European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 7, Issue 06, 2020

Mirna 326 Derived Exosomes Is A Potential Modulator Of Adiponectin In Diabetes Mellitus

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Abstract : ***Adiponectin is a biomarker for diseases associated with obesity, such as cardiovascular disease and type II diabetes mellitus. We assessed a correlation between blood exosome miRNA 326 and adiponectin in diabetic patients. One hundred diabetic patients and fifty healthy subjects were enrolled in the study. They were divided into three groups: Group I, (n= 50) apparently healthy volunteers as control subjects. Group II, (n= 50) type I diabetes patients Group III, (n= 50) type II diabetes patients. miRNA was extracted from isolated exosomes peripheral blood samples of both the diseased and control groups. miRNA 326 gene expression was quantified by qRT-PCR. Adiponectin and insulin levels were assessed by ELISA. Finally, HOMA is calculated for all subjects. There was a significant increase miRNA 326 expression in diabetic patients of type I and II relative to normal control subjects, miRNA326 increased significantly in diabetic patients of type II compared to diabetic patients of type I. While a significant decrease adiponectin level in diabetic patients of type I and II relative to normal control subjects and a significant decrease adiponectin level in diabetic patients of type II relative to diabetic patients of type I. Exosome miR-326 is significantly highly expressed in diabetic patients with significantly decrease in adiponectin.***
Keywords: miR-326, Adiponectin and Exosomes.

INTRODUCTION

Diabetes Mellitus (DM) is an endocrinal disorder that can arise from an irregularity in insulin secretions and insulin acts or both. (1) Insulin absence or reduction together with insulin resistance can lead to persistent abnormally high blood sugar and glucose intolerance. (2) Exosomes are nano vesicles with a size between 30 and 150 nm. (3) In cell-to-cell contact, exosomes play an important role and are implicated in both normal physiological processes, including such immune responses, and disease development, like cardiovascular disease. (4) Many types of cells, which include B cells, dendritic cells, T cells, platelets and tumor cells, secrete exosomes. (5) Exosomes can transport their genetic content in the form of mRNA, miRNA in addition to proteins. These varied exosome cargos offer novel possibilities for the discovery of biomarkers and the development of non-invasive tools of diagnosis. (6) Adipocytokines are cytokines that are released during chronic low-grade inflammation from the adipose tissue. (7) A group of immunoinflammatory cytokines which promote insulin-resistance are tumor necrosis factor-alpha (TNF-α), resistin and interleukin-6 (IL-6). In the existence of adipocyte hyperplasia and macrophage and lymphocyte penetration into adipose tissue, IL-6 and TNF-α are excessively secreted, which slows down the insulin signaling chain and influencing energy homeostasis and body mass, leading to the onset of DM attributed to insulin-resistance. (8) Adiponectin is known to be a regulatory adipokine with vascular protection-related antagonistic impacts, improved glucose absorption through better insulin sensitivity, reduced liver gluconeogenesis, and pro-inflammatory mediator (IL-6 and TNF-α) suppression. (9) miRNAs are an epigenetic gene regulator. miRNAs have been involved in regulation of multiple processes and in the regulation of insulin secretion and signaling. In addition to autoimmune diseases. (10) In the pathogenesis of type 2 diabetes, miRNA plays a significant role (T2D). Recent studies indicate that circulating miR-101; miR-375 and miR-802 levels have increased significantly in T2D patients versus control subjects, and could be new biomarkers for T2D patients. (11) miRNAs have been

suggested to have a biological function in the development of obesity. Obesity was closely related to diabetes, and a great deal of evidence revealed that in contrast to the PBMCs of non-obese people, peripheral blood mononuclear cells (PBMCs) of obese people substantially secreted more IL-6 and TNF-α. Importantly, in PBMCs of obese subjects, miR-21 expression was down-regulated and reduced expression seemed to be correlated with elevated cytokine levels in obese individuals, contributing to type II diabetes (T2D) incidence.[12]

MATERIALS AND METHODS

Study subjects

The study was conducted on 150 subjects matched sex and age; the patients were enrolled from the internal medicine department, Faculty of Medicine, Cairo University. They were divided into 3 groups: Group I, (n= 50) apparently healthy volunteers as control subjects, Group II, (n= 50) type I diabetes patients and Group III, (n= 50) Type II diabetes patients.

