

Evaluating the Sensitivity of (miR-378) as a Circulatory Screening Biomarker for Diabetic Cardiomyopathy: Comparative Study

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Abstract

Background: Diabetic cardiomyopathy is one of the serious complication of diabetes mellitus, with a silent development and it is often underestimated, Currently available diagnostic techniques are limited in their ability to identify patients who present with uncommon symptoms or silent myocardopathy in its early stages.

Aim: To evaluate role of miRNA-378 compared to GLS as a sensitive biomarker for early detection of subclinical diabetic cardiomyopathy.

Method: 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 and these patients were divided into (CD) group without cardiomyopathy, and (M)group with cardiomyopathy diagnosed by negative GLS and positive GLS, respectively. Group (F) include Hypertensive and/or ischemic diabetic patients with overt heart failure . conventional Echocardiography was performed to all patients and control groups, all of them were of normal EF, except group F who have EF less than 40%. Bloodsampling was done for all and miRNA extraction and analysis done according to the manufacturer recommendations.

Results: There is significant elevation in miR-378 Ct value between H in one side & other three diabetic gps. (CD, M, F) as presented in table (3-17) ($p < 0.05$). There is significant elevation in miR-378 Ct value in M than CD groups. There is significant elevation in Ct value in F than M gp. ($p=0.000$). In the ROC curve (AUC) was 0.965. The sensitivity and specificity were 0.978 and 0.646, respectively according to the chosen cut-off value.

Conclusion: This study showed that miRNA-378 could be regarded as a novel sensitive biomarker which could play a significant role for early detection of subclinical diabetic cardiomyopathy.

Keywords: Diabetic cardiomyopathy, screening, GLS, sensitivity, miRNA-378.

Introduction

Diabetic cardiomyopathy was first described by Rubler in 1972^[1,2]. The term describes several mechanisms involved in the pathogenesis of this entity

including changes in myocardial structure and metabolism that are not directly attributed to other co-morbidities such as coronary artery disease (CAD) or hypertension. Left ventricular hypertrophy, myocardial lipotoxicity, increased oxidative stress, cell death from apoptosis, impaired contractile reserve, altered substrate utilization as free fatty acids (FFA), mitochondrial dysfunction and fibrosis are among the mechanisms involved with these changes in structure and metabolism contributing to the progression of heart failure^[2]. In particular,

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fibrosis is one of the abnormalities that can be evaluated with the newer imaging techniques. Cardiac magnetic resonance (CMR) is the gold standard in the assessment of myocardial fibrosis given that T1 sequences separate normal from fibrotic tissue^[3]. Nonetheless, CMR is not widely available, and it is time and cost consuming with longer and exhausting protocols. Speckle tracking echocardiography has been validated against magnetic resonance imaging ^[4,5]. Global longitudinal strain (GLS) is the simplest deformation parameter specified by Speckle tracking echocardiography and probably the closest to routine clinical application ^[6]. Quantitative assessment of myocardial function is now possible with this technique ^[7]. Routine use of Speckle tracking echocardiography in daily clinical practice is not cost-effective in this scenario and currently impractical for large-scale population screening. Therefore, assessment of individual risk factors becomes very important in clinical practice especially in primary care settings. More than half of patients with heart failure have preserved ejection fraction (HFpEF) ^[8]. Monitoring of the serum biomarkers such as brain natriuretic peptide (BNP), cardiac troponins and MMPs is a cornerstone of clinical management of DbCM. However, these markers are expressed in <60% of cases, In several studies, BNP proved to be a suboptimal screening test to detect pre-clinical LV dysfunction or LVH ^[9, - 11]. For all these reasons novel sensitive markers are needed. Although many substances have been suggested as biomarkers for DbCM, none have qualified for clinical use. Diagnosis and therapies are difficult because of a lack of specific biomarkers and imaging techniques. There is a need to anticipate the progression of heart failure in patients with diabetes and for that reason we propose a strategy of non-invasive analysis of inflammation-fibrosis LVD. The detection of appropriate biomarkers could potentially permit routine population-wide screening, allowing early diagnosis and anticipation of cardiac

dysfunction, and stratify these subclinical cases^[12, 13]. MicroRNAs (miRNAs) are short noncoding RNAs that modify gene expression by regulating mRNA stability or translation during various disease processes. Each mRNA regulates expression of multiple genes. Dysregulated miRNAs are potentially involved in the pathogenesis of DbCM ^[14]. There are multiple miRNAs that have been implicated with the progression of disease. After release from the cell, these molecules remain stable in extracellular fluids and can be measured by quantitative polymerase chain reaction (qPCR)^[15]. Clinical studies have proposed peripheral blood microRNAs (miRNAs) as sensitive, specific and non-invasive biomarkers for the early monitoring of alterations in cardiac viability, structure, and function. Distinctive circulating miRNA signatures with clinical value have been described in various cardiomyopathies and type 2 diabetes and its related vascular complications^[16]. miRNA-378 has emerged as a new biomarker in diabetic heart failure and has a strong relationship with inflammation and fibrosis ^[17]. The goal of the present study was to evaluate the usefulness of miR-378, as a serum biomarkers of DbCM in an unselected patient sample. To determine whether this miRNAs would be equally appropriate as serum biomarkers, it was tested in this study in a group consisting of 75 diabetic patients. The utility of that miRNA as a serum biomarker was evaluated by comparing its sensitivity to that of GLS.

