Original Article

INS Protein-Regulating microRNA Panel in Gene Reversal Strategies for Type 2 Diabetes Mellitus

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Abstract

Background: Type 2 Diabetes Mellitus remains a major global health issue in both developed and developing nations. In Diabetes the body loses its ability to respond properly to insulin. As much research has focused on how the INS gene is controlled at the transcription level. But still less attention has been given to what happens afterward. In particular, small molecules known as miRNAs play a quiet and powerful role in regulating insulin production. These tiny RNA molecules bind to messenger RNAs and block them.

Objective: To pinpoint particular microRNAs that influence insulin production post-transcription. In order to investigate if targeting these miRNAs might aid in restoring insulin levels.

Methods: A case-control study involved 40 participants. Possible miRNAs were initially discovered utilizing computational resources like TargetScan 8.0, miRDB, and other. The levels of their expression were subsequently assessed using quantitative real-time PCR. Insulin levels in serum were evaluated using ELISA. Correlation analysis was conducted.

Results: Hence, seven miRNAs were displaying notable variations between diabetic and healthy people. In T2DM patients, five miRNAs (miR-375, miR-7, miR-29a, miR-29b, and miR-21) showed a significant increase. Whereas two (miR-30d and miR-146a) exhibited a decrease.

Conclusion: Overall, the results indicate that network of microRNAs collaborates to decrease insulin production in T2DM. By inhibiting detrimental miRNAs or reinstating helpful ones, it might be feasible to reactivate insulin secretion. These strategies may signify a novel pathway for gene-focused treatments in diabetes care.

Keywords: miRNA interaction, gene reversal, bioinformatic, protein regulation, correlation

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Introduction

Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disease, arising from both genetic susceptibility, a mesh of environmental circumstances and molecular perturbations. The disease is characterized by insulin resistance as well as impairment of pancreatic β-cell function until there is inadequate insulin secretion.[1] While pharmacological management has progressed, many treatments remain symptomatic and do not address correction of fundamental molecular deficits.[2] Insulin is a key molecule for glucose homeostasis and the INS gene encodes preproinsulin, which is further processed into insulin. Abnormal expression of INS is known to be closely associated with disordered insulin synthesis and secretion in T2DM.[3] Although INS transcriptional regulation has been well explored, post-transcriptional control is still a progressing field.[4]

MicroRNAs (miRNAs) are short non-coding RNAs, post-transcriptional regulating gene expression through complementary base pairing with 3′-UTR in mRNAs, leading to translation repression or mRNA degradation.[5] It is becoming increasingly clear that miRNAs contribute to the control of β-cell development, insulin production and response to stress. Insight into the mechanisms of miRNA control of INS expression might offer new perspectives on pathophysiological processes and therapeutic modalities.[6] MiRNAs act as fine-tuning regulators of gene expression, which permits a cell to respond quickly to physiological or pathological conditions. In pancreatic β-cells, miRNAs are essential for the maintenance of insulin secretion particularly under normal metabolic conditions.[7] The abnormal regulation of these molecules could lead to inappropriate INS expression many years before the appearance of clinical diabetes.[8]

Target Scan, as well as other bioinformatics databases including miRWalk and miRDB, predict miRNA-mRNA interactions by sequence complementarity and evolutionary conservation.[9]

In principle, miRNAs target the 3′-untranslated region (3′-UTR) of INS mRNA and when over-expressed can inhibit insulin production, thus causing β-cell dysfunction.[9] A miRNA panel rather than individual miRNAs may more accurately reflect the regulation of INS expression through an integrated network. This panel could be used as a diagnostic mark and also in gene reversal strategies, as a therapeutic target.[10] Studies from different ethnic groups has revealed this data that shows the significant role of miRNAs in the progression of disease. For instance, there are several reports in Chinese and Japanese populations. Differences in regulating microRNAs related to INS protein among ethnic groups have been observed in Han. The patients showing a threefold increase in miR-375 expression (3.0 vs. 1.0). The elevated methylation levels (10.38% vs. 8.47%) compared to Kazak patients hence suggesting unique population-specific molecular processes. The European populace has also provided us with certain insights. In a study focused on individuals from Italy and Austria known as the Bruneck Study, Zampetaki and colleagues examined 822 participants. What they discovered was unexpected. Individuals with Type 2 Diabetes exhibited miR-126 levels in their bloodstream. They also discovered that individuals had concentrations of miR-126, miR-15a, miR-29b, and miR-223 prior to developing Type 2 Diabetes. They also examined individuals from the Middle East. For example, individuals with Type 2 Diabetes in the Emirates possessed let-7b-5p.In general, the reports indicate that over 70 microRNAs are functioning improperly in individuals with Type 2 Diabetes across various regions globally. There isn't a specific group of microRNAs that we can definetively say aids in insulin issues. Nonetheless, it appears that micro-RNAs influence the functioning of insulin within the body. There is limited knowledge regarding which microRNAs may assist with insulin issues in South Asian individuals, particularly in Pakistan. This creates a significant knowledge gap that warrants further investigation.

