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Role of miR-496 in prediction of type 2 diabetes mellitus in Iraqi patients

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Abstract
Background: Diabetes mellitus is a metabolic disease. Non-insulin dependent diabetes mellitus which also known as type 2 diabetes (T2DM) is its common form, its percentage might proceed 90-95% of all diabetic cases. This disease increases rapidly over short period of time and now a days younger age people might facing this problem even with low body mass index. According to cultural, socioeconomic, ethnic condition and degree of urbanization, the epidemic is varied. MicroRNA-496 (miR-496) is one of the well-known miRs enrolled the inflammatory signaling pathway and recently it has been discovered as epigenetic changes contributor for the development of diabetes in the pre-diabetic stage.
Objectives: Evaluate the sera levels of miR-496 in prediabetic T2DM patients and investigate the relationship between prediabetic state and level of miR-496 gene expression.
Methods: This study was carried at Al-Hussan endocrinology center in Kerbala Provence in Iraq during the period from Oct, 2022 to April, 2023 on 70 patients with impaired fasting glucose level (34 males and 36 females) and 50 apparently healthy subjects (24 males and 26 females) who were dealt with as control group. Two ml of sera were collected from obese prediabetic patients (BMI $\geq$ 30, fasting serum glucose level 110-140 mg/dl) each patient / control subjects were investigated to detect miR-181b expression level with real time PCR.
Results: The results of the present study revealed a significant decline in mean of sera levels for miR-496 in prediabetic patients group in comparison with its levels in control group (p value $\leq$ 0.05).
Conclusion: miR-496 could be used as a novel diagnostic biomarker to predict diabetes in prediabetic state. Hence, to prevent diabetes by changing the life style or by using certain medications.

Keywords: miR-496, T2DM, diabetes mellitus

Introduction
With the economic development and transition in diet, the rates of overweight and obesity have been rapidly increased in Iraq, which correlate positively with prevalence of Type 2 diabetes mellitus (T2DM) and metabolic syndrome which are highly prevalent in Asians [1, 2].
MiRs are small non-coding RNAs constituting 19-24 nucleotides serving as gene regulatory network hubs by controlling so many targets by silencing of RNA and expression of gene in posttranscriptional level.
MicroRNA-496 is a well-known anti-inflammatory miR used in the signaling pathway of inflammatory process, recently a role of this miR as a contributor for epigenetic changes at gene level in the prediabetic state (Which might remain for 2 years before development of diabetes) [3].
Development of new epigenetic biomarkers for early detection of T2DM might be possible. MiRs, as revolutionary micro molecules that regulate gene expression at the post transcription level by inhibiting the translation of the target mRNA molecule. miR-496 serve as enhancer for insulin signaling, anti-inflammatory, and endothelial cell dysfunction by targeting endothelial PH domain and Leucine Rich Repeat Protein Phosphatase 2 (PHLPP2) [4-8].
The aim of this study is to estimate the level of expression of miR-496 in blood of pre-diabetic patients and healthy subjects to provide new noninvasive strategy for early diagnosis of T2DM, Hence, prevent its complications.

Methodology
This study was conducted at Al-Hussan endocrinology center in Kerbala Provence in Iraq.

Samples were randomly selected from obese prediabetic patients with BMI ≥ 30 and fasting serum glucose level 110-140 mg/dl (Impaired fasting glucose), who were visit the diabetic consultation unit during the period from Oct, 2022 until April, 2023. The patients and control groups were with age ranged between 20-75 years. Study was carried out on 70 patients with impaired fasting glucose level (34 males and 36 females) and 50 apparently healthy subjects (24 males and 26 females) who were dealt with as control group.

Two ml of whole blood has been collected from every patient and control subject and was put in EDTA tube with Trizol preservative material and stored in -70 °C until time of investigation. Work was done by Qiagen Rotor gene real time PCR.

This study have the approval by scientific committee of biochemistry department / faculty of medicine / university of Kerbala. A signed written consent was taken from each patient (or his / her relative) and each healthy individual participated in this study.

