Hypermethylation and Expression of MicroRNA 1285 and Sterol Carrier Proteins 2 in Type 2 Diabetes Mellitus

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Abstract

Diabetes mellitus (DM) is one of a severe metabolic disease found in all types of people living in developing as well developed countries. Specifically, Type 2 DM accounts for over 90% for older and urban population. It is has been suggesting that the prevalence of diabetes is increasing many countries and most of the cases are type 2 DM, clinical treatments are changed now to manage type 2 DM however, up to date there is no diagnostic, prognostic and therapeutic target for type 2 DM. MicroRNAs (miRNAs) is one of a non-protein coding RNAs have been regulating wide range cellular processes and induce the manifestation of many diseases. Most of the researchers concluding that miRNAs involvement is a important process in a broad range of signaling pathways such as cell proliferation, stem cell maintenance, migration, apoptosis and gene or protein expressions. Expressed sequence Tags (ESTs) are the best source of coding and non-coding sequences for the identification of miRNAs. Although DNA methylation is an important mechanism for microRNA up regulation, this has not been highly explored in type 2 DM. this study may useful to elucidate the molecular mechanism of miR-1285 in type 2 DM and its role in disease progression and we discovered miR-1285 as a novel prognostic, diagnostic and therapeutic target for type 2 DM.

Key Words: MicroRNAs; mRNA; MiR-1285; Type 2 Diabetes Mellitus; Methylation;gene expression
1. Introduction

Diabetes mellitus (DM) is one of a severe metabolic disease found in all types of people living in developing as well developed countries (1). It has been suggesting that DM is a multifactor disease resulting in altered glucose homeostasis and both type1 DM and type 2 DM, the beta cells of pancreatic organ cannot secrete proper amounts of insulin to control blood glucose level in the blood cells (2). Many reports suggesting that there will be increase of 300 million DM patient by 2025 and 366 million by 2025 and interestingly the majority of this patient from developing countries (3, 4, 5). Specifically, Type 2 DM accounts for over 90% for older and urban population (6). It is has been suggesting that the prevalence of diabetes is increasing many countries and most of the cases are type 2 DM, clinical treatments are changed now to manage type 2 DM however, up to date there is no diagnostic, prognostic and therapeutic target for type 2 DM.

MicroRNAs (miRNAs) is one of a non-protein coding RNAs have been regulating wide range cellular processes and induce the manifestation of many diseases (7,8,9). Single stranded mature miRNAs after processing cytoplasm induces negative regulation of gene expression in the cells. Most of the researchers concluding that miRNAs involvement is a important process in a broad range of signaling pathways such as cell proliferation, stem cell maintenance, migration, apoptosis and gene or protein expressions (10, 11). Expressed sequence Tags (ESTs) are the best source of coding and non-coding sequences for the identification of miRNAs (2). Based on athe above information the present study focused on the identification of novel miRNAs from DM ESTs using computational approaches and their involvement in type 2 DM. our results suggesting that a novel circulating hsa-mir-1285 from type 2 DM ESTs by using computational approaches involved in diminishing the expression of sterol carrier protein 2 SCP2 in type 2 DM patient samples through hypermethylation of promoter region. Furthermore, Although DNA methylation is an important mechanism for microRNA up regulation, this has not been highly explored in type 2 DM. this study may useful to elucidate the molecular mechanism of miR-1285 in type 2 DM and its role in disease progression and we discovered that miR-1285 as a novel prognostic, diagnostic and therapeutic target for type 2 DM.
2. Materials and methods

2.1 Creating local Nucleotide database using ESTs and miRNAs

Bioinformatics tools were used in search of miRNA responsible for types 2 DM through National Center for Biotechnology Information (NCBI) ESTs sequences (23189 (November: 10, 2018) are extracted by using the term type 2 “diabetic homo sapiens”. miRbase (http://www.mirbase.org/) online tool were used to retrieve mature and pre-miRNA and human miRNAs were used as a reference sequence. Local nucleotide database was formed for type 2 DM EST sequence after the elimination surplus and unwanted sequences. The nucleotide database was searched for their cognitive miRNAs. Figure.1 shows the flow chart for the identification of miRNAs from computational approach.

Figure.1 shows the flow chart for the identification of miRNAs from computational approach
2.2 Extracting Pre-miRNAs and their Precursor Sequences

Novel miRNA has been identified as previously described in priyanka et al 2018 (2). Using BLAST, BLASTx and clustal W sequences aligned with reference sequences and then aligned region was extracted and considered to be a Pre-miRNA sequence.

