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International Journal of Pharmaceutical Research & Allied Sciences, 2018, 7(1):15-24

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

MiR-216a in Diabetic Nephropathy: Relation with Autophagy and Apoptosis

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ABSTRACT

Background: Diabetic nephropathy (DN) is one of chronic diabetes complications and a major impulse of end-stage renal disease. However, the pathogenesis of DN is not well understood. Recently, several miRNAs were believed to contribute in the pathogenesis of DN while others revealed Reno-protective effects. Therefore, understanding the mechanisms by which miRNAs affect DN may provide a perfect prospect in the treatment of this disease. Objectives: To explore the possible impact of miR-216a in DN and to find out its relation with 2 important regulatory pathways in the cell; autophagy and apoptosis. Methods: This study was conducted on 60 subjects divided into three groups; 20 type 2 diabetic patients with nephropathy (DN), 20 type 2 diabetic patients without complications (T2DM) and 20 healthy controls. The miR-216a, light chain 3 (LC3) and caspase-3 serum gene expressions were assessed by quantitative real-time polymerase chain reaction (QRT-PCR). Results: Relative serum gene expressions of miR-216a and Caspase-3 were significantly higher by 32.99 and 32.11 folds, respectively in DN patients compared to T2DMgroup ($p \leq 0.001$) while relative serum gene expression of LC3 was significantly lower in DN compared to T2DM group($p \leq 0.001$). The diagnostic performance of miR-216a, caspase-3 and LC3 serum gene expressions showed efficiency of (91.7%, 88.3% and 83.3%), respectively. In the meantime, serum gene expression assessment of miR-216a and caspase-3 together revealed efficiency of 100% for recognition of DNpatients. Conclusions: Increased serum gene expression levels of miR-216a, caspase-3 and reduced that of LC3 collaborate in the pathogenesis of DN. Besides, dual assessment of miR-216a and caspase-3 gives powerful discrimination of DN patients.

Keywords: *Diabetic nephropathy; MiR-216a; Autophagy; LC3; Apoptosis; Caspase-3*

INTRODUCTION

The prevalence of diabetic nephropathy (DN) is increasing in a way threatening more diabetic patients worldwide [1]. Hence, studying the mechanisms underlying DN would result in better understanding and in turn could provide an avenue to inhibit or at least postpone DN.

Research on miRNAs has become a trouble spot because of their crucial role in modulating posttranscriptional levels of protein-coding genes leading to either mRNA degradation or inhibition of translation that may be implicated in many diseases pathogenesis [2].

Autophagy is a wise brilliant strategy to protect cells against prospective damage that is initiated by growth of signal deficiency, nutrient deprivation (e.g., lack of glucose or amino acids), genotoxic stress (e.g. DNA damage), hypoxic stress, endoplasmic reticulum (ER) stress, and/or reactive oxygen species (ROS) accumulation [3].The full mechanism of autophagy was described by Yoshinori Ohsumi caught Nobel Prize in Physiology and Medicine in 2016 for his discoveries in one of the most powerful quality-control pathways in cells [4]. Autophagy usually depicts the

degradation of cytoplasmic components inside lysosomes [5]. Simply, a part of cytoplasm, including organelles, is surrounded by an isolation membrane to form an autophagosome that subsequently merges with the lysosome; then the internal material is degraded and reused. Thus, autophagy is essential for constitutive turnover of cytoplasmic components [6]. Several molecular components contribute in autophagosome formation. These include UNC-51-like kinase (ULK) complex; phosphatidylinositol-3 kinase (PI3K) complex; two ubiquitin-like protein conjugation systems, they are ATG12 conjugation system and the microtubule-associated protein light chain 3 (LC3) conjugation system; in addition ubiquitinated proteins that selectively deliver these proteins to the autophagosome need a cargo receptor known as p62 [7]. Moreover, two transmembrane proteins, vacuole membrane protein 1 (VMP1) and autophagy-related gene 9 (ATG9) are also needed for autophagosome formation [8].

Apoptosis is the process of programmed cell death that occurs in multicellular organisms [9]. It occurs normally during development and aging and as a defense mechanism such as in immune reactions or when cells are deteriorated by disease or detrimental factors [10] forming cell fragments called apoptotic bodies then phagocytic cells can engulf them before the contents of the cell can spill out onto surrounding cells and cause damage to the neighboring cells [11]. As apoptosis cannot stop once it has begun, it is a perfectly regulated process [9, 12]. It can be launched through one of two pathways: intrinsic and extrinsic. In the intrinsic pathway, the cell kills itself when it senses cell stress; while in the extrinsic pathway, the cell kills itself as a result of signals from other cells [13, 14].

Defective apoptotic processes have been entangled in a wide assortment of diseases. Extravagant apoptosis causes atrophy, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer [15]. The key enzymes in apoptosis are caspases, which are proteases, expressed as inactive proenzymes in most cells and once activated, they can activate other procaspases authorizing initiation of apoptosis cascade. Ten major caspases have been identified and categorized into: initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) [16, 17] in addition to other caspases (caspase-11,-12,-13,-14) [18; 19, 20; 21].

