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Original Article

Clinical significance of microRNA 126 in diabetic retinopathy in type 2 diabetes mellitus

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ABSTRACT

Background: Diabetes mellitus (DM) is a group of metabolic disorders characterized by chronic hyperglycemia. It is caused by defective insulin production or resistance of the cells to insulin. The chronic hyperglycemia leads to damage of different organs, especially eyes, kidneys, nerves, heart, and blood vessels. MicroRNAs (miRNAs) are small non-coding regulatory ribonucleic acids (RNA). Many studies have showed the association between miRNA 126 and complications of DM including diabetic retinopathy (DR).

Objective: Assessing the ability of circulating miRNA 126 to be used as diagnostic biomarker of both proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR).

Methodology: This case-control study was conducted on 20 DR (PDR & NPDR) patients, 20 DM patients without DR and 20 apparently healthy controls that were recruited from Research Institute of Ophthalmology - RIO. Identification and quantification of plasma miRNA126 was performed by real-time PCR.

Results: MiRNA 126 expression is significantly decreased in PDR group when compared to healthy control. Its expression in PDR is less than in NPDR, expression in NPDR is less than in DMC, and expression in healthy people is higher than in other groups (P value < 0.001). ROC curve was done for healthy control group versus DMC, PDR and NPDR groups and showed that the area under the curve (AUC) was 1.0 for all groups with sensitivity and specificity 100%, confidence interval (CI) was 95% with upper and lower limit (1.0-1.0). Best cut off point of miRNA-126 was 5.44, 4.44, 4. 88 for DMC, PDR and NPDR respectively. There is also a high significant increase between each group and control regarding hemoglobin A1C (HbA1c). There is significant increase between PDR and control regarding triglycerides (TG).

Conclusion: miRNA 126 can differentiate between the PDR, NPDR, DMC and control group and could be considered a non-invasive diagnostic parameter.

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Keywords: Biomarker, DR, microRNA 126, diabetic retinopathy

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INTRODUCTION

Diabetes mellitus (DM) is a lifelong syndrome characterised by abnormally high blood glucose levels due to a defective insulin production or insulin insensitivity of the cells or a combination of both in type 2 DM. Diabetes is a problem that is increasing worldwide with increasing morbidity and mortality rates. DM prevalence is increasing mainly because of sedentary lifestyle and obesity, beside the genetic factors [1]. DM may cause serious complications such as cardiovascular, cerebrovascular, eye, renal disorders [2].

Diabetic retinopathy (DR) is a common complication of DM and it may lead to complete blindness. It has long been known as a microvascular disease. It is caused by a microvascular lesion in addition to retinal inflammation and neurodegeneration [3]. Clinically, DR has two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR is the early stage and characterized by increased vascular permeability and capillary occlusion, microaneurysms, haemorrhages and hard exudates can be detected by fundus examination. The patient may be asymptomatic. PDR is a more advanced stage. It is

characterized by neovascularization. The patient may complain from severe vision impairment because of vitreous haemorrhage or tractional retinal detachment [4].

MicroRNAs are short (~22 nucleotides) non-coding RNAs. Their function is to stop protein translation and/or degrade their messenger RNA targets [5]. MiRNA126 is widely studied in diabetes and its complications because of its important role in endothelial protection and angiogenesis. Levels of retinal miRNA126 are decreased in experimental DM [6]. This study aimed to assess the ability of miRNA126 to be used as a marker for diagnosis of PDR and NPDR.

SUBJECTS AND METHODS

Type, place, and duration of the study: This case-control study was conducted All studied patients were recruited from the Research Institute of Ophthalmology - RIO (Medical Retina Clinics). It was carried out in the period from December 2018 till May 2019.

Ethical approval: The study was approved by the Ethics Board of Al-Azhar University and an informed verbal consent was taken from each participant in the study.

Study participants

The study was conducted on 60 Egyptian adults who were divided into four groups:

- **Group (1):** included 20 non-diabetic subjects (control)
- **Group (2):** included 20 diabetic patients without evident DR (Diabetic control DMC)
- **Group (3):** included 12 diabetic patients with PDR
- **Group (4):** included 8 diabetic patients with NPDR

Exclusion criteria applied for cases and controls:

Type-1 diabetic patients, diabetic patients with stress conditions; acute complications of DM (e.g. diabetic ketoacidosis, ...), myocardial infarction, infections, recent surgery, cancer, patients with renal disease, pregnant women and patients with eye conditions that obscure retinal views (e.g. dense cataract, vitreous haemorrhage) or conditions affecting the visual field (e.g. glaucoma) were excluded from the study.