The difference in levels between, miR-496 in prediabetic patients and control group has been compared to find the effect of prediabetic state on that parameter. Coefficient t-test correlation used to describe the association between the variable studied parameter in this study; $p \leq 0.05$ was considered statistically significant. Data were statistically analyzed by utilizing SPSS, version 22. Data were expressed as mean ± standard deviation (SD), and to determine whether the studied parameter followed a Gaussian distribution, Shapiro–Wilk normality test have been used. Independent samples t-test was used to compare between means of the studied groups. For multiple comparisons after ANOVA tests, the categorical variables were analyzed by χ^2 tests. The Scheffé, Tukey, Hochberg's GT2 Post Hoc tests. The association degrees between variables were analyzed by Pearson correlation analysis. A two-tailed P-value less than 0.05 ($p \leq 0.05$) was considered significant.

Results

Seventy patients with prediabetes (36 females and 34 males), and 50 apparently healthy subjects (26 females and 24 males) with comparable percentage of both sex. The mean ± SD value of age of prediabetic patients (35±12 years), and healthy controls (40±16 years), without significant differences among them, (Table 1).

The nuclear control transcript (housekeeping gene) that have been used to search for miR expression in this study was miR-U6, which give information about the environment of storage of miR from day of blood collection to the day of laboratory work and about the normalization of the studying tools and materials. The results showed non- significant differences in mean ± SD value of Ct as well as in fold of gene expression of miR-U6 between each of prediabetic patients group and healthy controls, (Tables 2, 3 and figure 1).

The results of this study revealed a significant decline in mean ± SD of sera levels for miR-496 in prediabetic patients group in comparison with its levels in control group (p value ≤ 0.05), (Table 4 and figure 2).

The ΔCt of miR-496 in prediabetic patients = (means Ct of miR-496) – (means Ct of miR-U6). Subsequently, the fold of miR-496 gene expression in prediabetic patients was decrease (0.007 fold) in comparison with its levels in control group, with receiving operation characteristic ROC value of 92% (sensitivity =88%, specificity = 95%), cutoff value = 0.0558 (P value ≤ 0.05, 95%, AUC=0.92), (Table 5 and Figures 2 and 4).

Table 1: Mean ± SD value of age and number of studied subjects according gender.

Group	Age (Year) Mean (± SD)	Females No. (%)	Males No. (%)
Prediabetic patients (N=70)	35±12	36 (51.5)	34 (48.5)
Healthy subjects (N=50)	40±16	26 (52)	24 (48)

Table 2: Mean ± SD values of Ct of miR-U6 of studied groups.

Group	Mean Ct of miR-U6 ± SD	Range*
Prediabetic patients (N=70)	21.56±0.45	20-22
Apparently healthy (N=50)	21.55±0.43	20-22

* Accepted rang by supplied kit

Table 3: Comparison of miR-U6 fold expression between studied groups.

Group	Means ± SD Ct of miR-U6	2^{-Ct}	Experimental group/Control group	Fold of gene expression
Prediabetic patients (N=70)	21.57±0.44	3.21E-07	3.21E-07/3.24E-07	0.98
Healthy subjects (N=50)	21.55±0.43	3.24E-07	3.24E-07/3.24E-07	1

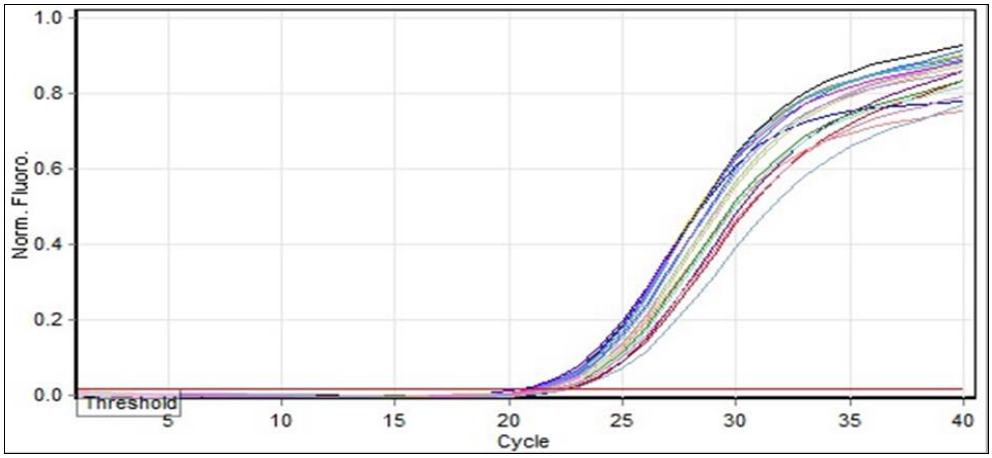

Fig 1: miR-U6 amplification plots by qPCR samples included all studied groups. The photograph was taken directly from Qiagen Rotor gene qrtPCR machine

