THE CLINICAL SIGNIFICANCE OF INSULIN RESISTANCE RELATED AUTOPHAGY GENE IN T2DM

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ABSTRACT:

Background: *Autophagy and Insulin resistance play a crucial role in pathogenesis of T2DM. Novel accurate strategies for detection of molecular pathogenesis of insulin resistance are distinctly needed.*

Aim of the work: *To retrieve potential mRNA associated with insulin resistance related autophagy from databases followed by validation of this network in patients' clinical samples.*

Material and Methods: *Bioinformatics' Analysis was done to retrieve a gene related to autophagy and IR in T2DM, then biomarker' verification was done through Gene Cards Database. Extraction of total RNA from PMNCs followed by reverse transcription PCR, then quantitative real time PCR for mRNA m-TOR in PMNCs of 123 patients with T2DM and 106 healthy normal volunteers.*

Results: *The mRNA m-TOR had high sensitivity and specificity for discriminating T2DM patients from healthy controls.*

Conclusion: *The mRNA m-TOR can serve as a potential biomarker for T2DM diagnosis and prognosis.*

Key Points: *The mRNA m-TOR has been revealed as a novel class of non-invasive disease biomarker with high specificity, sensitivity and stability for early detection of T2DM.*

INTRODUCTION:

Type 2 diabetes mellitus (T2DM) is a multifactorial disease. An individual's disease risk is based on a combination of genetic, epigenetic, environmental, and lifestyle risk factors. The pathophysiological changes are characterized by β-cell dysfunction, insulin resistance and chronic inflammation, all of which progressively disturb control of blood glucose levels and lead to the development of micro- and macro- vascular complications[(1)].

T2DM is a global health burden. Given that more than 415 million individuals are currently affected and the incidence is predicted to rise faster than the adult population growth[(2)]. Patients with type 2 diabetes (T2D) constitute approximately 90%-95% of all patients with diabetes worldwide and represent a growing epidemic rate[(3)]. The risk for type 2 diabetes increases with age, and nearly 26% of people in the United States older than 65 years have diabetes. In addition, because of the rising obesity rate in the United States, the incidence and prevalence of diabetes mellitus are increasing substantially[(4)].

The International Diabetes Federation (IDF) has identified Egypt as the ninth leading country in the world for the number of patients with T2D. The prevalence of T2DM in Egypt was tripled over the last two decades. The prevalence of diabetes in adults is 14.9%, and there are over 7.8 million cases of diabetes in Egypt in 2015. Moreover, the number of undiagnosed diabetic patients is more than 3.2 million

according to International Diabetes Federation (IDF)[5].

Insulin resistance is a complex metabolic disorder that participates in the development of type 2 DM and is a very early marker of diabetes risk and also predicts the development of the disease. Several molecular pathways contributing to insulin resistance and in the wake of the worldwide increase in type-2 diabetes, a major focus of research is understanding the signaling pathways impacting this disease for development of new strategies to treat diabetes and its complications[6].

Insulin signaling regulates glucose, lipid, and energy homeostasis, predominantly via action on liver, skeletal muscle, and adipose tissue. Precise modulation of this pathway is vital for adaption as the individual moves from the fed to the fasted state. The positive and negative modulators acting on different steps of the signaling pathway ensure a proper and coordinated biological response to insulin in different tissues[7]. Alterations in insulin receptor expression, ligand binding, phosphorylation, and kinase activity affect the downstream of insulin signaling resulting in insulin resistance[8].

Despite the availability of good medical treatment strategies for patients with insulin resistance, the majority of patients are far from achieving the combined target goals for glycemic, blood pressure and lipid levels. It could be argued that our current preventative and therapeutic strategies against this disorder are inadequate[9].

Autophagy is a degradative pathway critical in the maintenance of protein homeostasis (proteostasis) as well as the preservation of proper organelle function by selective removal of damaged organelles. Autophagy occurs constitutively but can also be induced in response to cellular stresses including limitations to various types of nutrients, such as amino acids, growth factors, oxygen, and energy, excessive ROS or DNA damage[10].