Methods

Diagnosis of DR was done by the ophthalmologist through proper history taking, dilated eye examination and using the ophthalmoscope and slit lamp. Some patients needed investigations such as optic coherence tomography (OCT) and fundus fluorescein angiography (FFA).

Laboratory investigations included: 1) Routine laboratory investigations: by fully automated analyzer "Xl-300" (ERBA) after passing the 2-levels internal quality control including: haemoglobin A1c (HbA1c) blood level (%) by turbidimetric assay, lipid profile (mg/dl) by enzymatic colorimetric method: Cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), Triglycerides (TG) and microalbumin in urine (mg/L) by turbidimetry. 2) Specific laboratory test: Measurement of plasma microRNA 126: by real time PCR

All subjects were instructed to fast (no caloric intake) 8-10 hours. A venous blood sample of 6 ml from antecubital vein was withdrawn from fasting subjects under sterile conditions then was divided into:

- 2 mL of venous blood were dispensed into a tube containing K-Ethylene Diamine Tetra Acetate (K-EDTA) for HbA1c level measurement.
- 2 mL of venous blood were dispensed into sterile plain tube with, left to clot at 37°C for 10 minutes then centrifuged at 3000 rpm for 5 minutes to separate the serum from the erythrocytes for lipid profile testing.
- 2 mL of venous blood were dispensed into a tube containing K-EDTA for microRNA 126 measurements, centrifuged at 3600 g for 20min, and the supernatants were transferred into Eppendorf tubes frozen at -80 ℃ pending RNA extraction.

Random urine samples were collected.

The miRNA assay steps were done as follows: 1. RNA Extraction: was performed using a miRNeasy Mini Kit (Qiagen). DNase treatment was carried out to remove any containing DNA. All serum RNA preparations were quantified by NanoDrop. 2. Reverse transcription and cDNA formation. 3. Quantitative polymerase chain reaction (qPCR) was carried out using the SYBR Premix kit. U6 snRNA was used as the endogenous control. 4. Detection of expression: The PCR amplification protocol was performed by the real-time cycler.

Statistical analysis

Data was analysed using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release22for Microsoft Windows. Numerical data were tested by Shapiro Wilk test. Comparison of numerical variables between the study groups was done using Kruskal Wallis test for comparing not-normal data. For comparing categorical data, Chi-square test was performed. Correlation between variables was done using Spearman correlation equation for non-normal variables/non-linear relation. Diagnostic accuracy was represented using the terms sensitivity, and specificity. Receiver operator characteristic (ROC) analysis was used to determine the optimum cut off value for ΔCT in differentiating DMC, NPDR, and PDR from control group. *P*-value ≤ 0.05 was considered statistically significant. *P-value* <0.01 was considered highly significant. *P*- value >0.05 was considered non-significant.

RESULTS

There was a high significant increase between PDR and control regarding HTN. There was significant increase between all diabetics groups regarding DM duration when compared to each other, while there was no significant difference between all groups regarding the remaining parameters (table 1). There was a high significant increase between each group and control regarding HbA1C. There was significant increase between PDR and control regarding TG, while there was no significant difference between all groups regarding the remaining parameters (table 2) MiRNA 126 expression is significantly decreased in PDR group when compared to healthy control. Its expression in PDR is less than in NPDR, expression in NPDR is less than in DMC, and expression in healthy people is higher than in other groups (P 0.001) (table 3). There was no significant correlation between plasma level of miRNA 126 and DM duration, CVD, HTN, smoking, HbA1C, CHO, TG, HDL, LDL in DMC group (table 4). There was no significant correlation between plasma level of miRNA 126 and DM duration, HTN, smoking, HbA1C, CHO, TG, HDL, LDL in NPDR group (table 4). There was no significant correlation between plasma level of miRNA 126 and DM duration, CVD, HTN, smoking, HbA1C, CHO, HDL, TG, LDL in PDR group (table 4). A receiver operating characteristic curve ((ROC curve) was done for healthy control group versus DMC, PDR and NPDR groups and showed that the area under the curve (AUC) was 1.0 for all groups with sensitivity and specificity 100%, confidence interval (CI) was 95% with upper and lower limit (1.0-1.0). Best cut off point of miRNA-126 was 5.44 for DMC, 4.44 for PDR and 4.88 for NPDR (table 5) and (figure 1).

