Assessment of miRNA-21 as an early detector of Diabetic Cardiomyopathy Compared to Echo Strain Study

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Abstract

Diabetic cardiomyopathy (DbCM) is one of the serious complication of diabetes mellitus, with a silent development and it is often underdiagnosed, due to few diagnostic possibilities in its early stages. The present study aims to evaluate role of miRNAs compared to GLS for early detection of subclinical DbCM. This case control study involved 75 DM2 patients from Merjan Medical City in addition to age and sex-matched 25 apparently healthy subjects. The echocardiographic assessment for GLS was done for control group (H) and normotensive diabetic patients who were divided into (CD) group without cardiomyopathy, and (M) group with cardiomyopathy diagnosed by negative and positive strain study, respectively. Group (F) include Hypertensive and/or ischemic diabetic patients with HF assessed by conventional Echocardiography as having EF%<40%. Blood sampling was done for all subjects; miRNA extraction and analysis done according to the manufacturer recommendations. This study showed that there is significant difference in GLS value between M on one side vs. both of CD and H gps (p=0.000) with non-significant difference in GLS between H and CD gps. (p=0.345). There was non-significant difference in miR-21 Ct value between H and CD gps with significant difference between H gp. on one side vs. M and F in other side (p=0.000 and 0.000) respectively. Present study concluded that miRNA could be regarded as a novel biomarker and plays a valuable role, alongside GLS echocardiographic assessment, for early detection of subclinical diabetic cardiomyopathy.

Key words: diabetic cardiomyopathy, strain echo, miRNA, GLS

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Introduction

Diabetic cardiomyopathy is defined by the existence of abnormal myocardial structure and performance in the absence of other cardiac risk factors, such as coronary artery disease, hypertension, and significant valvular disease, in individuals with diabetes mellitus. It was first described in 1972 (1) in postmortem pathological findings from 4 diabetic patients who manifested heart failure symptoms without evidence of coronary artery or valve disease and further confirmed in a 1974 Framingham Heart Study that demonstrated a higher incidence of heart failure in diabetic women (5-fold) and men (2.4-fold) after adjustment for other risk factors, such as age, coronary heart disease, and hypertension (1). In 2013, the American College of Cardiology Foundation, the American Heart Association, (2) and the European Society of Cardiology in collaboration with the European Association for the Study of Diabetes (3) defined diabetic cardiomyopathy as a clinical condition of ventricular dysfunction that occurs in the absence of coronary atherosclerosis and hypertension in patients with diabetes mellitus. In its early stages, diabetic cardiomyopathy includes a hidden subclinical period characterized by structural and functional abnormalities, including left ventricular (LV) hypertrophy, fibrosis, and cell signaling abnormalities. These pathophysiological changes of cardiac fibrosis and stiffness and associated subclinical diastolic dysfunction often evolve to heart failure with normal ejection fraction and eventual systolic dysfunction accompanied by heart failure with reduced ejection fraction (4).

Epidemiology of Diabetes Mellitus–Related Heart Failure
Clinical trials show the prevalence of heart failure in diabetic patients to range from 19% to 26% (5). The Framingham Heart Study found that the incidence of heart failure was increased in both male and female diabetic patients when compared with age-matched individuals, and this association was independent of obesity, hypertension, dyslipidemia, and coronary heart disease (1). One study found that the incidence of heart failure was higher in diabetic (39%) compared with nondiabetic (23%) patients, with a relative risk of 1.3 for developing heart failure after 43 months of observation (6).

**Pathophysiological mechanisms of diabetic cardiomyopathy**

Hyperglycemia, insulin resistance, and hyperinsulinemia induce cardiac insulin resistance and metabolic disorders that increase mitochondria dysfunction, oxidative stress, advanced glycation end products (AGEs), impairment of mitochondria Ca\(^{2+}\) handling, inflammation, activation of renin–angiotensin–aldosterone system (RAAS), autonomic neuropathy, endoplasmic reticulum stress, cardiomyocyte death, as well as microvascular dysfunction. These pathophysiological abnormalities promote cardiac stiffness, hypertrophy, and fibrosis, resulting in cardiac diastolic dysfunction, systolic dysfunction, and heart failure (7).

