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Role of circulating miRNA-9 as a diagnostic marker in a population with diabetic complications

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Abstract

Introduction: Diabetes mellitus is an autoimmune disorder which is caused by deregulation of blood glucose homeostatis accompanied by various other disturbances of carbohydrates, fat and protein metabolism. It also involves various other factors such as genetic, epigenetic and environmental factors. The deregulated metabolism of this disease is the failure of β cells to produce compensatory insulin which is a necessary step towards increased insulin resistance.
Material and methods: The present study was conducted over 50 diabetic patients and 50 non-diabetic healthy individuals. Briefly, reverse transcription was carried for cDNA synthesis using Applied Biosystems TaqMan *miRNA* RT kit (Thermo Fisher Scientific, USA). Statistical analysis used to compare the expression of *miRNA*s, clinical variables in cases and controls were done by SPSS software.
Results: Our study has revealed the significance of alterations and challenges in peripheral blood *miRNA*-9 as efficient biomarker for diabetes. The levels of *miRNA* 9 were found higher in diabetic patients in comparison to control healthy individuals. The mean age difference was found significant. In case of gender no significant difference was found between the two groups. ($p<0.05$). The glycemic status was found significantly higher in diabetic patients as compared to healthy individuals. No significant difference was found between the two groups in relation to blood pressure, triglycerides, total cholesterol and HDL.
Conclusion: The results revealed that however, a borderline significance and low diagnostic value of *miRNA*9 was evaluated through results still the fact that *miRNA*9 expression levels were higher in diabetes patients could not be surpassed and its link with the disease progression could not be foreseen.
Keywords: Diabetes, blood *miRNA*s, *miR*-9, biomarkers.

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1. Introduction

Diabetes mellitus is an autoimmune disorder of multiple etiologies that is basically due to deregulation of blood glucose homeostasis, thus causing hyperglaecimia accompanied by various other disturbances of carbohydrates, fat and protein metabolism [1,2]. The prevalence of this disease worldwide is a serious threat as it has increased substantially since last 50 years and has been estimated to affect 592 million people by 2035 [3]. The pathogenesis of this heterogenous disease involves various factors such as genetic, epigenetic and environmental factors [4]. The basic mechanism behind the development of this chronic disease is the failure of β cells to produce compensatory insulin which is a necessary step towards increased insulin resistance [5]. The vascular complication of the prolonged sustainability of

the disease is a serious manifestation of the disease and thus reduces the life expectancy of an individual [6].

The three principal forms of the disease are type I and Type II diabetes mellitus and gestational diabetes mellitus and other forms are linked to disorders of liver, pancreas, adrenal glands [7].

General signs and symptoms that mark the onset of the disease include blurred vision, polyurea, polydipsia, weight loss and polyphagia.

The disease pathophysiology is based on role of *microRNA* (*miRNA*s) that are associated with pancreatic β cells and insulin target tissues for this disease in particular [8,9]. These micro RNAs are endogenous small non coding RNAs present in cell free form in blood circulation [10] that regulate gene expression at post-transcriptional level by binding to complementary 3' UTR of the target mRNA and thereby restraining protein production [11,12]. Major signaling pathways could be adversely affected if associated *miRNA* are dysregulated and hence they are meant to control gene regulatory functions [13]. Indeed, these circulatory *miRNA*s are also involved in various physiological mechanisms such as cell proliferation, differentiation, apoptosis and metabolism of the cell hence, they are employed in various pathologies in the form of diagnostic, prognostic and predictive biomarkers as they can be easily be extracted through non- invasive procedures [14,15].

Among various *miRNA*s that govern the signaling pathway of insulin; the mechanistic regulation of its secretion is carried out by islet specific *miRNA* 375 and *miRNA* 9 which play a major role in the pathogenesis of the disease and blood glucose homeostatis [16]. Among the two *miRNA*s this research article basically focuses on *miRNA*-9. Regulation of insulin release in pancreatic β cells is carried out by *miR*-9 via interactions with transcription factor Oc-2.Inhibition of glucose stimulated insulin release is due to overexpression of *miR*-9. This leads to the negative regulation of granuphillin/Spl4, RabGTPase effectors related to insulin secretory granules. This *miRNA* reduces the levels of Sirt 1 and GSIS. Activation of Sirt1 causes downegulation of Ucp2 which negatively regulates GSI. Downregulation of Sirt1 may cause inhibition of *miRNA*9 and upregulation of Ucp-2 [17].