Subjects, Materials and Method

Subjects: From May 2018 to April 2019, a total of 75 patients with DM2 and 25 control subjects who were aged 40–65yr were prospectively enrolled from Diabetic Center, CCU, and Echo Unit in Merjan Medical City (M.M.C.) admitted as diagnosed cases of DM2 by expert physicians. Each 25 patients group has a definite characters as in table (1).

Table (1): Diagnostic criteria of study groups

CD group	Normotensive diabetic patients without cardiomyopathy diagnosed by negative strain echo study
M group	Normotensive diabetic patients with cardiomyopathy diagnosed by positive strain echo study
F group	Hypertensive and/or ischemic diabetic patients with heart failure diagnosed by conventional echo study
H group	Apparently healthy subjects with negative strain echo study

Clinical Evaluation: All subjects underwent a questionnaire include: medical history which involve: history of DM2 and its duration, ischemic heart disease,

hypertension, family history of DM2, medications already taken and alcohol consumption history. Previous history of ischemic heart disease (IHD) was considered

positive if there was any history of admission to CCU with records by physician suggestive of IHD. The absence of ischemia and structural abnormalities was verified in groups M, CD, and H. Nonischemic is defined by normal resting ECG and negative stress ECG test. Structural abnormalities were excluded by normal resting conventional echo study having EF% >50%.

The questionnaire includes also socio demographic measures involving: age, gender. In addition to those

the questionnaire includes anthropometric measures involving BMI which was calculated from the measured height and weight and categorized into the following categories: normal BMI <25, over weight BMI (25-29.9) and obese $\geq 30 \text{ kg/m}^2$. All were subjected to clinical examination including, heart rate and blood pressure. Further clinical details are shown in Supplementary Table (2).

Table (2): Clinical data for patients in all study groups and control subjects

Variables	Groups			
	CD group	M group	F group	H group
Total number (n)	25	25	25	25
Age/Mean \pm SD	52.76 \pm 6.3	53.04 \pm 6.2	58.44 \pm 5.9	50.88 \pm 8.1
Gender	Male	14	15	18
	Female	11	10	7
BMI (Kg/m ²)/Mean \pm SD	26.4 \pm 4.1	32.1 \pm 5.3	30.6 \pm 3.5	29.2 \pm 5.9
Family history of DM2	+ve	19	17	18
	-ve	6	8	7
HbA1c%	8.5 \pm 2.3	8.9 \pm 2.3	9.1 \pm 2.2	4.4 \pm 0.27

Method

Echocardiography: The gold standard biomarker of diagnosis was GLS% which was defined as the average longitudinal strain at end-systole in 18 segments. By regarding cut-off value of GLS = -18% according to (Islam E. Shehata et al^[18] who reported that a GLS cutoff of <-18.1% was able to accurately “predict subclinical LV systolic dysfunction”.

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 PLSL value for each participant were calculated as the average of values of the 16 segments^[18].

The adequacy of the tracking was verified visually, and if tracking was deemed suboptimal, a manual

adjustment of both the endocardial and epicardial border was performed. If tracking was still judged unsatisfactory, the subjects were excluded from the analysis. Strain curve were extracted from an average of three cycles of tissue Doppler imaging data, using an IBM computer and developmental software (Formtest V6.1; GE Vingmed).

Blood collection and Laboratory method: Venous blood was acquired by venipuncture without anticoagulant treatment. Samples were centrifuged and subsequently aliquoted and stored at -80°C prior to analysis. For RNA isolation, an miRNeasy Mini Kit (Sigma/USA) was used to extract total RNA from 500 μl of serum. Reverse transcription (RT) was performed using a TaqMan MicroRNA Reverse Transcription Kit (Sigma/USA). The RT product was preamplified, and level of miR-378-5p (assay MIRAP00354) were measured by qPCR using a TaqMan miRNA assay (Applied Biosystems). miRNA species with CT value ≥ 35 were considered below the detection threshold. The serum miRNA expression levels in individual samples were determined by a TaqMan probe-based RT-qPCR

on a 7300 Real-Time PCR Sequence Detection System (Applied Biosystems). Because U6 and 5S rRNA are degraded in serum samples and the lack of a consensus housekeeping miRNA for the RT-qPCR analysis of serum miRNAs, miRNA expression was normalized to serum volume [19]. For serum and exosome samples. Influence of haemolysis was discarded by analysing the Ct values of miR-23a and miR-451a [20].