Table (1): Comparison between healthy controls, DMC, PDR, and NPDR regarding age, sex, duration of DM, treatment of DM, smoking, CVD, and hypertension

Studied variables	Control groups No. = 40		Cases with diabetic retinopathy No.= 20		Test	P-value
	Healthy controls (n=20)	DMC (n=20)	PDR (n=12)	NPDR (n=8)		
Age/ yrs (mean± SD)	49.7±7.23	55.4±7.14	58±6.95	56.1±6.47	105.5[#] 38.0[#] 43.5[#]	P1: .010* P2:0.001** P3: 0.062
Sex: No. (%) Males Females	 9 (45%) 11(55%)	 5 (25%) 15(75%)	 8(66.7%) 4(33.3%)	 5(62.5%) 3(37.5%)	1.758[a] 1.414[a] 0.700[a]	P1: 0.320 P2: 0.234 P3: 0.678
DM duration/years (mean ± SD)	-	7.46±5.46	15.5±7.69	12.13±8.84	9.33ρ	P: 0.022*
Treatment of DM: No. (%): Insulin Oral hypoglycemic	-	 6 (30%) 14(70%)	 7(58.3%) 5(41.7%)	 4 (50%) 4 (50%)	2.558[a]	P: 0.260
Smoking: No. (%)	4 (20%)	4 (20%)	3 (25%)	3(37.5%)	0.000[a] 0.110[a] 0.933[a]	P1: 1.000 P2" 1.000 P3: 0.371
CVD: No. (%)	0 (0.0%)	1 (5%)	2(16.7%)	0 (0.0%)	1.026[a] 3.556[a]	P1: 1.000 P2: 0.133 ---
HTN: No. (%)	0 (0.0%)	3 (15%)	7(58.3%)	1(12.5%)	3.243[a] 14.93[a] 2.593[a]	P1:0.231 P2:0.001** P3: 0.286

DMC: Diabetic control, PDR: Proliferative diabetic retinopathy, NPDR: Non-proliferative diabetic retinopathy, DM: Diabetes mellitus, CVD: Cardiovascular disease, HTN: Hypertension. ρ: Kruskal Wallis test. #: Mann Whitney test. [a]: Chi square test. P1: comparison between control and DMC, P2: comparison between controls and PDR, P3: comparison between controls and NPDR, Kruskal Wallis (ρ), Mann Whitney (#) and Chi square tests [a]. * Statistically significant,

Table 2: Comparison between healthy controls, DMC, PDR, and NPDR regarding laboratory investigations

	Control groups No. = 40		Cases with DR No.= 20		Test#	P-value
	Healthy controls (n=20)	DMC (n=20)	PDR (n=12)	NPDR (n=8)		
HbA1c % (mean ± SD)	5.13±0.29	7.43±1.7	8.9±1.8	8.04±1.75	54.5 17.0 4.0	P1: 0.001* P2: 0.001* P3: 0.001*
CHO mg/dl (mean ± SD)	209.8±6.4	222.7±1.7	224±29.6	225.6±46.1	157.0 85.0 50.5	P1:0.245 P2: 0.173 P3: 0.133
TG mg/dl (mean ±SD)	107.4±35.8	126.4±39.4	138.3±32.5	139±48.8	144.0 52.5 50.0	P1:0.130 P2: 0.009* P3: 0.127
HDL mg/dl (mean ± SD)	55.5±11.9	54.3±8.2	51.0± 10	57.5±14.2	191.0 96.0 76.0	P1:0.807 P2: 0.350 P3: 0.839
LDL mg/dl (mean ± SD)	131.3±6.6	140.2±35.3	140.9±23.3	139.1±3.9	158.0 79.0 57.0	P1:0.256 P2: 0.110 P3: 0.242
Mic Alb in urine mg/L (mean± SD)	12.0±7.8	11.9± 8.7	13.6±10.1	10.0±8.7	191.0 112.0 67.0	P1:0.807 P2: 0.755 P3: 0.507

HbA1c: Hemoglobin A1c, CHO: Cholesterol, TG: Triglycerides, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, Mic Alb in urine: Microalbumin in urine. #: Mann Whitney test. P1: comparison between control and DMC, P2: comparison between controls and PDR, P3: comparison between controls and NPDR, using Mann Whitney test. * statistically significant.