**Evolution of Diabetic Cardiomyopathy to Clinical Heart Failure**

Diabetic cardiomyopathy is usually asymptomatic in the early stages of its evolution (8). One of the earliest manifestations is LV hypertrophy and decreased LV compliance characterized by impaired early diastolic filling, increased atrial filling, and prolonged isovolumetric relaxation (8). LV dilation and symptomatic heart failure occur after the development of systolic dysfunction (8). Indeed, the recent data support the notion that diastolic dysfunction, as observed by cine magnetic resonance imaging in rodents, is associated with impaired cardiac insulin metabolic signaling (9). Cardiomyocyte stiffness and hypertrophy, as well as myocardial fibrosis, all contribute to this cardiac abnormality. The Cardiovascular Health Study found that, in a cohort of 5201 men and women, the ventricular septal and left posterior myocardial wall thicknesses were greater in diabetic patients than in nondiabetic individuals and that this was associated with compromised systolic or diastolic function (10).

Underlying pathological factors include hyperglycemia, systemic and cardiac insulin resistance, increased free fatty acid (FFA) levels, systemic and tissue inflammation, oxidative stress, and activation of the renin–angiotensin–aldosterone system (RAAS) and the sympathetic nervous system (SNS)(11). Reduced calcium (Ca\(^{2+}\)) pump activity-induced inefficient sequestration of sarcoplasmic reticulum Ca\(^{2+}\) is regarded as an important contributor to the development of the cardiac diastolic dysfunction (12).

The second stage of diabetic cardiomyopathy is characterized by LV hypertrophy, cardiac remodeling, advancing cardiac diastolic dysfunction, and the consequent emergence of clinical indications of heart failure with normal ejection fraction (11). With progression of diabetic cardiomyopathy, diastolic dysfunction and reduced cardiac compliance may coexist with systolic dysfunction leading to reduced ejection fraction, prolonged pre-ejection performance, an enlarged LV chamber, shortened ejection period, and the latter by an increased resistance to filling with increased filling pressures (11).

Thus, cardiac dysfunction in diabetic hearts progresses from subclinical cardiac abnormalities, such as LV fibrosis, to diastolic dysfunction and eventually systolic dysfunction accompanied by reduced ejection fraction. Several noninvasive techniques, including echocardiography, computed tomography, and cinematic magnetic resonance imaging, have been applied to detect changes of cardiac structure (ie, fibrosis) and function (11).

**Role of strain echo-study in detection of Diabetic Cardiomyopathy**

Strain is a unitless measurement of dimensional or deformational change. Imaging-based techniques have been derived and refined in order to quantify myocardial strain in clinical practice. The most widely used technique is speckle-tracking echocardiography (STE). Speckle tracking is an offline technique that is applied to previously acquire 2D images (3, 4 and 2 apical views). The use of frame rates of 40 to 80 frames/sec have been used in various applications involving normal heart rates (13).

**Role of miRNAs in Promotion of Diabetic Cardiomyopathy**
Diabetic cardiomyopathy is associated with increased expression of miRNAs—a group of short single-stranded noncoding RNA molecules with an average length of 22 nucleotides. Importantly, miRNAs control the expression of transcriptional and post-transcriptional target genes through binding to the 3′-untranslated region and regulate mitochondrial function, ROS production, Ca^{2+} handling, apoptosis, autophagy, and fibrosis, all of which are regarded as important mechanisms in diabetes mellitus–induced cardiac hypertrophy, remodeling, and fibrosis, as well as heart failure progression (14)(15). miR-15a, -21, -24, -29, -30d, -103, -126, -146a, -150, -191, -223, -320, -375, and -486 have all been reported to be increased in type 2 diabetic individuals (14)(15). In cardiac tissue of a type 1 diabetic rodent model, expression of miR-21, -24, -142-3p, -195, -199a-3p, -700, -705, -208, -221, and 499-3p was upregulated whereas expression of miR-1, -20a, -29a, -143, -220b, and -373 was downregulated (16), miR103, 107, -143, and -181 play a role in insulin sensitivity and systemic glucose metabolism (17)(18).