2.3 Validation of Pre-miRNAs and Identification of Target mRNA for mature miRNA

Mfold (http://unafold.rna.albany.edu/) were used for finding Pre-miRNAs secondary structure and the criteria for selecting candidate miRNA was adopted from priyanka et al 2018 (2). The predicted miRNA was evaluated by higher energy MFEs and A+U contents and Target scan software were used for target prediction.

2.4 Type 2 DM Clinical samples and base line clinical data

Genetically related 40 Type 2 DM selected and divided in to two groups. Group I contains 20 healthy volunteers who are randomly involved in this studies and Group II consisted of 20 Type 2 DM. Table 2 shows that the data composed of each patient which including body mass index (BMI), family history, age, height and weight. With use of automatic biochemistry analyzer the levels of Fasting blood glucose (FBG), Post prandial (PP), glycosylated hemoglobin (HbA1c), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and C-reactive protein (CRP) were detected. Totally 7 ml of blood was collected from each subjects and a portion of each sample was stored for genomic DNA isolation and the remaining sample was processed for serum to determined the above parameters. All the parameters were calculated by the Friedewald formula. All experimental protocols were approved by a Narayana Medical College, Bangalore, India (approval Number: 2018/368) and all methods were performed in accordance with the relevant guidelines and regulations. Informed consent was also obtained from all human subjects.

2.5 RNA isolation and quantification of miR-1285 and SCP2

The expression pattern of miR-1285 was performed according to the procedure described by Krishnan et al 2017. The RNA was isolated using an RNeasy Mini Kit (Qiagen). Quantification of miR-1285 was performed by PCR using the following primers: Forward: 5’ TTTTTTTTACGTTTTTTTAAAGGTC-3’; reverse: 5’-CTATTACTAAACCCAACCTACACGC -
3’. MiR-1285 levels were normalized to 18s RNA levels using the $2^{\Delta\Delta Ct}$ model. The quantification of the target gene SCP2 was performed by the same method by using the primers:
Forward: 5′- TTTTTTATGTTTTTTTTAAGGTTGT -3’; Reverse: 5′-CCCTATTACTAAACCCCAACTACACAC -3’.

2.6 Bisulfite conversion and Methylation analysis

DNA was extracted from Type 2 DM using Qiagen mini kit and bisulfite-treated DNA was extracted EZ DNA Methylation-Gold Kit (Zymo Research) following the manufacturer's instructions. Hypermethylation analysis has been carried out by using the method described by Vogelsang et al (2014). The primers used in these experiments are MSP-miR-1285-Fw (GGTATGATTAAGGTAAATCGAGTAGC) and MSP-miR-1285-Rv(AACAATCGAAAATCAATACCGA) has been used to amplify CpG rich promoter region (450bp) from bisulfate treated DNA templates followed by second round of methylated and unmethylated specific primers amplification. MiRNA-1285 Fwd M: TTTGAGCTTTCGTAGTTTTGAAC,MirRNA-1285ReverseM: ATCCCTAATAATATCAACCTCCGAC,MirRNA-1285FwdU: TGAGTTTTTGTAGTTTTTGAAATGTMirRNA-1285reverseU: ATCCCTAATAATATCAACCTCCCAAC.

2.7 Statistical Calculation

Statistical analysis for all datas was carried out using SPSS software version 25.0 for Microsoft Windows. Two-tailed student’s t-test was performed and P < 0.05 was considered to be statistically significant. All data’s are expressed as the mean ± SD.

3. Results

3.1 Identification of miR-1285 and its targets through computational approach

After analyzing all the ESTs from NCBI, MiR-1285 has been identified by using BLAST, BLASTx and secondary structure evaluation procedure. Figure.2 depicts the predicted secondary structure.
MiR-1285 has been marked as identified MiRNA by looking at its sequence, precursor sequence length, minimum folding free energies and A + U content for the predicted MiRNA are provided in Table 1.