Ethical declaration

According to the 1964 Helsinki declaration, all procedures in the research including human subjects were conducted in compliance with ethical criteria. Written voluntary informed consent was obtained from each patient before entering the research. Laboratory investigations were conducted in Biochemistry & Molecular Biology Unit, Faculty of Medicine, Cairo University.

Peripheral blood exosomes isolation:

Exosomes have been isolated using an ultracentrifugation protocol from 5 ml of peripheral blood. For 15 minutes of anticoagulated peripheral blood tests, plasma specimens have been isolated by 1,200 x g centrifugation. In order to pellet larger cell debris and eliminate residual platelets, plasma specimens have been exposed to second centrifugation for 15 min at 1,500 x g. At 14,000 x g, 4 °C, the supernatant plasma specimen has been centrifuged for 35 min. The pellet has been suspended in 1,000 μL PBS, transferred to a tube of 1.5 mL and centrifuged at 100,000 x g, 4 C for 2 hours. For subsequent miRNA isolation, Supernatant has been aspirated and resuspended in 400 μL Trizol reagent exosome pellets.

Exosomal miRNA-326 expression:

All plasma exosomes samples were subjected to miRNA extraction using the Zymoresearch Quick-gRNA™ MiniPrep kit, Catalog No. D3024. With the TaqMan micro-RNA kit (Catalogue no. 4427975, ID 000397, Applied Biosystems), miRNAs have been converted into cDNA as directed by the manufacturer. The RT reaction was followed by a real-time PCR with TaqMan assay performed on an Applied Biosystems (Step One System. SDS software v2.1 and RQ Manager 1.2). To acquire the value of the comparative threshold cycle (CT) from the plot of amplification. For normalization control, RNU6B has been used as an endogenous reference control gene. With any real-time RT-PCR assay to remove any contamination, negative controls have been used. We amplified the cDNA by the real-time PCR in the presence of miRNA326 forward primer (5′AACTCAAGGTTCTTCCAGTCACG-3′), reverse primer (5′-CCTCTGGGCCCTTCCTCCAG-3′), (gene bank accession number:NR_031132.1) and RNU6B forward primer (5′-CGCGTTCGGTTTCCCCAGA-3′), reverse primer (5′-TGTCACAGACCCTGAGAAAT-3′), (gene bank accession number: NM_021177.5) was used as a house keeping gene.

Estimation of Adiponectin by ELISA Technique:

Adiponectin has been evaluated by RayBio Human Adiponectin ELISA Kit Catalog: ELH-Adiponectin using a commercially available enzyme linked immunosorbent assay (ELISA) kit.

Estimation of Insulin by ELISA Technique:

Insulin has been evaluated by a commercially available enzyme linked immunosorbent assay (ELISA) kit provided by The DEMEDITEC DE2935 ELISA Diagnostics GmbH 24145 Kiel (Germany) for the quantitative in vitro diagnostic measurement of insulin in serum.

HOMA IR calculation: HOMA-IR= fasting blood insulin (microU/L) x fasting blood glucose (nmol/L)/22.5 IR% = (20 X FBI) / (FBG-3.5).

Statistical analysis

The data was encoded and entered using the SPSS version 22 statistical package. Data is statistically defined as average, standard deviation for quantitative variables and frequency for categorical variables, Variance analysis (ANOVA) and multiple post-hoc test comparisons were done to compare quantitative variables between the studied groups and chi X^2 for comparing categorical variables. A probability value (P value) below 0.05 has been deemed to be statistically significant.

Results

The incidence of type I diabetes is significantly more common in male patients when compared to the normal control and type II diabetic patients, while it is more common in females among type II diabetic patients. The age of type I diabetic patients showed statistically significant decrease compared to the normal control subjects. Type II diabetic patients showed statistically significant increase in age relative to the normal control subjects and type I diabetic patients (p value< 0.001) significant increase in BMI in type II diabetic patients relative to normal control people and type I diabetic patients ($p < 0.001$), while no significant difference in BMI among type I diabetic patients and the control subjects ($p = 0.06$). table1.