Increased expression of miR-454, 500,-142-3p/5p, and 1246 has been identified in cardiac diastolic dysfunction (19). Other miRNAs, such as miR-113a, -133a, -150 have been found to be involved in the regulation of cardiomyocyte hypertrophy and interstitial fibrosis (20).

Recent scientific evidence for the potential use of circulating miRNAs as biomarkers for detection of diabetic cardiomyopathy highlights emerging methods for the diagnosis and prevention of diabetes mellitus and cardiomyopathy. The American Diabetes Association recommends screening patients with type 2 diabetes mellitus for the prevention of diabetic cardiomyopathy, but the results of the recently published Detection of Ischemia in Asymptomatic Diabetics trial indicate that screening of asymptomatic patients with nuclear imaging does not improve cardiac event rates (21). Unfortunately, screening approaches including B-type natriuretic peptide, exercise stress testing, and echocardiographic assessment do not seem to be sufficiently sensitive to identify subclinical dysfunction in diabetic patients (22). Therefore, further studies are vital to the understanding of the precise mechanisms involved in the initiation and progression of diabetic cardiomyopathy and to the development of novel strategies to reduce the risk of heart failure in diabetic patients.

Detection of myocardial dysfunction recommend this new non-invasive diagnostic method for routine clinical use. In this study, the global LV myocardial functions was evaluated by Strain echocardiography in asymptomatic patients with type 2 DM as a method of early detection of any subclinical myocardial dysfunction as a gold standard; then we tried to investigate whether miR-21 Ct value detected by Real time PCR can be a new screening marker and therapeutic target for this disease.

Materials and Methods:

Subjects: Between 1st May 2018 to 1st April 2019; 100 subjects were recruited to this case control study, 75 DM2 patients from Merjan Medical City in addition to age and sex-matched 25 apparently healthy subjects. The overall mean age of normotensive DM2 patients and control were (52.76±6.3) and (50.88±8.1) years old, respectively.

The echo study done by ( VIVID 9 GE, with a 3.5 MHz transducer) in Echo unit of Merjan Medical City. The diabetic patients divided in to three groups; as in Table 1

<table>
<thead>
<tr>
<th>Table (1): characteristics of subjects’ study groups</th>
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<tbody>
<tr>
<td>H group</td>
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<tr>
<td>CD group</td>
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<tr>
<td>M group</td>
</tr>
<tr>
<td>F group</td>
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</tbody>
</table>
The biochemical part of this study was performed in the laboratories of Biochemistry Department in faculty of Medicine /University of Babylon.

**Exclusion criteria**

**Group CD and M exclusion criteria include:** Patients having any systolic dysfunction observed through echocardiography, evidence of prior or current myocardial infarction, known valvular or congenital heart disease or any resting electrocardiography abnormalities, CAD excluded by –ve TMT study (stress electrocardiogram)(23) in spite of the limited accuracy. The study does not depend on more sensitive tool like CT angiography depending on WHO criteria for CAD screening from the work of Wilson and Jugner in addition to the application of WHO criteria for a good screening test (23). Hypertensive diabetic patients, significant comorbidities, including malignancy, renal failure, hepatic, or thyroid disease, Age< 40 and >65 years, Patients coming from outside the governorate, Pregnant women. Incapacity to provide vocal consent, Prior inclusion in the present study, Anemic patients (Hb<9g/dl for male and Hb<8g/dl for female), Drug and alcohol abusers and obstructive lung disease patients.