Statistical Analysis: Statistical analysis was performed using SPSS version 20. Data were expressed as (mean \pm SD). ANOVA test was used to compare means of more than 2 groups. P values less than (0.05) were considered significant. Receiver operating characteristic (ROC) analysis was performed to evaluate the discriminatory power of the markers analyzed. We chose Ct = 20.06 as the cutoff value to evaluate sensitivity and specificity of miRNA-378 in this study.

Results and Discussion

The difference in miRNA-378 circulating level in different study groups: There is significant elevation in the Ct value of miR-378 with the progression of the diabetic cardiomyopathy as presented in table (4) indicating its down regulation as the disease progress. This result agreed with (Rui Guo et al) who classify miR-378 as anti-hypertrophic miRNAs as it is downregulated with the development of DbCM [21].

This result disagreed with (Sarah Costantino,) who stated that miR-378 is one of key initiators of apoptosis, were significantly overexpressed in the diabetic heart and intensive glycemic control was unable to revert these changes [22]. The complex biology of miRNAs may also influence the respective findings. The miRNA profile of a given cell is highly specific to the stressor which it is exposed and suggests that miRNA expression in the human heart is dynamically regulated as a function of the pathophysiological context [23].

There is significant elevation in miR-378 Ct value between H & other three diabetic gps. (CD, M, F) as presented in table (5) $p < 0.05$; which represent its relevance to DM2. This result agreed with (Ivana Knezevic et al) who reported that in tissues such as fibroblasts and fetal hearts, where insulin-like growth factor receptor-1 (IGF1R) levels are high, we found either

absent or significantly low miR-378 levels, suggesting an inverse relationship between these two factors [24]. IGF-R are hybrid receptors which are more abundant when insulin receptors are down-regulated in response to the hyperinsulinemia as seen in insulin-resistant patients with DM2 as stated by Liam J. Murphy [25] Also it agreed with (Assmann et al [26]) who stated that it is downregulated with DM2. miR-378 down regulation relevance to DM2 approved by (Yong Zhang et al [27]) when they found that Tg mice had a significant overall increase in body O₂ consumption, CO₂ production, and energy expenditure.

The significant elevation in miR-378 Ct value in M than CD groups indicate its downregulation with the development of cardiomyopathy in its subclinical state as that miR-378 downregulated in case of hypertrophy development and oxidative stress increase [28]. These findings confirm the potential role of these modulators in identifying DbCM in relevance to their role in mitochondrial metabolism of cardiomyocyte via FoxO-1 as presented by (Puthanveetil, P. [29]) who stated that overexpression of FoxO1 mimics a hyperglycemic effect on vascular endothelium and also that hyperglycemia-induced endothelial dysfunction is mediated through FoxO1. miR-378 mimics reduced Akt phosphorylation also resulted in decreased phosphorylation of FoxO1, ultimately leading to FoxO1 activation. This effect was also perceived in a H₂O₂-induced oxidative stress model in rat cardiomyocytes. So downregulation of miR-378 resulted in increased expression of FoxO1 which resulted in mitochondrial dysfunction trigger cardiac cell death in diabetic heart disease.

There is significant elevation in Ct value in F than M gp. ($p = 0.000$) which indicate more downregulation of miR-378 in F gp. than M gp. as presented in table (5). This lower expression may be due to different pathophysiological background related to hypertension & ischemia as concluded by (Jan Fiedler et al [30]) who published that presence of miR-378 attenuated ischemia-induced apoptosis by inhibiting caspase-3 expression in cardiac myocytes and blunted cardiac hypertrophy and dysfunction upon cardiac overload by targeting Ras signaling. So may be the ischemic hypertensive metabolic changes resulted in downregulation of miR-378.

Table (4) ANOVA–All Groups with Ct of miR-378

Descriptive				
Ct of miR-378				
Groups	Mean	Std. Deviation ±	95% Confidence Interval for Mean	
			Lower Bound	Upper Bound
CD	19.6	2.1	18.80208	20.54192
F	30.4	3.4	28.97827	31.89006
H	17.6	2.9	16.30735	18.89526
M	25.9	3.4	24.39589	27.45138

Table (5): Comparison between groups

Multiple Comparisons					
Dependent Variable: Ct of miR-378					
LSD					
Groups		Mean Difference	p-value	95% Confidence Interval	
				Lower Bound	Upper Bound
H	CD	-2.070	.020	-3.80996-	-.33143-
	F	-12.832	.000	-14.58941-	-11.07632-
	M	-8.322	.000	-10.11752-	-6.52714-
M	CD	6.251	.000	4.49190	8.01137
	F	-4.510*	.000	-6.28734-	-2.73372-