Table 1.-Represents the information about Mir-1285 after computational Prediction

<table>
<thead>
<tr>
<th>Source miRNA</th>
<th>Source Organism</th>
<th>PL</th>
<th>MFEΔG</th>
<th>MS</th>
<th>ME</th>
<th>SE</th>
<th>Strand</th>
<th>A+U %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp-mir-1285-1</td>
<td>Homo sapiens</td>
<td>84</td>
<td>-58.20%</td>
<td>GAUCUC</td>
<td>38/40</td>
<td>AJ318808.1</td>
<td>5'</td>
<td>52.38%</td>
</tr>
</tbody>
</table>

PL = Pre-miRNA Length, MEF = Minimal Free Energy, MS = Mature sequence, ME = Match Extent, SE = Source EST.

We found some targets for MiR-1285, among the list sterol carrier protein 2 is the top most target for MiR-1285 linked to diabetes and cholesterol metabolism (12). Table.2 represents
various target mRNA for miR-1285. So it has been understand that miR-1285 directly involved in the regulation of DM.

Table.2 Shows that the various target mRNA for MiR-1285.

<table>
<thead>
<tr>
<th>SL.NO</th>
<th>Target mRNA</th>
<th>Representative transcript</th>
<th>Molecular Function</th>
<th>Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sterol carrier protein 2</td>
<td>ENST00000488965.1</td>
<td>fatty-acyl-CoA binding, catalytic activity, signaling receptor binding</td>
<td>Positive regulation of intracellular cholesterol transport. Protein targeting to peroxisome</td>
</tr>
<tr>
<td>2</td>
<td>glycoprotein hormones, alpha polypeptide</td>
<td>ENST00000369582.2</td>
<td>hormone activity, protein binding, follicle-stimulating hormone activity</td>
<td>Regulation of transcription by RNA polymerase II. positive regulation of cell proliferation</td>
</tr>
<tr>
<td>3</td>
<td>testis expressed 30</td>
<td>ENST00000376027.1</td>
<td>Hydrolase activity</td>
<td>Not yet known</td>
</tr>
<tr>
<td>4</td>
<td>Nucleosome assembly protein 1-like 6</td>
<td>ENST00000373518.1</td>
<td>Nucleosome assembly protein</td>
<td>Nucleosome Assembly</td>
</tr>
</tbody>
</table>
3.2 Down regulation and hypermethylation of miR-1285 enhance the type 2 DM

In our study group 40 Type 2 DM patients and 40 Healthy controls were added. From the sample, 20 (50%) were men, and 20 (50%) were women. Baseline clinical data shows that (Table.3) there is no significant between Type 2 DM and controls with respect to BMI, total cholesterol, HDL, LDL and triglycerides (P > 0.05). HbA1C, CRP blood glucose levels showed high differences between patients and controls (P < 0.05). According to our previous publication (2, 9) hypermethylation is one of the key molecular mechanisms for the induction of type 2 DM and it has been documented that MiRNAs follows the same molecular mechanism like classical genes including epigenetic events. Many MiRNAs involved in the progression of type 2 DM but the exact mechanism behind its action is still questionable. So in this study we intend to analyze and evaluate hypermethylation and expression status of identified miR-1285 from available Type 2 DM ESTs. In order to test this hypothesis, we evaluated the expression nature of mature miR-1285 in blood cells from Type 2 DM and healthy samples. Interestingly, our results shows that the miR-1285 in control sample was significantly lower than that in the Type 2 DM patients (Fig. 3).

Table.3 Represents the baseline clinical Parameters for the study population

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Typ2 DM</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>60±5.3</td>
<td>61.2±5.5</td>
</tr>
<tr>
<td>Gender(M/F)</td>
<td>25/15</td>
<td>25/15</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>34±6.5</td>
<td>32.5±5.5</td>
</tr>
<tr>
<td>Duration of T1DM, yr</td>
<td>5.2±5.3</td>
<td>0</td>
</tr>
<tr>
<td>Glycemia (mmol/L)</td>
<td>7.5±5.1</td>
<td>4.5±4.1</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.8±7.1</td>
<td>5.0±4.1</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>4.8±3.2</td>
<td>4.3±3.1</td>
</tr>
<tr>
<td>LDL - cholesterol (mg/dl)</td>
<td>90±5.2</td>
<td>87±4.2</td>
</tr>
<tr>
<td></td>
<td>Type 2 DM</td>
<td>Control</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>HDL - cholesterol (mg/dl)</td>
<td>38.2±4.5</td>
<td>34±3.2</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>135±7.4</td>
<td>134±6.2</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>11.9±1.1</td>
<td>5.1±1.0</td>
</tr>
</tbody>
</table>

Figure. 3. Represents expression level of circulating miR-1285 Type 2 DM by quantitative real time PCR (q PCR). Unpaired t-test data P value<0.005*Vs Control Samples.