**Group (F)** patients were chosen to be diabetic patients with hypertension and/or IHD, but without renal failure, malignant or thyroid disease, admitted to the CCU as cardiomyopathic patients with EF<40% (The American guidelines defined that HFpEF is EF ≥50%, whereas EF between 41 and 49% was considered as Bborderline^ HF and finally EF ≤40% defined HFrEF ) (24)

**Group (H) Control group:** Twenty five (25) subjects were recruited from colleagues, medical staff or their relatives. The subjects age ranged between (40-65yr) include 15 male (60%) and 10 female (40%). This group was matched in age and sex to patients group.

Each person who contributed to the control group underwent full history and physical examination including: age, gender, TMT to exclude cardiac ischemia, conventional and strain echo study to exclude any systolic dysfunction observed through echocardiography, evidence of prior or current myocardial infarction, known valvular or congenital heart disease. Exclusion criteria for the patients group were applied on control group.

**Clinical evaluation:** All the patients were subjected to full history taking including age, gender, diabetes duration, drugs for glycemic control, and to clinical examination including, body mass index, heart rate and blood pressure, see table 2.

**Materials**

**Table (2): miRNA-21 detection kits**

<table>
<thead>
<tr>
<th>No</th>
<th>Item</th>
<th>Cat no. and SKU-Pack Size</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RNAzol® RT</td>
<td>R4533-200ML</td>
<td>Sigma/USA</td>
</tr>
<tr>
<td>2</td>
<td>MystiCq® microRNA cDNA Synthesis Mix</td>
<td>MIRRT-100RXN</td>
<td>Sigma/USA</td>
</tr>
<tr>
<td>3</td>
<td>MystiCq® microRNA cDNA Synthesis Mix</td>
<td>MIRRT-25RXN</td>
<td>Sigma/USA</td>
</tr>
<tr>
<td>4</td>
<td>MystiCq® microRNA® SYBR® Green qPCRReadyMix</td>
<td>MIRRM00-500RXN</td>
<td>Sigma/USA</td>
</tr>
<tr>
<td>5</td>
<td>MystiCq® microRNA® SYBR® Green qPCRReadyMix</td>
<td>MIRRM00-100RXN</td>
<td>Sigma/USA</td>
</tr>
<tr>
<td>6</td>
<td>MystiCq® Universal PCR Primer</td>
<td>MIRUP-500RXN</td>
<td>Sigma/USA</td>
</tr>
<tr>
<td>9</td>
<td>MystiCq® microRNA qPCR Assay Primer hsa-miR-21-5p</td>
<td>MIRAP00047-250RXN</td>
<td>Sigma/USA</td>
</tr>
</tbody>
</table>
Methods

**Echocardiography:** Echocardiographic evaluation: conventional Echocardiography was performed to all patients and control group the recordings were obtained in the left lateral decubitus position using (VIVID 9 GE, with a 3.5 MHz transducer) machine and from standard apical and parasternal views the following parameters were measured and averaged from three cardiac cycles, the LV end-diastolic dimensions (LVEDD), LV end systolic dimensions (LVESD), and EF calculated from the M-mode echocardiography. And pulsed-wave Doppler of mitral inflow velocities were obtained to measure diastolic early filling velocity (E) wave and late diastolic velocity (A) wave, E/A ratio and E wave deceleration time (DT). And Pulsed wave TDI was obtained after placement of the sample volume at the level of the septal and lateral mitral annuli. From these recordings, myocardial systolic (s’), early diastolic (e’), and E/ e’ ratio were measured and averaged (13).

Assessment of global longitudinal strain by STE is a semiautomatic method, which requires manual definition of the myocardium. Furthermore, the sampling region of interest was adjusted to ensure that most of the wall thickness is incorporated in the analysis, while avoiding the pericardium. When automated tracking does not fit with the visual impression of wall motion, regions of interest was adjusted manually until optimal tracking is achieved. For the left ventricle, because end-systole can be defined by aortic valve closure as seen in the apical long-axis view, this view was analyzed first (25).