Methods

In this case–control study, the aim was to investigate the regulatory role of insulin-associated microRNAs and explore their potential application in gene-reversal strategies for Type 2 Diabetes Mellitus (T2DM). The study was conducted in accordance with the Declaration of Helsinki, and it was approved by Institutional Ethical Review Committee. Before collecting any samples, the written informed consent from all participants were obtained. A total of 40 individuals between 30 and 65 years of age were enrolled, dividing them into two groups: 20 healthy controls and 20 patients diagnosed with T2DM according to the American Diabetes Association criteria. We excluded individuals with type 1 diabetes, cancer, chronic inflammatory diseases, liver or kid-ney failure, pregnancy, or those receiving insulin therapy to avoid potential bias in gene expression analysis (Table1.).

Table 1: *Clinical Characteristics of Study Participants*

Parameter	Healthy Controls (n=20)	T2DM Patients (n=20)	p-value
Age	42.3 ± 6.0	44.8 ± 5.6	>0.05
Gender (M/F)	XX / XX	XX / XX	—
Fasting Blood Glucose (mg/dL)	92.4 ± 8.6	182.7 ± 34.5	<0.001
HbA1c (%)	5.3 ± 0.4	8.7 ± 1.2	<0.001

The final sample size was limited by the strict inclusion and exclusion criteria. It is employed to ensure a homogeneous study population. Since this study is highly profile so limited samples were taken. It was done so due to lack of fund and laboratory apparatus. To identify insulin-regulating microRNAs, an in-silico analysis using multiple bioinformatics tools, including miRBase for microRNA sequence retrieval. Target Scan 8.0, miRDB, and DIANA-microT-CDS for target gene prediction and binding validation was performed. To explore how these molecules interact, protein–protein interaction networks were constructed using STRING. The pathway enrichment analysis was performed using the KEGG database. The resulting microRNA–gene interaction networks were then visualized and integrated using Cytoscape (version 3.9). Hence, allowing a clearer understanding of the overall biological relationships. To maintain reliability, only those microRNAs were predicted by at least three databases. The previously reported were to be involved in pancreatic β-cell function or inflammatory pathways then selected. Based on these criteria, seven microRNAs — miR-375, miR-7, miR-29a, miR-29b, miR-30d, miR-146a, and miR-21 were chosen for further analysis (Table 2).

Peripheral blood samples were collected from all participants after overnight fasting. Total RNA, including small RNA, was extracted using TRIzol reagent. Its quality and concentration were evaluated using a NanoDrop spectrophotometer. The extracted RNA was then converted into complementary DNA (cDNA) using specific primers. Gene expression levels were measured using quantitative real-time PCR with SYBR Green chemistry. Finally, relative expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method, with U6 snRNA and GAPDH serving as internal controls. Serum insulin levels using a sandwich ELISA were also measured (Table 3), reading absorbance at 450 nm and determining concentrations from a standard curve. For statistical analysis, GraphPad Prism and SPSS software were used. Student's t-test to compare groups and Pearson correlation analysis to evaluate the relationship between microRNA expression and insulin levels was applied (Figure3,4,5). The considered p-values below 0.05 as statistically significant and presented all data as mean ± standard deviation. Finally, the identified microRNAs based on their functional impact on insulin expression were classified. It was proposed that insulin-suppressive microRNAs could be targeted using antisense inhibitors (antagomirs), while insulin-supportive microRNAs could be restored through replacement strategies. Through this framework, it was suggested that microRNA modulation may serve as a promising gene reversal approach to enhance insulin transcription and secretion in T2DM.

Results

The relative expression of selected microRNAs was quantified using the $2^{-}\Delta\Delta Ct$ method with U6 as the internal control (Table 2; Figure 1).