Table 3: Comparison between healthy controls, DMC, PDR, and NPDR regarding expression of miRNA 126 in plasma (ΔCT)

	Control groups No. = 40		Cases with diabetic retinopathy No.= 20		Test#	P-value
	Healthy controls (n=20)	DMC (n=20)	PDR (n=12)	NPDR (n=8)		
ΔCT miRNA 126 in plasma	8.12±1.40	4.55±0.31	1.68±0.87	3.53±0.32	210.0 78.0 36.0	P1:0.001* P2:0.001* P3:0.001*

CT: Cycle threshold. #: Mann Whitney test. P1: comparison between control and DMC, P2: comparison between controls and PDR, P3: comparison between controls and NPDR, Mann Whitney test. * statistical significance

Table 4: Correlation of delta CT miRNA 126 with clinical and laboratory findings among the studied groups (DMC, NPDR PDR)

	Delta CT					
	DMC group		NPDR group		PDR group	
	r	p value	R	p value	r	p value
DM Duration	-0.125	0.600	0.323	0.435	0.035	0.913
HbA1C	0.232	0.326	0.012	0.978	0.007	0.983
CHO	-0.397	0.083	-0.262	0.531	0.105	0.744
TG	0.029	0.905	-0.048	0.911	-0.305	0.336
HDL	-0.320	0.170	0.168	0.691	0.344	0.274
LDL	-0.346	0.135	-0.333	0.420	0.308	0.330

Table (5): ROC curve analysis of miRNA-126 for discrimination between healthy controls, diabetic controls and diabetic retinopathy groups:

	AUC	Cut-off point	Sensitivity	Specificity	CI (upper-lower limit)
Control vs. DMC	1.0	5.44	100%	100%	95% (1.0-1.0)
Control vs. PDR	1.0	4.44	100%	100%	95% (1.0-1.0)
Control vs. NPDR	1.0	4.88	100%	100%	95% (1.0-1.0)

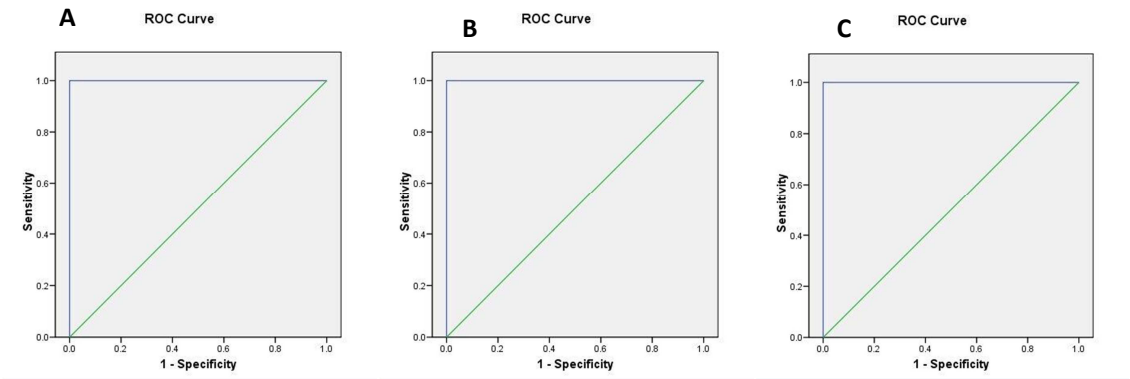

Fig.1: ROC curve analysis of plasma miRNA-126 for discriminating: A: Healthy control from DMC. B: Healthy control from PDR. C: Healthy control from NPDR. This means that miRNA-126 has the potentiality to differentiate between DMC, PDR, NPDR groups and controls and could be considered a non-invasive biomarker for diagnosing diabetic retinopathy

DISCUSSION

Type 2 DM represents about 90% of all cases of diabetes. Although most cases are of age more than 45, the incidence is increasing in children and young adults because of increasing levels of obesity, sedentary life, and fast food [7]. DR is the most common and severe microvascular complication in type 2 DM, and a major cause of blindness. The incidence of retinopathy in the world is increasing from 126.6 million to 191 million in only two decades [8]. Half of all cases of blindness can be prevented by early diagnosis and management. The gold standard of diagnosis of DR is fundus fluorescein angiography (FFA). It is important to find a sensitive and simple diagnostic tool for early diagnosis of DR [9]. Many studies showed that miRNAs have key roles in the development of many diseases including DR. So, assessment of miRNA levels in plasma maybe an important tool for diagnosing diseases and their progression [10].