Strain echocardiography: Speckle tracking strain mode was selected on the echo-machine during apical 4, 2 and 3-chamber imaging and three consecutive cycles were recorded at a frame rate of 60 to 80 frame/sec. The LV is divided into 6 walls (inferoseptum, lateral, anterior, inferior, posterior and anteroseptal walls) every wall is divided into basal, mid and apical segments except the anteroseptum and posterior wall divided into basal and mid segments only, the global PSLS value for each participant were calculated as the average of values of the 16 segments (26).

**Blood sample collection:** Five ml of blood were obtained from each subject by vein puncture in sitting or lying position, the blood pushed slowly into disposable plane tubes. Blood in them was allowed to clot at room temperature for 30 minutes and then centrifuged at 1000 ×g for approximately 15 minutes then the supernatant were obtained and stored in crayon tubes at -170°C in liquid nitrogen until analysis.

**miRNA extraction and analysis:** done according to the manufacturer recommendations.

**Statistical analysis:** The collected data were tabulated and analyzed by using the Statistical Package for Social Sciences (SPSS) for Windows version 20th version. Data were expressed as (mean ± SD). Independent sample t-test was used to compare means between two groups. ANOVA test was used to compare means of more than 2 groups. P values less than (0.05) were considered significant.

Results and Discussion

**Difference of patients and control in GLS value**

Echocardiographic strain imaging is a promising tool for the evaluation of myocardial function and in this study, global LV myocardial function was evaluated by strain echocardiography in asymptomatic patients with type 2DM as a method of early detection of any subclinical myocardial dysfunction and it was found a subclinical global LV systolic dysfunction in diabetic patients (27).

By regarding cut-off value of GLS = -18% according to Islam E. Shehata et al.,(28) who reported that a GLS cutoff of > −18.1% was able to accurately “predict subclinical LV systolic dysfunction”. There is significant difference in GLS value between M gp on one side and both of CD and H gps. in the other side (p=0.000) which indicate the ability of strain study to discriminate subclinical cardiomyopathy in spite of normal EF% detected in conventional echo study as presented in table (3) and (4). This finding agree with Murtaza et al.,(29) who presented that subclinical LV longitudinal dysfunction is preferentially and frequently observed in asymptomatic DM patients with normal EF on speckle echocardiography. Speckle derived global longitudinal strain measurements can increase the detection of subclinical DbCM.
The non-significant difference (matching) in GLS between H gp. and CD gp. (p=0.345) presented in table (4) indicate that CD group has no cardiomyopathic changes in spite of the matching with M gp. in age, diabetic period and HbA1c%. This result has agreement with Ramiro Flores-Ramírez et al., (30) who postulated that there was no difference in GLS between diabetics with pEF compared to controls (-19.4 ± 3.2 vs -20 ± 2.6; p = .70). Abnormal global longitudinal strain (GLS) is a sensitive marker of systolic dysfunction, identifying abnormalities of longitudinal deformation when the EF is still preserved.

Table (3): GLS mean values in (M,CD,H) groups

<table>
<thead>
<tr>
<th>GLS</th>
<th>Groups</th>
<th>Mean GLS</th>
<th>Std. Deviation</th>
<th>95% Confidence Interval for Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>±</td>
<td>Lower Bound</td>
<td>Upper Bound</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>-14.9</td>
<td>1.2</td>
<td>14.4337</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>-20.7</td>
<td>1.6</td>
<td>20.0769</td>
</tr>
<tr>
<td></td>
<td>CD</td>
<td>-20.3</td>
<td>1.7</td>
<td>19.6144</td>
</tr>
</tbody>
</table>