Table (1): Demographic and biochemical laboratory data among the groups studied

Variable	**Control Group (I) n=50**	Type I diabetes **Group (II) n=50**	Type II diabetes **Group (III) n=50**	**P1 value***	**P2 value#**
Sex **Females: n (%)** **Males: n (%)**	 27 (54%) 23 (46%)	 12 (24%) 38 (26%)*	 34 (68%)*# 16(32%)	 *< 0.001*	 *< 0.001*
Age (years)	38±10	20±5*	49±11*#	*< 0.001*	*< 0.001*
BMI	23.9±3.1	21.3±2.2	37.5±4.4*#	*< 0.001*	*< 0.001*

The data was presented as Mean ± SD, p value <0.05 was significant
(*)Indicates significant variations versus control subjects
(#)Indicates significant variations versus type I diabetes patient

As regard the glycemic parameters, there was significant increase in FBS diabetic patients of type I and type II relative normal control subjects ($p <0.001$). Significant decrease in FBS in diabetic patients of type II relative to diabetic patients of type I ($p < 0.001$) (figure 1a). Significant increase in HbA1C in type I diabetic patients compared to normal control subjects ($p < 0.001$) and significant decrease in HbA1C in diabetic patients of type II relative to diabetic patients of type I ($p < 0.001$) (figure 1b). Significant decrease in fasting insulin level in type I diabetic patients relative to normal control subjects ($p < 0.001$),

while there is significant increase in fasting insulin level in diabetic patients of type II relative to normal control subjects and diabetic patients of type I ($p < 0.001$) (figure 1c). Significant increase in HOMA level in diabetic patients of type II relative to normal control subjects and diabetic patients of type I ($p < 0.001$), but no significant difference in HOMA level among type I diabetic patients and normal control subjects ($p = 0.64$) (figure 1d).

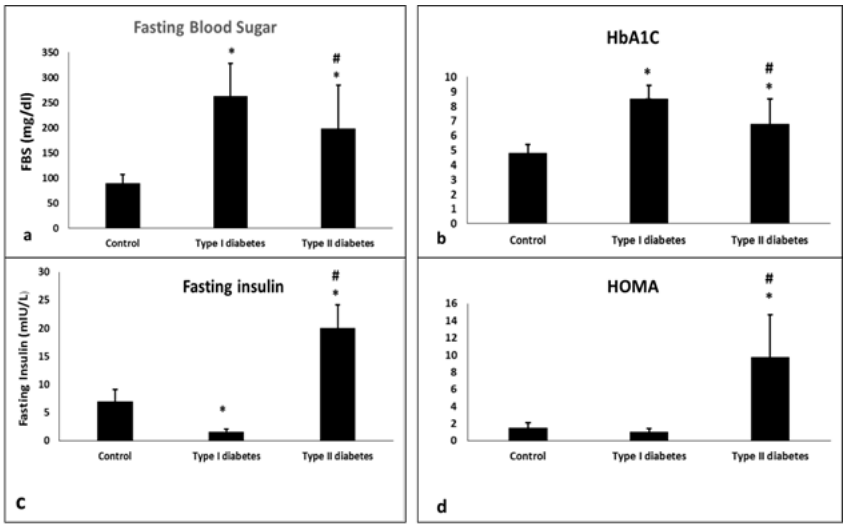

Figure 1: a-Fasting blood glucose level, b- HbA1c level, c- Fasting insulin level and d- calculated HOMA among all the studied groups

The data was presented as Mean ± SD, p value <0.05 was significant
(*)Indicates significant variations versus control subjects
(#)Indicates significant variations versus type I diabetes patient

miRNA 326 is significantly highly expressed in diabetic patients

Among diabetic patients, significant increase in miRNA 326 expression in Diabetic patients of type I and type II relative to normal control subjects ($p < 0.001$) Significant increase in miRNA326 in diabetic patients of type II relative to diabetic patients of type I ($p = 0.04$) (figure 2a).

Adiponectin is significantly decreased in diabetic patients

The adiponectin level in diabetic patients of type I and type II have decreased significantly relative to normal control subjects ($p<0.001$) and significant decrease in adiponectin level in patients with type II diabetes relative to diabetic patients with type I ($p <0.001$) (figure 2b).