*The mean difference is significant at the 0.05 level

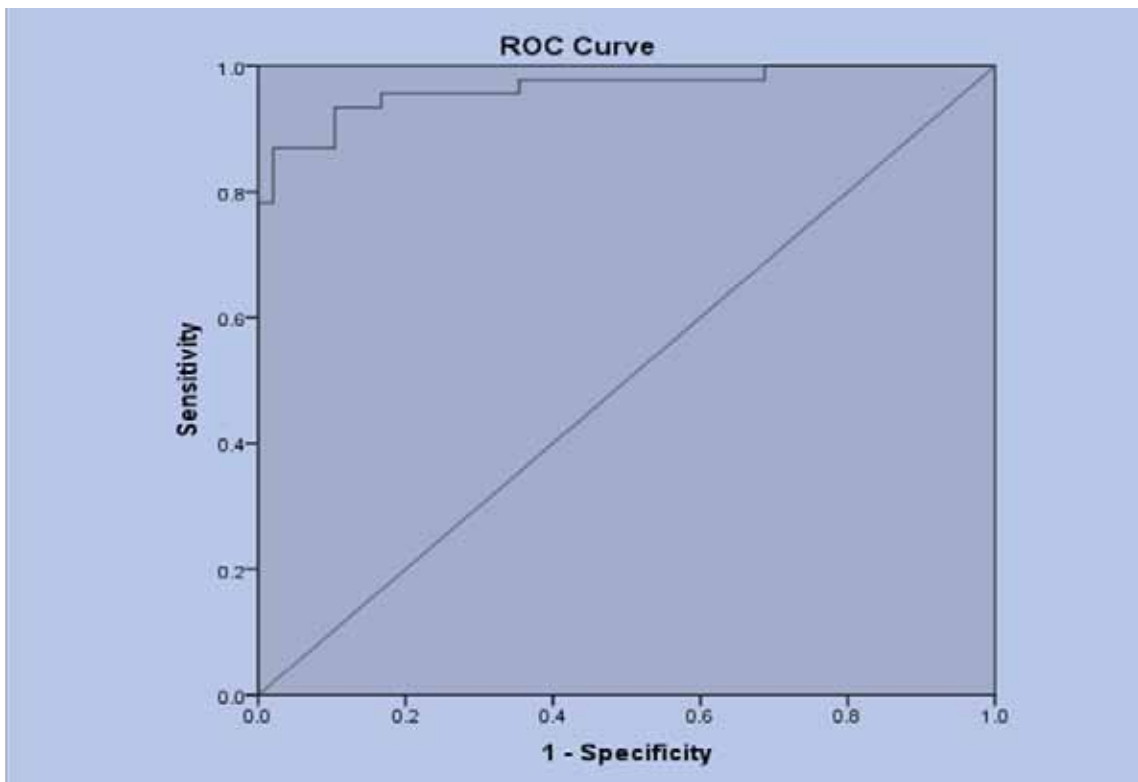


Figure (1): ROC curves for miRNA-378 Ct value in all study groups

miRNA-378 as a screening marker: To evaluate the potential diagnostic value of miRNA-378, the ROC curve has been used. For cut-off value, values exceeding the cut-off are positive, while those below the cut-off are negative.

It is found that the area under the ROC curve (AUC) was 0.965 (Fig. 1). The sensitivity and specificity were 0.978 and 0.646, respectively. The cut-off Ct value was 20.06, it was appeared to have independent associations with the clinical end-points that are studied. As a blood-based marker, the miR-378 characteristics as highly sensitivemay improve the screening values of DbCM. These results suggested that miR-378 may be highly sensitive and moderately specific enough to detect DbCM.

The extremely high sensitivity (0.978) of miR-378 to identify DbCM patients from non-symptomatic diabetic individuals exceeded our most optimistic expectations.

These findings strengthen the clinical applicability of circulating miR-378 as a biomarker of diabetic cardiomyopathy in type 2 diabetes patients.

Conclusion

Serum miR-378 expression level can be regarded as a non-invasive tool to improve the detection, prediction, and monitoring of cardiac-related complications in the early stages of diabetes.

Ethical Issues:

- a. Approval of scientific committee of the Clinical Biochemistry Department in Babylon Medical College/University of Babylon/Iraq.
- b. Approval of Babylon Health Directorate/Ministry of Health & Information Center for Research & Development of Babylon Province.
- c. The objectives and methodology were explained to all participants in the current study and their vocal consent was gained.

Conflict of Interest: The authors declare no competing interest on this paper.

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