In the current study, we identified a novel gene related to autophagy and IR in T2DM; then biomarker' verification was done through Gene Cards Database. Then characterize the expression of coding RNA to evaluate its usefulness as diagnostic biomarker and the relationship between the selected RNA marker and pathological changes of patients.

We have selected m-TOR as a gene highly correlated to T2DM based on bioinformatics' Analysis through more than one data bases to confirm the expression of m-TOR in T2DM and to decrease the false discovery rate through Expression Atlas database (Available at https://www.ebi.ac.uk/gxa/home). And GeneCards Database (Available at http://www.genecards.org/cgi-bin/ carddisp.pl? gene=m-TOR).

AIM OF THE WORK:

To retrieve potential mRNA associated with insulin resistance related autophagy from databases followed by validation of this network in patients' clinical samples.

MATERIAL AND METHODS:

Patients and samples:

123 T2DM patients were enrolled in the current study; they were diagnosed based on American Diabetes Association (ADA) practice guidelines. All blood samples were collected before any therapeutic procedures including surgery, chemotherapy and radiotherapy. Complete follow up data for each patient was available. 106 healthy normal volunteers were recruited during their routine medical checkup.

PBMNCs were isolated from EDTA test tube containing venous blood of both groups, both genders and various ages, using a Lymphoprep™ (Axis-Shield PoC AS,

Oslo, Norway). Blood collected into a tube containing anticoagulant (EDTA) diluted by addition of an equal volume of 0.9% NaCl, 6 ml of the diluted blood was carefully layered over 3 ml Lymphoprep™ in a 15 mm centrifuge tube without mixing then centrifuged at 800 x g for 20 minutes at room temperature (approximately 20°C) in a swing-out rotor. After centrifugation the mononuclear cells formed a distinct band at the sample/medium interface, the plasma was removed with a transfer pipet, the WBCs removed from the interface using a Pasteur pipette and put into a 2 ml tube, the harvested fraction diluted with 0.9% NaCl or medium to reduce the density of the solution and pellet the cells by centrifugation for 10 minutes at 250 x g. From all the participants of this study, written informed consent was obtained, which was performed according to Declaration of Helsinki, and was approved by the Research Ethical Committee at Faculty of Medicine, Ain Shams University, Egypt (ethical approval number; FWA 000017585). Clinical and demographic data of all the participants are summarized in (Table 1).

Bioinformatics' Analysis:

The identification of the mRNA biomarker included two steps: (i) we retrieved a gene related to autophagy and IR in T2DM namely *m-TOR*. The *mRNA m-TOR* gene was down-regulated in T2DM compared to normal, fold change was ≥ 1 and *P*-value < 0.05 according to the data from Expression Atlas database(Available at https://www.ebi.ac.uk/gxa/home); then to enhance the data reliability, we verified the expression of the *mRNA m-TOR* gene in T2DM by Gene Cards Database (Available at http://www.Genecards.org/cgibin/card disp.pl? gene=m-TOR

Total RNA extraction and Reverse Transcription:

Total RNA was extracted from PMNCs samples of all participants using miRNeasy kit (Cat no.217004, Qiagen, USA) according to manufacturer's instructions, the miRNeasy Mini Kit combines phenol/guanidine-based lysis of samples with silica-membrane–based purification of total RNA. QIAzol Lysis Reagent, included in the kit, is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis of tissues or cellular pellets, to inhibit RNases, and also to remove most of the cellular DNA and proteins from the lysate by organic extraction followed by a solid phase extraction procedure on silica columns[11].

The extracted total RNA was reverse transcribed into cDNA as soon as possible with a miScript II RT Kit (Cat nos. 218160, 218161, Qiagen, USA), following the manufacturer protocol using ThermoHybaid PCR express (Thermo Scientific, USA).