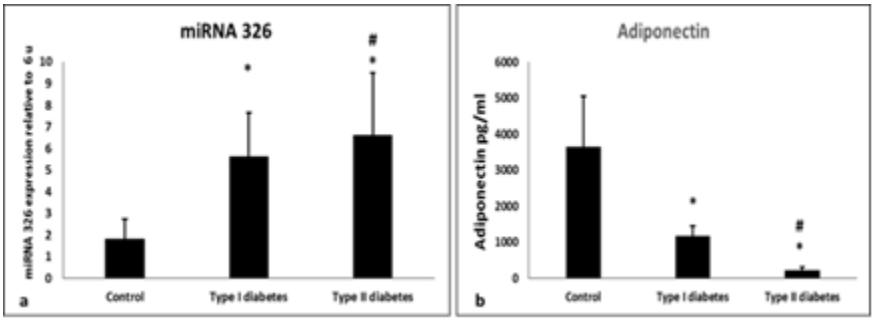

Figure 2: a-Relative quantitation of miRNA326 gene expression among all the studied groups and b-Adiponectin level among all the studied groups

The data was presented as Mean ± SD, p value <0.05 was significant
(*)Indicates significant variations versus control subjects
(#)Indicates significant variations versus type I diabetes patient

Correlation between miRNA 326, Adiponectin and glycemic state in diabetic patients.

In type I diabetic patients, the miRNA 326 is significantly positively correlated with FBS, HbA1c (p value <0.001) and inversely correlated with each of fasting insulin, adiponectin, BMI and HOMA (p value <0.001).

Adiponectin is significantly inversely correlated with FBS, HbA1c (p value <0.001) and significantly positively correlated with fasting insulin, BMI and HOMA (table 2).

Table (2): Correlation analysis between miRNA326, adiponectin the different studied parameters in type I diabetes patient

		FBS	HbA1C	BMI	Fasting insulin	HOMA	miRNA326	Adiponectin
miRNA326	r	.778**	.796**	-.364**	-.682**	-.364**	1	-.608**
	P value	.000	.000	.000	.000	.000		.000
Adiponectin	r	-.669**	-.696**	.411**	.618**	.289**	-.608**	1
	P value	.000	.000	.000	.000	.004	.000	

The data was presented as Mean ± SD, p value <0.05 was significant
(*)Indicates significant variations versus control subjects
(#)Indicates significant variations versus type I diabetes patient

In type II diabetic patients, the miRNA 326 is significantly positively correlated with FBS, HbA1c, BMI, fasting insulin and HOMA (p value <0.001) and inversely correlated with adiponectin (p value <0.001). While, adiponectin is significantly inversely correlated with FBS, HbA1c and BMI, fasting insulin and HOMA (p value <0.001) (table 3).

Table (3): Correlation analysis among miRNA326, adiponectin and the different studied parameters in type II diabetes patient

		FBS	HbA1C	miRNA326	Adiponectin	BMI	Fasting insulin	HOMA
miRNA326	r	.644**	.603**	1	-.679**	.678**	.618**	.656**
	P value	.000	.000		.000	.000	.000	.000
Adiponectin	r	-.570**	-.511**	-.679**	1	-.738**	-.787**	-.662**
	P value	.000	.000	.000		.000	.000	.000

The data was presented as Mean ± SD, p value <0.05 was significant
(*)Indicates significant variations versus control subjects
(#)Indicates significant variations versus type I diabetes patient

Discussion

The risk of insulin resistance and type 2 diabetes mellitus was significantly raised by the wide spread of overweight and obesity worldwide. It has been reported that obesity alters microRNA (miRNA) expression in addition to being mediators in the pathogenesis of several diseases. Several pathways are regulated by miRNAs, which include insulin signaling, immune-mediated inflammation, adipogenesis, adipokine expression, lipid metabolism, and regulation of food intake. MiRNA-based therapeutics are therefore a novel treatment modality.[13]

In various physiological processes, which include cell proliferation, apoptosis, differentiation and metabolism, miRNAs have a regulatory role.[14] miRNA dysregulation was involved with numerous disorders, including cancer and diabetes.[15]

In our study there was significant increase in miRNA 326 expression in type I and type II diabetics relative to control subjects and there was a substantial increase in miRNA326 in type II diabetics compared to type I. We found that in type I diabetics there was significant positive correlation between miRNA 326 expression and FBS , additionally between miRNA 326 and HBA1c, while there was inverse correlation between miRNA 326 expression and fasting insulin . As regard type II diabetics there was positive correlation between miRNA 326 expression and each of FBS, HbA1c and fasting insulin.