Their quantification is made possible through quantitative Reverse Transcription PCR (RT-qPCR) which is accompanied by both sensitivity and specificity to visualize the expression of diabetic patients in comparison to control group.

2. Material and Methods

2.1 Study design and sample collection

The present study was conducted over 100 subjects including 50 diabetic patients and 50 non-diabetic healthy individuals. The blood samples were collected from both patients recruited as cases and controls attending Department Of Endocrinology Era's Lucknow Medical College and Hospital Lucknow. Written informed consent was obtained from each participant in advance for the study and was approved by Ethic committee of Era's Lucknow Medical College and Hospital Lucknow.

Diabetic patients were defined as FG levels (5.6-6.9mmol/L), IGT as 2 h PG after 75g OFTT levels (7.8-11.0mmol/L, hbA1 levels <6.5%. Healthy individuals were those with no previous history ranging between levels stated above.

The participants were enrolled in accordance with the inclusion and exclusion criteria as stated below:

2.2 Inclusion Criteria

1) Blood samples from patients who fulfilled the diagnostic criteria of diabetes were used at the time of the study.
2) Patients willing to get enrolled in the study.

2.3 Exclusion criteria

1) Subjects suffering from tuberculosis, pancreatic disease and other autoimmune diseases which could affect the *miRNA* expression levels were excluded from the present study.
2) Subjects not willing to participate in the study.

2.4 Blood collection, storage of serum and RNA isolation

The patients were categorized according to World Health Organization Guideline. Basic demographic and clinical information was fetched from each subject participating in the study. For serum preparation 5 ml of peripheral blood was collected in a clot activator tube and allowed to coagulate at room temperature for 45-50 minutes until further processing according to Qiagen kit (Germany). This step was followed by centrifugation at 3000 rpm for 10 minutes at 4C (REMI). Further removal of contaminants such as, cellular debris including erythrocytes, the supernatant was centrifuged at 13500 rpm for 10 minutes at 4C to obtain better concentration for RNA isolation. It was aliquoted and stored at -80 until *miRNA* detection. Total RNA, including *miRNA* was collected from serum samples using blood *miR*Neasy Mini kit (Qiagen kit, Germany) according to manufacturer's instructions. Moreover, *miRNA* extraction was carried by using *miR*Neasy Serum/Plasma Kit (Qiagen kit, Germany). The purity and concentration of the sample was quantified using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

2.5 Reverse transcription

Briefly, for reverse transcription cdna synthesis was done using Applied Biosystems TaqMan *miRNA* RT kit (Thermo Fisher Scientific, USA). The reaction was carried out with total volume of 20 µl, reverse transcription HiSpec buffer (1.5 µl), dNTPs (4 µl), miSript master mix (2 µl) and nuclease free water (10.5 µl). The PCR conditions were kept

as 95^0C for 5 minutes and 37^0C for 60 minutes. cDNA samples were stored at -20 C until analysis.

2.6 Real time PCR

Each reaction was performed in a final volume of 25 µl with 1 µl of 1/100 dilution of cDNA in water, 2.5 µl of universal primers, 2.5 µl PCR primers and 12.5 µl 2X Quanti Tech SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific) along with RNAse free water. The reactions were amplified for 95^0C for 15^0C and 30 secs at 60^0C for 40 cycles. The thermal denaturation protocol was used to evaluate the total number of products present in the reaction. All reactions were performed in triplicates using SYBR Green Master Mix (Qiagen, Germany) and included no templates and no reverse transcription controls were specifically used for any gene. All data was analyzed using 7900 HT real time PCR system (Apllied Biosystems; Thermo Fisher Scientific). Analysis of relative gene expression of *miRNA* 9 was done using Livak Method $2^{-\Delta\Delta Ct}$. The CT value corresponds to the number of cycles required to generate signal to reach a definite threshold.

2.7 Statistical analysis

All statistical analysis used to compare the expression of *miRNA*s, clinical variables in cases and controls were done by statistical t-test and on way ANOVA (analysis variance) as appropriate through SPSS software. Receiver operating characteristic (ROC) analysis was used as a statistical method to evaluate the diagnostic accuracy of *miRNA*9 unveiling the trade-offs of the true-positive rate (sensitivity) and false-positive rate (1-specificity). All the data presentations are in the form of mean ± standard deviation (SD). Probability (p) value was taken <0.05 was considered statistically significant.