Real time-PCR (qPCR) quantification of *mRNA m-TOR*

Expression of *mRNA m-TOR* in samples from T2DM patients were estimated using Quanti Tect SYBR Green PCR Kit (Cat no. 204143, Qiagen, Germany) and QuantiTect Primer Assay (Hs_MTOR_1_SG QuantiTect Primer Assay) (Cat no. QT00056133).

All the PCR primers were taken up from Qiagen, MD. Using Leviak method, relative quantification of mRNA biomarker expression was calculated, where RQ= $2^{-\Delta\Delta Ct}$ method [12]. Hs_GAPDH_1_SG QuantiTect Primer Assay (NM_001002) was used as housekeeping gene to normalize our raw data as the invariant control for the samples, and compared with a reference sample. The PCR program for Applied Biosystems^{Tm} 7500 Real-Time PCR system was as follow: firstly, denaturation at 95°C for 15 min; followed by 45 cycles of denaturation for 15 sec at 94°C; then annealing for 30 sec at 55°C, then extension for 15 sec at 72°C.

Using the Applied Biosystems^{Tm} 7500 Real-Time PCR system (Foster city, California, united States), the threshold

cycle (Ct) value of each sample was calculated. Any Ct value more than 36 was considered negative. The results were analyzed by Data Assist (QIAGEN) Software version 3.2. Amplification plots and Tm values were analyzed to confirm the specificities of the amplicons for SybrGreen-based PCR amplification. All the samples were analyzed in duplicate to confirm the results.

Statistics and Analysis:

All Statistical analysis was performed by Statistical Package for the Social Sciences (SPSS software version 20). Comparisons were performed using Krausakul-Wallis, one-way analysis of variance (ANOVA test), and chi-square test, as appropriate. The receiver operating characteristics (ROC) curve was performed to explore the predictive value of selected *mRNA m-TOR* biomarker for T2DM. The association between expression of *mRNA m-TOR* biomarker and clinicopathological data were assessed with the Spearman rank correlation.

RESULTS:

Description of study population:

There was no significant difference as regard age and sex among the two study groups (p>0.05), details of the clinical data are presented in (Table 1).

Table (1): The Clinicopathological Factors between 2 Groups of the Study.

Demographic data	Type 2 DM N (%)	Healthy N (%)	P	$\chi^{2(a)}$
Mean age:				
≥ 54 yrs (113)	65 (52.8%)	48 (45.3%)	NS	
< 54 yrs (116)	58 (47.2%)	58 (54.7%)	0.254	1.303
Sex:				
Male (87)	47 (38.2%)	40 (37.7%)	NS	0.005
Female (142)	76 (61.8%)	66 (62.3%)	0.941	
Smoking:				
Smoker: (102)	86 (69.9%)	16 (15.1%)		
Non-smoker:(117)	30 (24.4%)	87 (82.1%)	0.000*	76.568
x-smoker: (10)	7 (5.7%)	3 (2.8%)		
Family history of diabetes:				
Positive:(98)	98 (79.7%)	0 (0%)	0.000*	147.636
Negative:(131)	25 (20.3%)	106 (100%)		
Oral Anti Diabetic Medications (OAD):				
Metformin: (44)	44 (35.8%)	0 (0%)		
SU: (24)	24 (19.5%)	0 (0%)	0.000*	105.960
Combined: (12)	12 (9.8%)	0 (0%)		
Not Taking OAD: (149)	43 (35%)	106 (100%)		

(a): Chi- square test, p: NS; not significant (>0.05), **p: is highly significant (< 0.01),
*p: is significant (<0.05).

Expression of *mRNA m-TOR* biomarker among the study groups:

The RQ value of *mRNA m-TOR* biomarker in PMNCs is shown in (Table 2). The median (RQ) were 0.224 and 13.107 for *mRNA m-TOR* in T2DM group and healthy control group respectively. Compared with the non-diabetic group, the diabetic group had a lower expression of *mRNA m-TOR* (p< 0.01). The positivity rate of the *mRNA m-TOR* was 97.6% in the diabetic group. However, it was 23.6% in normal individuals (p< 0.01), as shown in (Table 2).