Sterol Carrier Proteins 2 (SCP2) is the top most target for MiR-1285, according to the literature SCP directly involved in diabetes and cholesterol metabolism. We analyzed the expression status of SCP2 in all above clinical samples control samples. As we expected SCP2 gene expression was found to be higher in control samples compared to Type 2 DM (Fig. 4).
Based on our gene expression data, miR-1285 levels was higher in Type 2 DM and it may relevant to the initiation and development of Type 2 DM. In contrary, SCP2 gene expressions lower in Type 2 DM (Fig.4). In order to clarify the molecular mechanism behind the expression status of miR-1285 and SCP2. We performed the hypermethlation experiments on the same samples since methylation and expressions are inversely proportional stated by many research articles (13, 14). As per the literature the epigenetic regulation of miRNAs is a widespread phenomenon (2,13,14). In order to evaluate the hypermethylation status of MiR-1285 in Type2 DM and control samples, we used methylation-specific PCR (MSP) or nested MSP. Our study revealed that the miR-1285 promoter region was unmethylated in Type 2 DM samples, but in contrast the control sample was found to be methylated (Fig. 5).
On the other hand, the SCP2 promoter region was methylated in Type 2 DM and unmethylated on control samples (Fig. 6).

Fig. 6. Represents the methylation status of SCP 2 promoter region in Type 2 DM samples and the control samples by Methylation sensitive PCR (MSP).

We also evaluated the methylation frequency of all the samples for the above candidates (Figs. 7 & 8)

Fig. 7. Represents the methylation frequency for the promoter MiR-1285 was compared between Type 2 DM and normal samples. The number of Methylated (M) and Unmethylated (U) samples is indicated above the bars. Unpaired t-test data P value<0.005 Vs*Control.
Fig. 8. Represents the methylation frequency for the promoter SCP 2 was compared between Type 2 DM and normal samples. The number of Methylated (M) and Unmethylated (U) samples is indicated above the bars. Unpaired t-test data P value<0.005 Vs*Control.

and we found that the methylation frequency was statistically significant between these samples (55.0 vs. 85.0%, and 57 Vs 80% P ≤0.05). Over all our data suggesting that expression nature of miR-1285 corroborate with methylation status. MiR-1285 was found to be a candidate MiRNA for Type 2 DM additionally it need more investigation to prove the above mechanism.

4. Discussion

It has been suggesting that the mechanisms in which miRNA repress the targets are diverse, for example, the miRNAs binds to complementary seed region and also in the central region causing mRNA cleavage and subsequent degradation of mRNAs. Many articles demonstrated that miRNAs reduce expression of their cognate target genes by degrading m RNA, inducing decapping, inducing deadenylation, altered cap protein binding, and reduced ribosome occupancy. MiRNAs regulate wide range of molecular mechanism such as transcriptional activation or inhibition, epigenetic repression, cell proliferation, differentiation and other cellular function. It is considered to be non-invasive biomarkers since it is found in many body fluids. Computational approach is the best method of identifying miRNA through conditional specific
ESTs. Type 2 DM is an insulin independent autoimmune disorder and miRNAs is a key player in regulating Type 2 DM. up to date there is no prognostic, diagnostic or therapeutic target for Type 2 DM.

Our computational approach found that SCP2 is a direct target for MiR-1285 and interestingly, SCP2 involved in cholesterol and diabetic complications. From our results miR-1285 expression directly corroborated with the promoter methylation in Type 2 DM. Surprisingly only few studies reported on the SCP2 expression status in diabetes and importantly the association with miR-1285 has not been revealed, moreover the molecular mechanism behind their role has not been elucidated. Our study revealed that miR-1285 may act as a candidate miRNA in Type 2DM, still future studies needed to support this investigation.

5. Additional Information

There is no competing financial and non-financial Interest

Author contribution statement

A. Lian Bai – Execution of the hypermethylation experiments and all data processing
B. Junwu Li – preparation of figure 1,2 and bioinformatics analysis
C. Mani Panagal – preparation of figure 3,4 and primers synthesis
D. Biruntha M - writing materials and methods and miR-target analysis
E. Durairaj Sekar – concepts, manuscript writing and follow up each experiment.

6. References