Table (4) ANOVA comparison of GLS mean values in (M,CD,H) groups

<table>
<thead>
<tr>
<th>Multiple comparison</th>
<th>Dependent variable GLS</th>
<th>Mean Difference</th>
<th>p-value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>M</td>
<td>H</td>
<td>-5.8</td>
<td>.000</td>
<td>-6.7284</td>
</tr>
<tr>
<td>CD</td>
<td></td>
<td>-5.3</td>
<td>.000</td>
<td>-6.3084</td>
</tr>
<tr>
<td>H</td>
<td>CD</td>
<td>0.42</td>
<td>.354</td>
<td>-.4780</td>
</tr>
</tbody>
</table>

* The mean difference is significant at the 0.05 level

The difference between patients’ groups and control in Ct value of miRNA-21

The non-significant difference in miR-21 Ct value between H and CD gps. (p=0.793) as presented in Table (5) indicate that these groups are identical in miR-21 copy no. which give an indication that there isn't downregulation or upregulation in this marker between diabetics having normal cardiac function and control; meaning it is not affected by DM2 as entity. In spite of the controversy among studies whether miR-21 is protective or deleterious when it is upregulated; it is found in this study that its downregulation as seen in groups M and F is associated with symptomatic and subclinical cardiomyopathy agreed with Beibei Dai et al., who reported that miR-21 protected against the H2O2-induced damage in cardiac myocytes via programmed cell death protein 4 (PDCD4) and activation protein-1 (AP-1) pathway (31). While Thomas et al. showed that miR-21 promoted cardiac fibroblast survival, which led to fibrosis, hypertrophy and cardiac dysfunction (32). So it was supposed that the same miRNA from different cell sources may exert various effects on the same disease (33).

Table (5): mean of Ct miR-21 values in all groups

<table>
<thead>
<tr>
<th>descriptive</th>
<th>Ct miR-21</th>
</tr>
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<tbody>
<tr>
<td>Groups</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>16.9</td>
</tr>
<tr>
<td>F</td>
<td>26.7</td>
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<tr>
<td>H</td>
<td>17.20</td>
</tr>
<tr>
<td>M</td>
<td>23.5</td>
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</tbody>
</table>

Table (6): ANOVA comparison of mean Ct miR-21 values in all groups
In addition, there is a significant difference in miR-21 Ct value between H gp. on one side with M and F on the other side (p=0.000 and 0.000) respectively, as indicated in Table 5. This indicates the relationship of miR-21 serum level with symptomatic and subclinical cardiomyopathy. The higher the Ct value in F gp. and the lower the Ct value in CD gp. are indicative of the downregulation of miR-21 in cardiomyopathy, which improves its positive role in protection against cardiomyopathy as presented by (Li, H. et al) who revealed a positive function of miR-21 in mitochondrial translation, which is sufficient to reduce blood pressure and alleviate cardiac hypertrophy in spontaneous hypertension rat (34). The significant difference between M and CD gps. in miR-21 Ct value (p=0.000) indicates its worthiness in detection of diabetic cardiomyopathy even in its subclinical state as cleared by Dai et al., who provide a sum of data suggesting that miR-21 might protect against diastolic dysfunction by inhibiting cardiac hypertrophy via decreasing ROS level and increasing eNOS induced-NO release in db/db mice (31). Also, the previous study provides data that indicate cardiac specific overexpression of miR-21 protected against diastolic dysfunction, which was the key early phase sign of diabetic cardiomyopathy in db/db mice, independent of systemic metabolic improvements, such as blood glucose and lipid levels (31).

The significant difference between M and F gps. (p=0.05) as indicated that the pathological background of the two cardiomyopathic states is different as this miRNA is involved in the metabolic changes associated with the disease. This finding agrees with the data suggested by (Dai B. et al) who recognized that miR-21 did not participate in diastolic dysfunction via regulating fibrosis, apoptosis or lipid accumulation in db/db mice (31). It is an interesting issue that miR-21 plays different roles in different cell types.

**Conclusion**

miR-21 can be used as a valuable non-invasive screening marker concomitantly with GLS value to detect subclinical diabetic cardiomyopathy in asymptomatic DM2 patients.

**Acknowledgement**

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