3. Results

3.1 Basic characteristics of diabetic and non-diabetic (healthy) subjects

This study constituted 50 diabetic patients and 50 non diabetic patients. Basic characteristics of the subjects are compared in the table given below. The age of the diabetic patient ranged from 53±15 years and healthy patients was 50±6. The mean age difference was found significant ($p<0.001$).The ratio of male to female diabetic patient was 23/27 and that of healthy patient was 26/24. In case of gender no significant difference was found between the two groups. ($p<0.05$). The glycemic status including FG, HbA1C, OGGT was found significantly higher in diabetic patients as compared to healthy individuals. For parameters such as mean blood pressure, triglycerides, total cholesterol and HDL, no significant difference was found between the two groups. LDL was significantly higher in diabetic patients as compared to control. Table 1 illustrates the basic characteristics of diabetic and non-diabetic (healthy) subjects.

Table 1: Basic characteristics of diabetic and non-diabetic (healthy) subjects The data is presented in the form of

Characteristics	Diabetes Type 2	Control (healthy subjects)
Number of subjects	50	50
Age(years)	53±15	50±6
Gender(Male/Female)	23/27	26/24
Diabetes duration	18±4.7	-
BMI(Kg/m^2)	25.9±(5.2)	23.6±(4.4)
FG(mmol/L))	9.1± (14.3)	5.1±(0.7)
HbA1c (1%)	8.44± (2.5)	4.81±(0.66)
2h OGTT(mol/L)	13.65± (2.01)	5.71± (0.71)
Mean blood pressure(mmHg)	89.1± (5.3)	87.5± (4.02)
Trigylceride	1.47± (0.4)	1.52± (0.5)
Total cholesterol	4.59± (1.11)	4.44± (0.6)
LDL(mmol/L)	2.48± (1.1)	2.13± (0.8)
HDL(mmol/L)	1.33± (0.2)	1.47± (0.3)

(i) Either numbers, percentages (%) for categorical data (ii) mean ± standard deviation for parametrically distributed data or median (interquartile range) for non-parametrically distributed data.

BMI- body mass index; FG- fasting glucose; HbA1c- glycated haemoglobin; OGTT- oral glucose tolerance test; HDL- high density lipoprotein; LDL- low density lipoprotein; $p < 0.05$, $p < 0.001$ compared with the non-diabetic subjects; # $p < 0.05$, ## $p < 0.001$ compared with T2D patients.

3.2 Expression analysis of circulating *miRNA*-9 in diabetic and non-diabetic (healthy) patients

The expression of circulating *miRNA*-9 and its normalization control *miRNA*-16 were determined by SyBr Green based RT-qPCR in blood of diabetes patients and non-diabetic patients as control group. The result predicted the significant up-regulation of *miRNA*-9 in diabetic patients (2.5± 1.3) as compared to non-diabetic patients (4.9± 1.6). It was found to be 2 fold (calculated as the mean ± SD) up-regulated in diabetic patients as compared to control group ($p < 0.001$) (Figure 1).

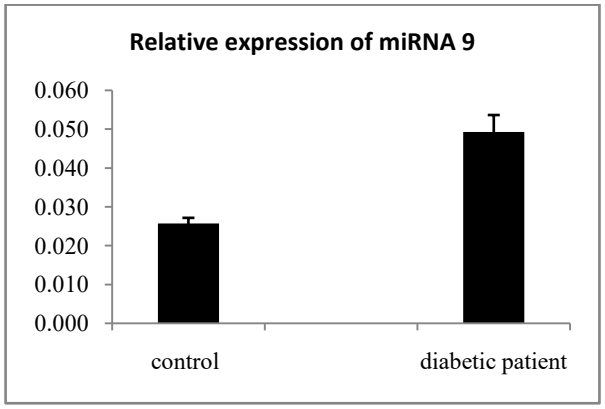

Figure 1: Expression analysis of circulating *miRNA*-9 in diabetic and non-diabetic (healthy) patients

The expression of *miR*-9 normalization relative to the expression of *miR*21 were determined by TaqMan-based RT-qPCR in peripheral whole blood of diabetes patients and healthy control individuals. Data is shown as mean ± SD. * $p < 0.05$ compared to healthy controls

3.3 Evaluation of the Diagnostic Values of Blood *miR*-9

The conventional approach of dichotomous (positive/negative test results), diagnostic test evaluation uses sensitivity and specificity as measures of accuracy of test. To elucidate the feasible role of *miR*-9 as biomarker for diabetes, its diagnostic value was determined using the receiver operating characteristic (ROC) curve analysis. Through ROC analysis it was evaluated that *miR*-9 revealed a poor aptitude and uncertain significance in distinguishing between the subject groups. The AUC was 0.50 (95% 0.301 to 0.514 p = 0.075) in discriminating T2D patients from healthy subjects.