Table (2): The median (RQ) and the positivity rate of mRNA m-TOR biomarker among the study groups.

	Type 2DM	Healthy Control	P	$U^{2(a)}$
RQ of *mRNA m-TOR gene*	0.224	13.107	.000**	2542
Positivity rate of *mRNA m-TOR gene*	120 (97.6%)	25 (23.6%)	.000 **	134.147

Relation between *m-TOR mRNA expression* and different clinicopathological factors in diabetic group (N=123):

There was no statistical significant difference between fold change (RQ) value of *m-TOR mRNA* and the different clinicopathological factors within the diabetic group (P >0.05) except for the intake of oral antidiabetic drugs which shows significant difference (p<0.05) as shown in (Table 3A). And also there was no statistical significant correlation between fold change (RQ) value of *m-TOR mRNA* and the different laboratory parameters in diabetic group (P >0.05) as shown in (Table 3B).

Table (3A): Relation between m-TOR mRNA expression and different clinicopathological factors in diabetic group (N=123):

`	*m-TOR mRNA*					
Clinicopathological factors	Median	Mean rank	Statistics	No. of patients ≤ 4.19 (%)	P	$\chi^{2\,(c)}$
Mean age:			NS		NS	
≥54 years (65)	0.207	56.11	P=.052	64(53.3%)	.493	.470
<54 years (58)	0.556	68.60	$U^{(a)}$=1502	56(46.7%)		
Sex:			NS		NS	
Male (47)	0.433	63.19	P=.770	46(38.3%)	.860	.031
Female (76)	0.207	61.26	$U^{(a)}$=1730	74(61.7%)		
Smoking:						
Smoker (86)	0.23	62.41	NS	84(70%)	NS	.280
non-smoker (30)	0.19	60.87	P=.979	29(24.2%)	.869	
x-smoker (7)	0.43	61.79	$\chi^{2\,(b)}$=.042	7(5.8%)		
Family history of diabetes :			NS			
Positive (98)	0.26	64.95	P=.069	96(80%)	NS	.321
Negative (25)	0.18	50.44	$U^{(a)}$=936	24(20%)	.571	
Oral Anti Diabetic Medications (OAD):					NS	
Metformin (44)	0.20	58.03	P=.011**	43(35.8%)	.501	2.360
SU (24)	0.18	49.98	$\chi^{2\,(b)}$=	24(20%)		
Metformin + SU (12)	1.40	90.21	11.092	12(9.2%)		
Not Taking OAD (43)	0.26	64.90		43(35%)		
HOMA-IR cutoff			NS		NS	
≥ 2.3	0.209	58.76	P=.026	101(84.2%)	.021	5.342
<2.3	0.450	77.74	$U^{(a)}$=740.5	19(15.8%)		

Table (3B): Correlation of RQ of *mRNA m-TOR gene* with the different laboratory parameters within the diabetic group.

Spearman's rho								
		Duration of Diabetes	Fasting Glucose	Post Prandial Glucose	Fasting Insulin	HbA1c	HOMA_IR	BMI
RQ (*mRNA m-TOR gene*)	Correlation Coefficient	.009	-.085	-.063	.000	.095	-.084	.134
	Sig. (2-tailed)	.922	.352	.491	1.000	.296	.356	.141

Accuracy of parameters for predicting T2DM by ROC analysis:

ROC curve analysis and value of the area under the curve (AUC) was used to evaluate the diagnostic value of the selected *m-TOR mRNA* as shown in Figure (2).

