5p	0.900977998
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-186-5p	0.900563673
ENSG00000169047 (IRS1)	ENST00000305123	hsa-miR-3176	0.900508917
ENSG00000117394 (SLC2A1)	ENST00000426263	hsa-miR-8055	0.964693003
ENSG00000117394 (SLC2A1)	ENST00000426263	hsa-miR-5580-5p	0.936394805
ENSG00000117394 (SLC2A1)	ENST00000426263	hsa-miR-330-5p	0.928204283
ENSG00000117394 (SLC2A1)	ENST00000426263	hsa-miR-6838-3p	0.911798905
ENSG00000117394 (SLC2A1)	ENST00000426263	hsa-miR-4758-5p	0.904632891
ENSG00000117394 (SLC2A1)	ENST00000426263	hsa-miR-4436b-3p	0.900956221
ENSG00000117394 (SLC2A1)	ENST00000426263	hsa-miR-3914	0.900763351
ENSG00000213996 (TM6SF2)	ENST00000389363	hsa-miR-5681a	0.951298187
ENSG00000213996 (TM6SF2)	ENST00000389363	hsa-miR-488-3p	0.932333681
ENSG00000167608 (TMC4)	ENST00000449860	hsa-miR-5683	0.974055157
ENSG00000167608 (TMC4)	ENST00000449860	hsa-miR-668-5p	0.967374267
ENSG00000167608 (TMC4)	ENST00000449860	hsa-miR-7-5p	0.917351563

Table 3: The above table shows candidate genes with miRNAs targeting them along-with significant miTG scores (≥ 0.90) from DIANA microT analysis.

					Validation methods		
					Strong Evidence		
ID	**Species (miRNA)**	**Species (Target)**	**miRNA**	**Target**	**Reporter gene**	**Western blot**	**qPCR**
MIRT731658	Homo sapiens	Homo sapiens	hsa-miR-548p	APOB	+	+	+
MIRT003807	Homo sapiens	Homo sapiens	hsa-miR-7-5p	IRS1		+	
MIRT000731	Homo sapiens	Homo sapiens	hsa-miR-145-5p	IRS1	+	+	+
MIRT004355	Homo sapiens	Homo sapiens	hsa-miR-126-3p	IRS1	+	+	+
MIRT006859	Homo sapiens	Homo sapiens	hsa-miR-148a-3p	IRS1	+	+	
MIRT006860	Homo sapiens	Homo sapiens	hsa-miR-152-3p	IRS1	+	+	
MIRT437910	Homo sapiens	Homo sapiens	hsa-miR-105-5p	IRS1	+	+	
MIRT732116	Homo sapiens	Homo sapiens	hsa-miR-1225-5p	IRS1	+	+	+
MIRT734155	Homo sapiens	Homo sapiens	hsa-miR-144-3p	IRS1	+	+	+
MIRT735025	Homo sapiens	Homo sapiens	hsa-miR-628-5p	IRS1	+		
MIRT735485	Homo sapiens	Homo sapiens	hsa-miR-384	IRS1	+	+	+

MIRT438642	Homo sapiens	Homo sapiens	hsa-miR-1291	SLC2A1	+	+	+
MIRT699417	Homo sapiens	Homo sapiens	hsa-miR-22-3p	SLC2A1	+	+	+
MIRT734315	Homo sapiens	Homo sapiens	hsa-miR-148b-3p	SLC2A1	+	+	+
MIRT735435	Homo sapiens	Homo sapiens	hsa-miR-132-3p	SLC2A1	+	+	+
MIRT735450	Homo sapiens	Homo sapiens	hsa-miR-150-5p	SLC2A1	+	+	
MIRT054541	Homo sapiens	Homo sapiens	hsa-miR-200b-3p	HFE		+	+
MIRT054542	Homo sapiens	Homo sapiens	hsa-miR-200a-3p	HFE		+	+
MIRT054543	Homo sapiens	Homo sapiens	hsa-miR-200c-3p	HFE		+	+

Table 4: Candidate genes showing experimentally validated miRNA-target interaction in miRTarBase and showing validation methods with only strong evidence w.r.t experimental methodology.

Gene Name	mi-RDB	T-CDS	miRTarBase Strong evidence
APOB	**hsa-miR-548p**	**hsa-miR-548-3p**	+
IRS1	**hsa-miR-7-5p**	**hsa-miR-7-5p**	+

Table 5: Comparative data of candidate genes along-with their cognate miRNAs from all three databases used in the miRNA elucidation pipeline. miRNAs common in both miRDB and DIANA as well as having strong experimental evidence as per miRTarBase are identified in bold.