In the present study, there was a high significant decrease in miRNA-126 expression in plasma in the diseased groups (PDR, NPDR and DMC) compared to control, and its level is less in PDR than NPDR, DMC and control respectively. This result is in agreement with Qin et al., [11] study which reported a higher expression of miRNA-126 in healthy people than in DMC, NPDR, PDR respectively. Our result also agrees with Bai et al., [12] a study that reported severe vascular retinal impairment in mice after miRNA-126 deletion, and decreased levels of VEGF, IGF-2 after intra-vitreal injection of miRNA-126, which proved the protective effect of miRNA-126 in DR. Our study also agrees with a study done by Wang and Yan [13] who reported that treatment with Niaspan, which normalised retinal miRNA-126 levels, prevented overexpression of VEGF and reduced haemorrhage and apoptosis in the retina.

HbA1c in the red blood cells (RBC) is the product of non-enzymatic glycation of haemoglobin and its amount is directly dependent on the amount of plasma glucose. Also, measured HbA1c is also directly related to RBC life span. All RBC contribute to the measured level of HbA1c. Although older RBC is exposed longer to blood glucose, younger RBC is more abundant. So, HbA1c is considered a good method of measurement of the average blood glucose levels along the past 120 days [14]. In the present study, there is high significant increase in HbA1c between the diseased groups as compared with control. This result agrees with a Chinese study done by Wat et al., [15] who demonstrated an association between higher levels of HbA1C and increased incidence and progression of DR. Another study done by Raum et al., [16] showed that elevated levels of HbA1C were associated with increased risk of DR.

In our study, there is a significant difference between the diseased groups regarding the duration of DM. This agrees with a study done by Zhang et al., [17] who reported that patients with DR had a longer duration of DM than those without DR.

In the present study, there is no significant difference between all groups regarding CVD. In this study, there is a significant difference between PDR and control regarding HTN, and no significant difference between the diseased groups and control regarding smoking. These results are consistent with a study done by Sliwinska-Mosson and Milnerowicz [18] that reported that DR was associated with HTN and not smoking. Another study done by Cheng et al., [19] reported that HTN had a positive association with DR.

In the present study, there is a significant difference between PDR and control regarding TG levels. This is consistent with Cheng et al study that found an association between high TG and DR in type 2 DM. In contrast, Tomic et al., [20] study reported no association between high TG and DR.

In this study, there was no significant difference between the diseased groups and control regarding CHO levels. This disagrees with Yau et al., [21] study that demonstrated an association between high CHO and the development of diabetic macular edema.

In the present study, there is no significant difference between diseased groups and control regarding sex. This is consistent with a study done in Nepal by Thapa et al., [22] that found no significant difference between males and females regarding the prevalence of DR.

In this study, we found no significant difference between all groups regarding micro-albumin in urine. In the present study, we found no significant difference between diseased groups regarding the type of treatment, whether tablets or insulin, in contrast with Zhang et al., [17] study that found that DR patients were significantly more likely to be using insulin.

In the present study, ROC curve was done for healthy control group versus DMC, PDR and NPDR groups and showed that the area under the curve (AUC) was 1.0 for all groups with sensitivity and specificity 100%, confidence interval (CI) was 95% with upper and lower limit (1.0-1.0). Best cut off point of miRNA-126 was 5.44 for DMC, 4.44 for PDR and 4.88 for NPDR. This means that miRNA-126 has the potentiality to differentiate between DMC, PDR, NPDR groups and controls and could be considered a non-invasive biomarker for diagnosis of diabetic retinopathy. Another study done by Qin et al., [11] showed that serum miR-126 levels differentiated DR patients from healthy controls, with an AUC of ROC curve of 0.932 [95% confidence interval (CI), 0.913 to 0.951]. The cut-off value was 8.43, which was associated with sensitivity and a specificity of 84.75% and 93.60%, respectively. Serum miR-126 levels could also differentiate NPDR patients from healthy controls with an AUC of ROC curve of 0.919 (95%CI 0.879 to 0.959). At the cut-off value of 8.43, suggested 84.75% sensitivity and 94.41% specificity. Serum miR-126 levels differentiated PDR patients from healthy controls, with an AUC of ROC curve of 0.976 (95%CI 0.960 to 0.992). The cut-off value was 5.02, which was associated with sensitivity and specificity of 81.21% and 90.34%, respectively

In the present study, there is no significant correlation between plasma levels of miRNA-126 in diseased groups and levels of HbA1C and other parameters. This is inconsistent with a study done by Rezk et al., [23] who demonstrated a negative significant correlation between expression of miRNA-126 in DR patients and HbA1C levels which may be explained by the greater number of cases included in that study (186 cases).