Our results was in agreement with some studies that showed correlations between miRNA 326 and T1D and other autoimmune diseases, in contrast to controls, they strongly indicated the overexpression of miR-20a and miR-326 in the PBMCs of T1D patients and indicated that these two miRNAs could be used in the diagnosis and treatment of T1D as targeted therapies.[16] In T1D patients with ongoing islet autoimmunity, miR-326 levels were also upregulated relative to antibody negative patients measured in peripheral blood lymphocytes from type 1 diabetic patients.[17] Higher levels of miR-326 therefore tend to be associated with islet autoimmunity and may be a biomarker of autoimmunity for type 1 diabetes.[18] Interestingly, some reports showed that the miRNA serum profile is deregulated prior to the development of overt type 2 diabetes in the prediabetic state.[15] As regard type 2 diabetes, our results agreed with previous study which provided the first proof of miR-326 participation in type 2 diabetes, despite the reality that it plays a possible major role in the differentiation of adipocytes,[19] MiR-326 has so far been discovered to be up-regulated only in type 1 diabetes lymphocyte patients with positive auto-antibodies,[17] another research found that poorly controlled type 2 diabetes was correlated with a reduction in let-7a/let-7f and a rise in circulating miR-326 levels.[20] The most concentrated peptide hormone secreted by adipocytes is Adiponectin. It has been reported that adiponectin can be considered as a biomarker for obesity-related disorders such as metabolic syndrome, cardiovascular disease, and type 2 diabetes mellitus. [21] Our study shows significant decrease in adiponectin level in type I and type II diabetics relative to control subjects and significant decrease in adiponectin level in type II diabetics when compared to type I diabetics.

We found that there was inverse correlation between miRNA 326 expression and adiponectin in both types I and II diabetics. Also there was inverse correlation between adiponectin and HbA1c and FBS in type I and type II diabetics. In type I diabetes the correlation between adiponectin expression and fasting insulin was positive correlation while it was negative in type II diabetic patients.

These results agreed with Mendelian randomization study that demonstrated an inverse correlation between adiponectin and insulin-resistance.[22]

We agreed with previous study which not only found that the level of miR-326 in diabetics was increased, but also showed an inverse association among the circulating miR-326 and its expected target adiponectin, moreover, they also observed that gene expression of adiponectin receptors was lower in diabetic patients' PBMCs, another expected target of miR-326. Higher levels of circulating miR-326 may therefore be a new possible modulator of the adiponectin pathway in diabetic patients.[20] Concerning insulin resistance, several studies have confirmed the association between insulin resistance and type 2 diabetes. Evaluating the degree of insulin resistance may assist to decide the best way to prevent the development of diabetes; HOMA is a homeostatic model assessment based on algorithms using fasting glycaemia and fasting insulinaemia measurements to estimate beta cell function and insulin sensitivity.[23] Our study showed that sever insulin resistance was significantly occurring in type 2 diabetics. We showed significant increase in HOMA level in type II diabetics compared to control subjects and type I diabetics, while no substantial difference in HOMA level among type I diabetics and control subjects. The correlation between miRNA 326 expression and HOMA is significant correlation in type 2 DM, and also between HOMA and fasting insulin is positive correlation while significant negative correlation between HOMA with adiponectin. This was agreed with a previous study reported a substantial up-regulation of miR-326 and down-regulation of let-7a and let-7f in diabetic patients together with inverse negative correlation between circulating miR-326 and adiponectin.[20] In conclusion, severe insulin resistance is most significantly occurring in type II diabetic patients which might be related to deregulated miRNA 326 and adiponectin, as there was significant increase in miRNA 326 expression in diabetic patients of type I and type II relative to normal control subjects and in diabetic patients of type II compared to type I diabetic patients. Furthermore, adiponectin is significantly decreased in diabetic patients of type I and type II relative to normal control subjects and in diabetic patients of type II when compared to type I diabetic patients. miRNA 326 and adiponectin were significantly correlated with the glycemic state of diabetic patients.

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Acknowledgement:
There are no previous presentations of the information reported in the article.

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Competing of interest

The authors declare that they have no competing interests.