Table 4: Fold of miR-496 expression depending on $2^{-\Delta Ct}$ method

Groups	Means Ct of miR-496	Means Ct of U6	ΔCt (Means Ct of miR-496	$2^{-\Delta Ct}$	Experimental group/ Control group	Fold of gene expression	p-value
Patients	27.09	21.57±0.44	5.52	0.021657	0.021657/2.770218	0.007	≤ 0.05
Control	20.92	21.55±0.43	-1.47	2.770218	2.770218/2.770218	1.00	

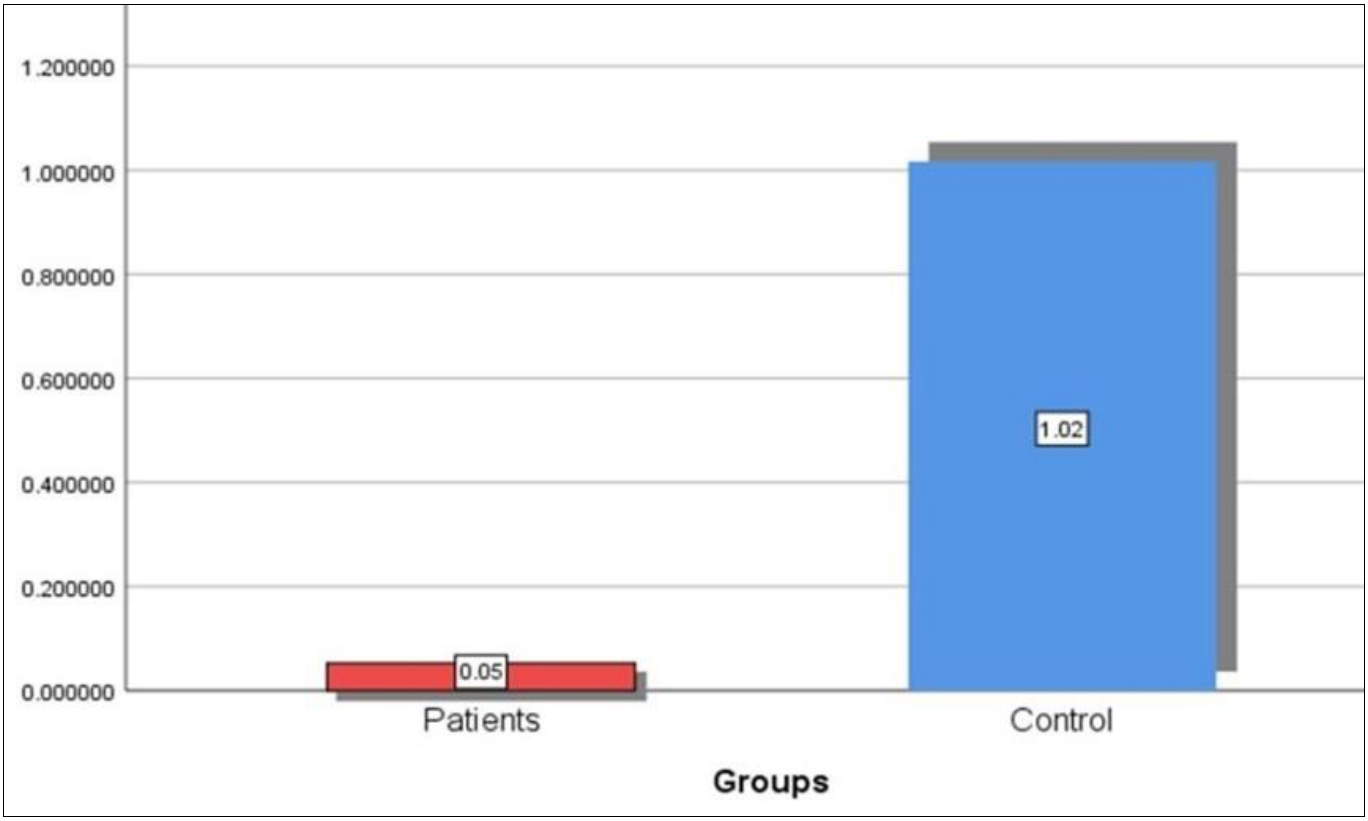