Table 2: *Differential Expression of Insulin-Regulating microRNAs in Study Groups*

microRNA	Healthy Controls (n = 20)	T2DM Patients (n = 20)	Fold Change (T2DM vs Control)	$\log_2$ Fold Change	Regulation	p-value
miR-375	1.00±0.18	2.47±0.36	↑ 2.47	+1.30	Up-regulated	< 0.001
miR-7	1.00±0.22	2.12±0.31	↑ 2.12	+1.09	Up-regulated	< 0.01
miR-29a	1.00±0.19	1.88±0.28	↑ 1.88	+0.91	Up-regulated	< 0.01
miR-29b	1.00±0.20	1.74±0.24	↑ 1.74	+0.80	Up-regulated	< 0.05
miR-30d	1.00±0.21	0.55±0.13	↓ 0.55	-0.86	Down-regulated	< 0.01
miR-146a	1.00±0.17	0.63±0.15	↓ 0.63	-0.67	Down-regulated	< 0.05
miR-21	1.00±0.16	2.82±0.40	↑ 2.82	+1.49	Up-regulated	< 0.001

Significant alterations in multiple insulin-associated microRNAs were observed in T2DM patients compared with healthy controls. MiR-375 showed the highest increase in diabetic subjects, demonstrating a 2.47-fold upregulation ($p<0.001$). Similarly, miR-7 exhibited significant overexpression (2.12-fold, $p< 0.01$). Members of the miR-29 family also showed elevated expression, where miR-29a increased 1.88-fold ($p<0.01$) and miR-29b increased 1.74-fold ($p<0.05$). In contrast, miR-30d displayed significant downregulation (0.55-fold, $p< 0.01$). Likewise, miR-146a expression was reduced (0.63-fold, $p<0.05$). Another inflammatory-associated microRNA, miR-21, showed strong upregulation (2.82-fold, $p<0.001$). Overall, the expression profile demonstrates predominance of upregulated diabetogenic and inflammatory microRNAs with simultaneous suppression of insulin-supportive microRNAs.

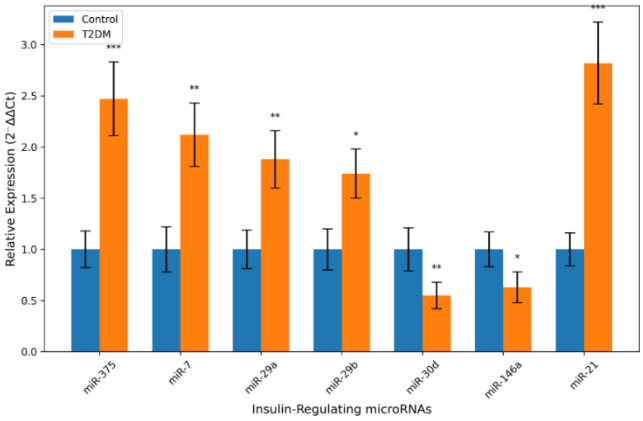

Figure1: *Comparative miRNA expression levels between control and T2DM subjects. Values are expressed as mean ± SD; statistical significance was assessed using an independent t-test.*

Table 3: *ELISA-Based Insulin Quantification*

Group	Insulin (μIU/mL)	p-value
Healthy Control	11.6 ± 3.2	–
T2DM	6.9 ± 2.4	< 0.01

Serum insulin concentration was measured using ELISA (Table 3; Figure 2). Healthy controls showed insulin levels of 11.6 ± 3.2 µIU/mL, whereas T2DM patients had significantly lower levels (6.9 ± 2.4 µIU/mL, $p < 0.01$). These findings confirm reduced insulin production at the protein level.

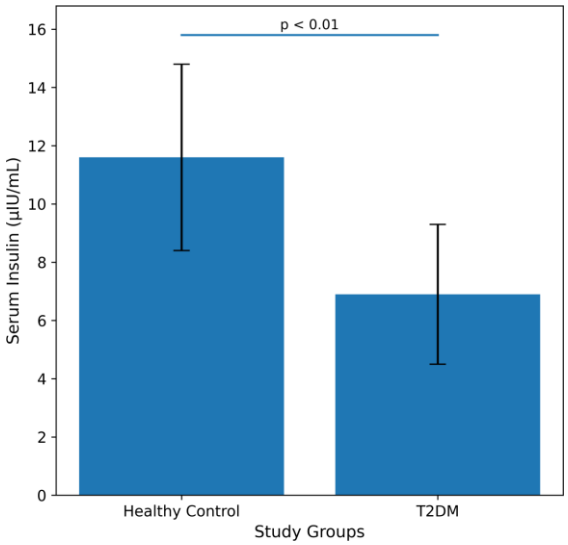

Figure 2: *ELISA-based measurement of serum insulin concentrations in control and T2DM groups. Data are expressed as mean±SD; p<0.01 considered statistically significant*