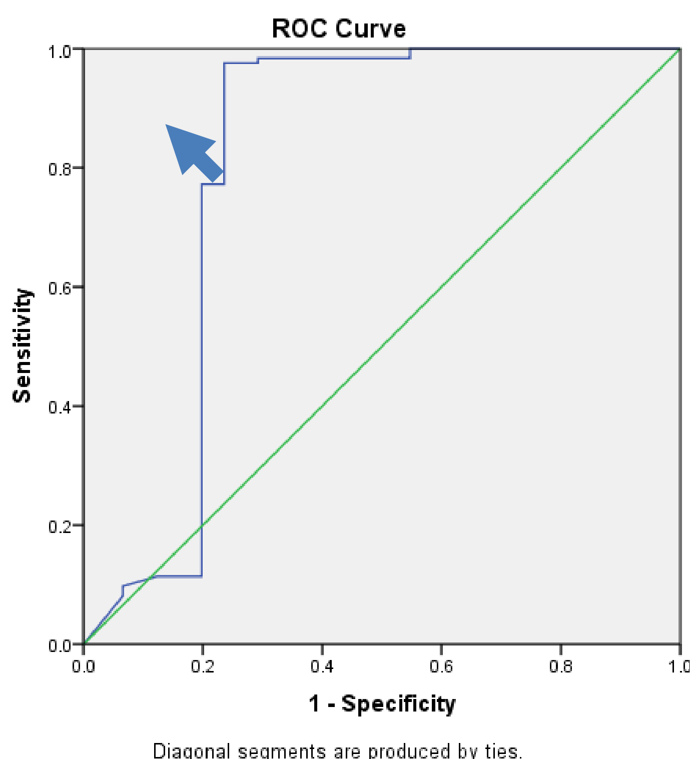

Figure (1): ROC curve analysis for m-TOR mRNA

The analysis of ROC curve for *mRNA m-TOR gene* to calculate the best cutoff point that discriminates between diabetic and non-diabetic groups. Best cutoff point of *mRNA m-TOR gene* is ≤ 4.19 [sensitivity = 97.6% and specificity = 76.4%. Area under the curve (AUC) = 0.805, Accuracy = 87.77, *P*= .000**].

As regard diabetic patients versus healthy control, the best discriminating cutoff value of *mRNA m-TOR* was ≤ 4.19. Accordingly, the sensitivity was 97.6% and specificity was 76.4% which indicated that these threshold values could be used to discriminate diabetic group (T2DM) from non-diabetic group (healthy subjects) as shown in (Table 4).

Table (4): Cutoff values, Sensitivity, Specificity and Accuracy of mRNA m-TOR biomarker.

RQ	AUC	Std. Error	95% CI	Sensitivity	Specificity	ACC.	P	Sig.
m-TOR mRNA Positive if ≤ 4.19	.805	0.036	0.735-0.875	97.6%	76.4%	87.77%	.000**	S

DISCUSSION:

Type 2 diabetes mellitus (T2D) is the predominant form of diabetes which accounts for nearly 90% of all diabetes cases. It is a complex metabolic disease in which concomitant insulin resistance and beta-cell impairment lead to hyperglycemia, which is the hallmark of the disease [12].

Common mechanisms in T2DM include aberrant redox regulation, oxidative stress, and active inflammatory processes resulting in impaired insulin secretion and signaling Mitochondrial dysfunction seen in altered autophagy result in the accumulation of reactive oxygen species (ROS), possibly leading to insulin resistance. Additionally, autophagy is required for the homeostasis of glucose tolerance and β-cell hyperplasia under high-fat diet conditions[13, 14, 15]. It is widely postulated that lipid peroxides modify mitochondrial proteins and critical components of the insulin signaling pathway, leading to impaired stimulation of glucose uptake, mitochondrial damage, and additional oxidative stress, thereby propagating a deleterious cycle[16]. Lipid-associated insulin resistance has also been shown to be linked to GLUT4 translocation defects[17]. Inflammation is a key player in insulin resistance progression and the establishment of type 2 DM (T2DM). In response to inflammatory stimuli, an increased migration and infiltration of macrophages may occur in peripheral tissues, so cells and tissues malfunction results including reductions in insulin sensitivity[18]. As obesity is established and body weight increases with age, a parallel state of chronic inflammation, characterized by an elevation of proinflammatory cytokines, can induce changes and switch the metabolic homeostatic set points, leading to T2DM.