Fig 2: miR-496 in prediabetic patients group and control group

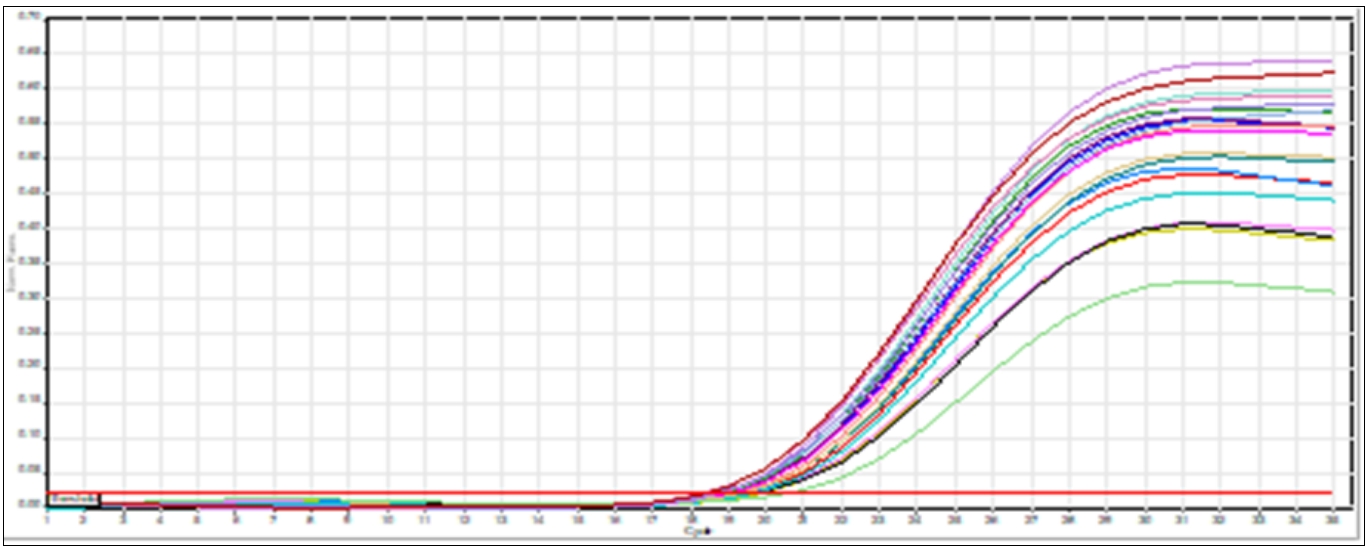

Fig 3: MiR-496 amplification plots by qPCR Samples included studded groups. The photograph was taken directly from Qiagen Rotor gene qrtPCR machine

Table 5: Receiver operating characteristic curve data of the studied genes

Parameters	AUC	Explanation	P-value	The best Cut off	Sensitivity %	Specificity %
miR-496	0.92	Excellent	≤ 0.05	0.0558	88	95