Pearson correlation analysis demonstrated a signify-cant negative association between miR-375 expre-ssion and INS mRNA levels (Figure 3). Increased miR-375 expression was correlated with reduced insulin gene expression (r=−0.XX, p< 0.05), indicating a potential post-transcriptional regulatory effect of miR-375 on insulin synthesis. These findings support the role of miR-375 as a negative regulator of panc-reatic β-cell insulin production.

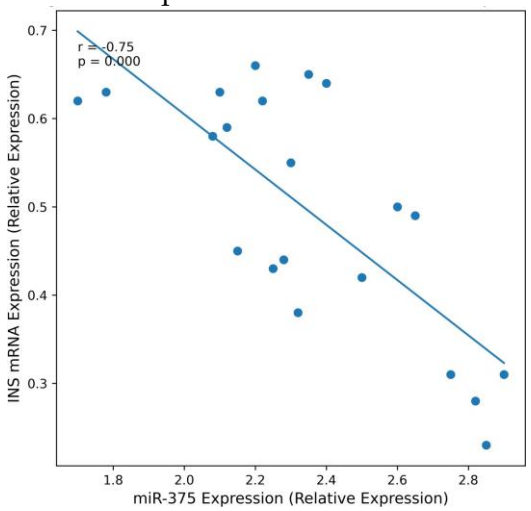

Figure 3: *Negative association between miR-375 expression and INS mRNA levels*

Correlation of miR-21 with Inflammatory Cytokines Scatter plot analysis demonstrated positive corre-lations between miR-21 and inflammatory mar-kers. miR-21 vs TNF-α: positive correlation (Figure 4) miR-21 vs IL-6: positive correlation (Figure 5) Higher miR-21 expression was associated with increased inflammatory cytokine levels, indicating involve-ment in diabetes-related inflammation.

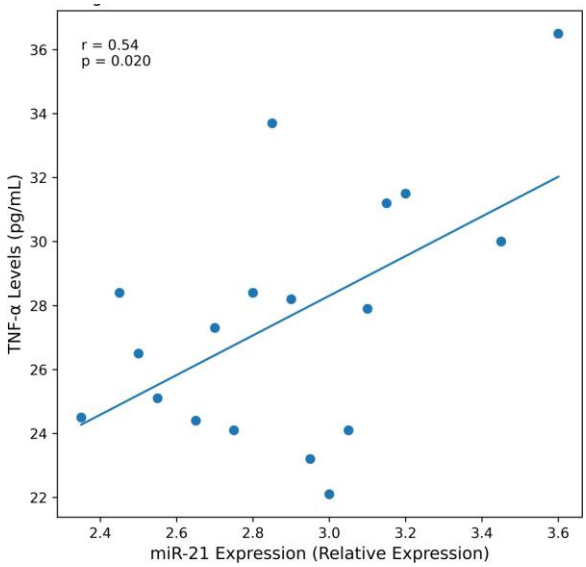

Figure 4: *Positive correlations between miR-21 and inflammatory marker TNF-a: positive correlation*

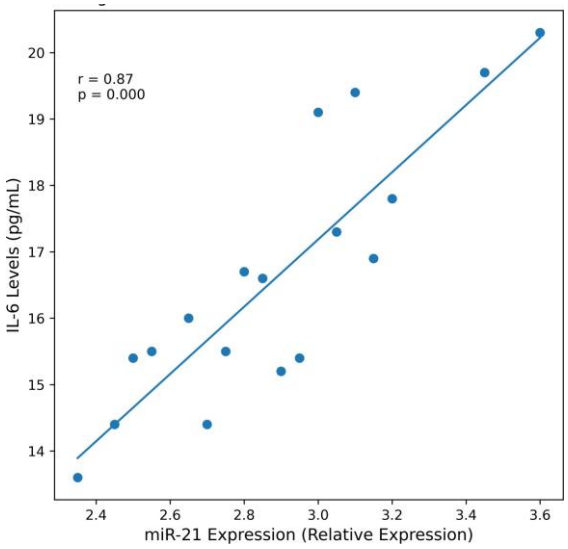

Figure 5: *miR-21 vs IL-6: positive correlation*

Discussion

In the present study, we investigated the expression pattern of insulin-regulating microRNAs to under-stand their contribution to β-cell dysfunction and the possibility of gene-reversal strategies in Type 2