The mammalian target of rapamycin (mTOR), an atypical multidomain serine/ threonine kinase of the phosphoinositide 3-kinase (PI3K)-related kinase family, elicits a significant role in diverse signaling cascades responsive to changes in intracellular and environmental conditions. Activation of mTOR has been implicated in an increasing number of pathological conditions, including cancer, obesity and diabetes, cardiovascular diseases, and neurodegenerative disorders[19].

The present study is the first to report that mRNA m-TOR gene expression was down-regulated in T2DM patients compared with normal healthy control by quantitative real time PCR so mRNA m-TOR could be used as potential novel biomarker in the diagnosis of T2DM.

As regard levels of the mRNA m-TOR gene when compared T2DM patients with healthy controls, there was statistically significant difference between them with lower expression in T2DM patients ($p<0.01$) which indicated that level of mRNA m-TOR gene threshold could be used to discriminate T2DM patients from healthy normal people.

So the findings of this study demonstrated that estimation of mRNA m-TOR level could have a potential diagnostic and screening target in patients with T2DM in the future.

Conclusion

Collectively, this study provides evidences that level of *mRNA m-TOR* may have great clinical value as accurately

promising biomarker in Type 2 DM. As the down-regulation of *mRNA m-TOR* play key roles in the development of T2DM by regulating cell signaling and sensitivity to insulin.

Compliance with Ethical Standards: This study was accomplished in compliance with Ethical Standards.

Conflict of interest: All the authors; Sanaa Eissa, Marwa Matboli, Miram Mohamed and Marwa Mostafa Kamel stated that they have no competing interest.

Ethical approval & informed consent: Written informed consent was taken from all the participants of this study, which was done in accordance with Declaration of Helsinki, and was approved by the Ethics Committee of Faculty of Medicine, Ain Shams University, Egypt (ethical approval number; FWA 000017585).

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الأهمية السريرية للجينات المتعلقة بالاتهام الذاتي ذات الصلة بمقاومة الانسولين في مرض النوع الثاني من البول السكري

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نبذة مختصرة

الخلفية: يلعب الالتهام الذاتي ومقاومة الأنسولين دورًا مهمًا في التسبب في مرض النوع الثاني من البول السكري. هناك حاجة واضحة إلى استراتيجيات دقيقة جديدة للكشف عن التسبب الجزيئي لمقاومة الأنسولين.

الهدف من العمل:الكشف عن الاحماض النووية الريبوزية الناسخة المحتملة المرتبطة بالالتهام الذاتي ذات الصلة بمقاومة الأنسولين من قواعد البيانات تليها التحقق من صحة هذه الشبكة في العينات السريرية للمرضى.

المواد والطرق: تم إجراء تحليل المعلوماتية الحيوية للكشف عن الجينات المتعلقة بالالتهام الذاتي في مرض النوع الثاني من البول السكري ، ثم تم التحقق من العلامات البيولوجية من خلال قاعدة بيانات اخرى ثم استخراج الحمض النووي الريبوزي الكلي من خلايا الدم البيضاء ذات النواه تليها تفاعل البلمرة المتسلسل

و النسخ العكسي ، ثم تفاعل البلمرة المتسلسل في الوقت الحقيقي الكمي للحمض النووي الريبوزي m-TOR في خلايا الدم البيضاء ذات النواه من ١٢٣ مريضا مع مرض النوع الثاني من البول السكري و ١٠٦ من المتطوعين العاديين الأصحاء.

النتائج: كان لـm-TOR حساسية عالية وخصوصية لمرضى النوع الثاني من البول السكري متكافئة مع الضوابط الصحية.

الخلاصة: يمكن أن يكون الحمض النووي الريبوزي m-TOR بمثابة علامة حيوية محتملة لتشخيص مرض النوع الثاني من البول السكري .

النقاط الرئيسية: تم الكشف عن الحمض النووي الريبوزي m-TOR كجين جديد محتمل و مرتبط بمقاومة الانسولين و الالتهام الذاتي ذات الدقة العالية والحساسية للكشف المبكر عن النوع الثاني من البول السكري.