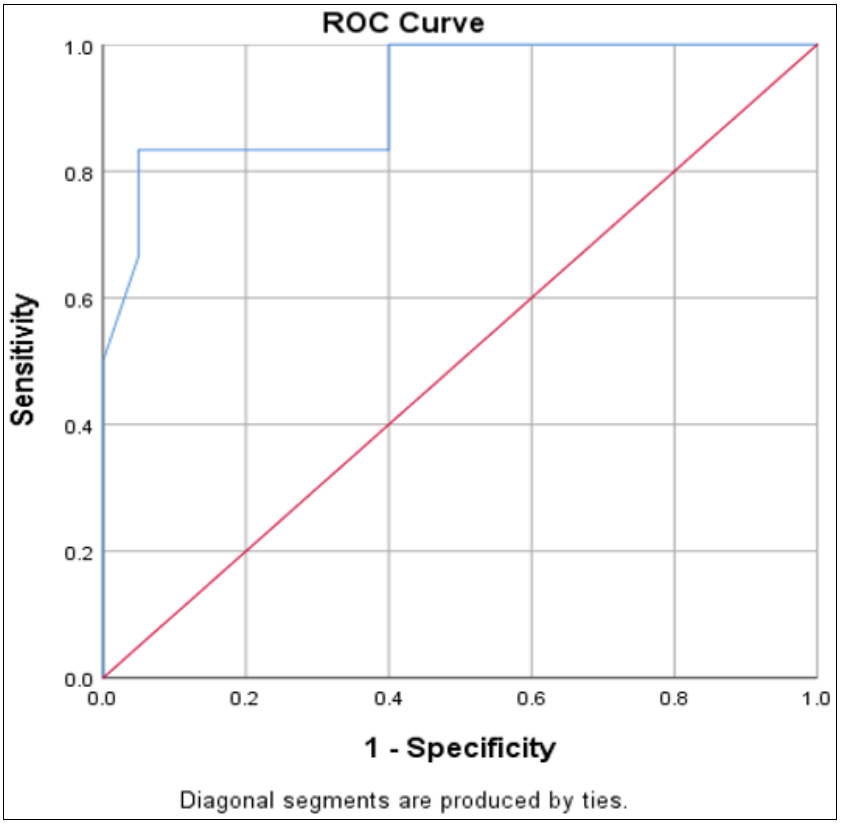

Fig 4: Receiver operating characteristic curve of the miR-496

Discussion

The known vital cause of type 2 diabetes mellitus development is insulin resistance, the process that start with adipose tissue dysfunction and low-grade inflammation in adipocyte [9-12]. Imbalance dietary habit and excessive diet and its metabolic substrate end product expose adipose tissue and other tissues to primary low-grade inflammation [13], enhancing the release of cytokines [14,15]. Recent studies prove the hypothesis (In white adipose tissue, the chronic inflammatory process is enrolled in pathogenesis of obesity-associated insulin resistance [16-19].

Furthermore, endothelial cells dysfunction is a big contributor for insulin resistance development, Metabolic syndrome and T2DM [20-22], which was experimentally established.

Over production of these cytokines and leptin hormone might play an additional impact on inflammatory process that have been noticed in obese patient as we mentioned above because the 3′ UTR region of the interlukin-6 targated as a binding site for miR-496 and reduces the expression of interleukin-6 which act as anti-inflammatory agent during inflammation [23].

In consistence with our study results, Tomé-Carneiro, found that miR-496 expression levels increased significantly in the patients who consumed one daily dose of resveratrol (an anti-inflammatory drug [24]. It was recently identified that miR-496 plays an important role in inhibiting the NF-κB (Transcription factor that regulates genes responsible for both the innate and adaptive immune response) [25]. Overexpressing miR-496 improved glucose uptake and glucose homeostasis in adipocytes via paracrine mechanisms with adipocytes.

In other hand, miR-496 targets PHLPP2, a phosphatase that dephosphorylates Rho-associated serine/threonine kinase ROCK1 [26]. ROCK1 that is a key downstream target of the small GTPases. ROCK is involved in diverse cellular activities including actomyosin and cytoskeleton dynamics contractility and organization that is involve in inflammatory process in insulin resistance [27]. The ROCK signaling pathway plays a critical role in a range of diseases [28]. MiR-496 effect on glucose homeostasis, insulin sensitivity. The elevated level of ROCK1mRNA may be due to its major cytoskeletal role in proliferation and adhesion of especially in malignant cells [29-30]. Hallgren *et al*. demonstrated that ROCK signaling pathway act as a tissue compliance sensor [31]. Elevated level of ROCK1mRNA may also be due to down regulation of miR-496 resulted in the present study as the demonstrated unambiguously that miR-496 targeted specifically the 3′UTR region of ROCK1.

Conclusion

MiR-496 could be used as a novel diagnostic biomarker to predict diabetes in prediabetic state.

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