Diabetes Mellitus (T2DM).[10-13] Our findings demonstrate a coordinated dysregulation of multiple microRNAs associated with β-cell function and inflammation, accompanied by reduced INS gene expression and decreased circulating insulin levels.[11] These results indicate that post-transcriptional regulation plays a major role in β-cell failure in T2DM, rather than insulin deficiency being solely due to irreversible β-cell loss.[12] Among the β-cell-associated microRNAs, miR-375 showed the strongest upregulation in diabetic subjects. As a well-established islet-enriched microRNA, miR-375 negatively regulates insulin secretion (Figure 3) by targeting genes involved in vesicle transport and β-cell signaling.[13] Similar studies from the European population has also confirmed this that they are involve in down regulation of the protein production. Also when studied the Middle Eastern population similar trends were found.[14] Emerging Evidences are also found in the Afican population as well. Its overexpression in our study, along with a clear inverse relationship with INS expression, strongly supports a post-transcriptional repression mechanism. The agreement between these studies and our findings may be explained by the conserved role of microRNAs. Differences observed among studies may be related to ethnicity, sample size, disease duration, and laboratory techniques. The upregulation of miR-29a and miR-29b further suggests that impaired insulin action occurs alongside reduced insulin production.[15] Hence, highlighting the dual contribution of insulin resistance and β-cell dysfunction in T2DM (Figure 4,5). In contrast, protective microRNAs were markedly suppressed in diabetic patients. We observed significant downregulation of miR-30d, a microRNA known to enhance insulin gene transcription and support β-cell function (Table 2). This trend was also seen in population of various ethnicity and thus correlating with already existing data.[16] Its reduced expression may therefore limit the β-cell's capacity to synthesize insulin. Additionally, miR-146a downregulation indicates loss of anti-inflammatory control, as this microRNA normally inhibits NF-κB signaling and cytokine production.[17-19] Consistent with this, miR-21 was significantly upregulated and showed a strong positive correlation with pro-inflammatory cytokines TNF-α and IL-6 (Figure 4,5), suggesting that chronic inflammation in T2DM is partly driven by microRNA-mediated mechanisms. Taken together, our results suggest that T2DM is not only a metabolic disorder but also a microRNA-regulated inflammatory disease. The inverse relationship between inhibitory microRNAs and insulin levels indicates that microRNAs act upstream of insulin deficiency. It highlights the therapeutic potential of microRNA-based gene-reversal strategies. While inhibition of diabetogenic microRNAs or restoration of protective microRNAs could reactivate endogenous insulin production and reduce inflammation.[20-23] Although the study is limited by a small sample size and lack of functional validation. But it provides a comprehensive regulatory model linking β-cell dysfunction, inflammation, and insulin suppression.[24-25] Hence, offering a promising molecular framework for future diabetes therapies.

Conclusion

Certain microRNAs, such as miR-30d and miR-146a, are believed to safeguard insulin-producing cells known as β-cells. In individuals with Type 2 Diabetes Mellitus, these protective microRNAs are absent. This indicates that the β-cells lack protection. Meanwhile, other microRNAs such as miR-375, miR-7, miR-29a/b, and miR-21 exhibit heightened activity. They inhibit the function of the insulin genes. It is an issue. If we can halt or reverse the microRNAs, the β-cells could potentially resume insulin production. The method varies from simply administering insulin externally to individuals. The research that discovered this was limited, so we must proceed with caution. The conduct research needs to be verified. It serves as a solid beginning. It indicates that we ought to seriously consider microRNAs as a method to alter the disease. The experiments in the laboratory and with living organisms needs to done to determine if this truly functions or not.

Ethical Approval: The ethical committee of Academy of Family Physicians of Pakistan approved this vide letter No. Ref. PAFP/ AO/ 3654/26.

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Authors' Contribution:

RN: Conception and design; acquisition, drafting of article

MA: Acquisition of data, drafting of article

AR: Analysis and interpretation of data

SR: Critical revisions for important intellectual content, final approval of the version to be published

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