CONCLUSION

The miRNA 126 can differentiate between the PDR, NPDR, DMC and control group and could be considered an early non-invasive diagnostic parameter.

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Conflict of interest: the authors declare no conflict of interest.

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الملخص العربي

الأهمية السريرية للحمض النووي الريبوزي متناهي الصغر 126 في اعتلال الشبكية السكري في النوع الثاني من مرض السكري

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ملخص البحث:

الخلفية: مرض السكري هو مجموعة من الاضطرابات الأيضية التي تتميز بفرط□كر الدم المزمن. يحدث بسبب خلل في إنتاج الأنسولين أو مقاومة الخلايا للأنسولين. ويؤدي فرط□كر الدم المزمن إلى تلف الأعضاء المختلفة، وخاصة العينين والكلى والأعصاب والقلب والأوعية الدموية. إن الأحماض النووية الريبوزية متناهية الصغرهي أحماض تنظيمية غير مشفرة وقد أظهرت العديد من الدرا□ات الارتباط بين الحمض النووي الريبوزي متناهي الصغر 126 ومضاعفات السكر بما فيها اعتلا□ الشبكية السكري.

الهدف: تقييم القدرة على تعميم الحمض النووي الريبوزي متناهي الصغر 126 لا□تخدامها كمؤ□ر بيولوجي تشخيصي لكل من اعتلا□ الشبكية السكري التكاثري واعتلا□ الشبكية السكري غير التكاثري.

الطرق: تم إجراء درا□ة الحالات والشواهد هذه علي 20 مريضا باعتلا□ الشبكية السكري التكاثري وغير التكاثري, و20 مريضا بالسكر بدون اعتلا□ الشبكية, و20 من الضوابط الطبيعية. وتم جمعهم من معهد بحوث امراض العيون. وتم تحديد وقياس مستوي الحمض النووي الريبوزي متناهي الصغر 126 في البلازما بوا□طة الوقت الحقيقي للتفاعل المتسلسل للبلمرة.

النتائج: مستوي الحمض النووي الريبوزي متناهي الصغر 126 انخفض بشكل ملحوظ في مجموعة اعتلا□ الشبكية السكري التكاثري مقارنة بالضوابط السليمة. وانخفض مستواه في مرضي اعتلا□ الشبكية السكري التكاثري عن مرضي اعتلا□ الشبكية السكري غير التكاثري. وانخفض مستواه في مرضي اعتلا□ الشبكية السكري غير التكاثري عن مرضي السكري بدون اعتلا□ الشبكية. وكان مستواه في مجموعة الضوابط السليمة أعلي من المجموعات الأخرى. وتم عمل منحني عامل الا□تقبا□ لجميع المجموعات وتبين أن المنطقة تحت المنحني تساوي 1 لكل المجموعات والحسا□ية والخصوصية تساوي 100% وفاصل الثقة يساوي 95% والحد الأعلى والأدني (1-1) وأفضل نقاط قاطعة هي 5.44 , 4.44 , 4.88 لكل من مجموعات مرضي السكري بدون اعتلا□ الشبكية, ومرضي السكري باعتلا□ الشبكية التكاثري, ومرضي السكري باعتلا□ الشبكية غير التكاثري بالترتيب. هناك أيضا زيادة ملحوظة بين كل المجموعات مقارنة بمجموعة الضوابط السليمة فيما يخص الهيموجلوبين السكري. وهناك زيادة ملحوظة فيما يخص الدهون الثلاثية في مجموعة اعتلا□ الشبكية السكري التكاثري مقارنة بالضوابط السليمة.

الاستنتاجات : يمكن أن يستخدم قياس مستوي الحمض النووي الريبوزي متناهي الصغر 126 في بلازما الدم في التمييز بين اعتلا□ الشبكية السكري التكاثري, وغير التكاثري, ومرض السكري بدون اعتلا□ الشبكية, والأ□خاص الطبيعيين. وقد يكون علامة بيولوجية تشخيصية غير جراحية.

الكلمات المفتاحية: علامة بيولوجية-اعتلا□ الشبكية السكري- الحمض النووي الريبوزي متناهي الصغر 126

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