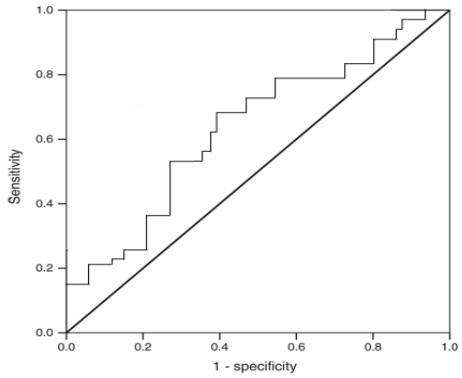

Figure 2: Receiver operating characteristic curve analysis

Receiver operating characteristic (ROC) curves constructed to assess the diagnostic value of *miR*-9 as biomarkers for diabetes and control group

4. Discussion

The rapid elucidation of the elementary role of micro RNAs (*miRNA*s) in the commencement of a wide range of human oncological and non-oncological diseases and their presence in the blood stream have fetched the interest of most of the researchers to explore their potential as biomarkers. There exists a vast quantitative and qualitative difference in the levels of these micro RNAs due to specific alterations in diseased and healthy individuals which remarkably highlight their sensitivity in various pathologies. In context of diabetes, biomarkers heralding the early diagnosis of the disease progression allow the researchers to avail preventive measures and increase the life expectancy of an individual. Circulating *miRNA*s, may be used as novel diagnostic and prognostic tool, risk monitors and safety biomarkers.[18]

Our study has revealed the significance of alterations and challenges in peripheral blood *miRNA*9 as efficient biomarker for diabetes and other diseases.[19] Previous evidences have illustrated that *miRNA*s *miR*-7, *miR*-124a, *miR*-9, *miR*-96, *miR*-15a/b, *miR*-34a, *miR*-195, *miR*-376, *miR*-103, *miR*-107, and *miR*-146 regulate the protein cascades in insulin signaling pathways. Among these *miRNA*s, *miRNA*9 is a islet specific *miRNA* showing high expression levels during islets differentiation in pancreas.

Plaissance *et al* discovered exocytosis mechanism initiated by glucose which is reduced by overexpression of *miR*-9 in insulin secreting cells. It was also illustrated that *miR*-9 poses a negative control on insulin release by inhibiting the expression of the transcription factor Onecut-2 thereby increasing the level of Granuphilin/Slp4, a Rab GTPase effector associated with β-cell secretory granules [20]. Several studies have depicted the profound alterations of *miRNA*9 in blood glucose homeostatis and insulin secretion therefore this study focuses on the expression analysis of this particular *miRNA* through RTqPCR in peripheral blood of diseased and healthy subjects and grade this *miRNA* in profiling and monitoring disease progression. The level of *miRNA* 9 was found higher in diabetic patients in comparison to control healthy individuals. Kong *et al* also reported close association of *miRNA*9 with disease susceptibility and suggested it as a probable biomarker for prediabetes as well as diabetes [21]. The metabolic abnormalities such as impaired insulin secretion associated with diabetes may be carried out through years leading to the progression of disease. The correlation of disease progression and its pathogenicity with their important regulatory *miRNA* is strengthened through the current study. Several epidemiological parameters like age, dyslipidemia, obesity, hypertension, uncontrolled hyperglycemia contributed to the advancements of the ill effects of the disease [22,23].

The results imply a positive link between the susceptibility of an individual with the disease and the associated *miRNA*9. A positive correlation was found between elevated levels of *miRNA*9 and various parameters such as FG, hBa1, mean BP, age and BMI. However, a borderline significance and low diagnostic value of *miRNA*9

was evaluated through results and hence another *miRNA* could be used in combination, to improve the significant diagnostic ability. Despite these results, the fact that *miRNA*9 expression levels were higher in diabetes patients could not be surpassed and its link with the disease progression could not be foreseen.

5. Conclusion

This study demonstrated that *miR*-9 was found closely related with the disease and higher expression levels in diabetic patients as compared to healthy controls and was found progressively enriched in former group of individuals. It was also positively interrelated with glycemic status of the individuals revealing its association with the initiation of the disease. However, a borderline significance and low diagnostic value of *miRNA*9 was evaluated through results still the fact that *miRNA*9 expression levels were higher in diabetes patients could not be surpassed and its link with the disease progression could not be foreseen.

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