In this work, we focused on the link between serum miR-216a gene expression and DN through exploring how miR-216a might bridge the crosstalk between two important types of cell deaths; autophagy and apoptosis by detecting LC3and caspase-3 serum gene expression levels.

SUBJECTS AND METHODS

Study population:

This study is a case control study that was conducted on sixty subjects. Forty type 2 diabetic patients were recruited from the outpatient clinic of Al-zahraa Hospital, Cairo, Egypt from January 2017 to April 2017. They were diagnosed clinically before sample collection and categorization into two groups; twenty DN patients (stage 2) and twenty T2DM patients without any complications. Diabetes was defined as a fasting blood glucose (FBG) $\geq$ 126 mg/dl [22]. Glycated haemoglobin (HbA1c) was measured by HPLC to indicate glycemic control. Nephropathy was determined based on clinician diagnosis, serum creatinine, estimated glomerular filtration rate (eGFR) and urinary albumin concentration where microalbuminuria is diagnosed when albumin is between 30–299 mg/day [23]. Twenty healthy volunteers (sex and age matched with the patient groups) were taken as a control group. A written consent was obtained from all participants. Patients with history of tumors, hepatic disease, cardiac disease or any metabolic disorders that may affect the studied biochemical parameters were excluded.

All individuals were subjected to full clinical examination, assessment of FBG and serum creatinine was done using fully automated chemistry analyzer (Cobas Integra 400 plus).HbA1c was estimated by HPLC. Urinary albumin was assessed then eGFR was calculated according to Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [24]. Also, QRT-PCR was performed for serum gene expression of miR-216a, LC3, and caspase-3.

Sample collection:

Eight ml of blood were withdrawn from every participant under complete aseptic conditions after an overnight fasting (8-12 hours) divided in three vacutainer tubes. Two ml in a fluoride coated tube used for determination of fasting blood glucose. Another two ml were collected in EDTA tube for glycated haemoglobin assay on fresh whole blood.

The remaining part of blood sample was left to clot at room temperature, then serum was separated by centrifugation at 3000 rpm for 15 minutes then divided into aliquots and stored at -80℃ until estimation of serological markers; creatinine and gene expression of miR-216a, LC3 and caspase-3. Moreover, 24 hours' urine samples were collected from each subject for the determination of urinary albumin by turbid metric method.

Quantitative real-time polymerase chain reaction for serum gene expression of miR-216a, LC3 and caspase-3:

Total RNA was extracted from frozen serum samples using RNeasy Mini kit (Qiagen, Germany; Cat. No. 217184) according to instructions of manufacture. The isolated total RNA (2µg) was reverse transcribed into cDNA using miScriptII Reverse Transcription Kit (Qiagen, Germany; Cat No. 218161). QRT-PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA) in 20 µl reaction mixture consisting of 10 µlSYBR® Green PCR Master Mix (Qiagen, Germany; Cat. No. 204141), 1 µl forward primer (nM), 1 µl reverse primer (nM), 3 µlcDNA, and 5 µlRNase free water with performing the following thermal cycling conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The sequences of PCR primer pairs utilized for each gene are shown in table 1. All values of miR-216a were normalized to the RU6 gene as an invariant endogenous control (housekeeping gene) while, GAPDH gene was used as a reference gene for normalization of values of LC3 and caspase-3. Relative expression of the studied genes was calculated according to Applied Bio system software.

Table 1: Primers Sequences of the studied genes:

Genes	Primers sequence	Gene bank accession number
miR-216a	Forward primer: 5′-GTTGGCTTAATCTCAGCTGGC-3′ Reverse primer: 5′-GCATAATCCCAGAGACCACTGT-3′	NR_049437.1
LC3	Forward primer: 5′-CAACAGTGCAGGGGAGCTTA-3′ Reverse primer: 5′-AATGCCAGCACTGCATACCT-3′	XM_005073109.3
Caspase-3	Forward primer 5′-TTGGAACGGTACGCGAAGAA-3′ Reverse primer -3′TAGTAACCGGGTGCGGTAGA5′-	XM_006253130.3

Statistical methods:

IBM SPSS statistics (V. 24.0, IBM Corp., USA, 2016) was used for data analysis. Data were displayed as median and percentiles for quantitative non-parametric measures, in addition to number and percentage for categorized data.

The following tests were performed:

Comparison between two independent groups for non-parametric data using Wilcoxon Rank Sum test.

Comparison between more than 2 groups for non-parametric data using Kruskall Wallis test.

Ranked Sperman correlation test to study the possible association between each two variables among each group for non-parametric data. The probability of error≤0.05 was considered significant while ≤0.01 and 0.001 were considered highly significant.

Diagnostic validity test: the receiver operating characteristics (ROC) curve was constructed to obtain the most sensitive and specific cutoff for each biochemical marker. To evaluate the most discriminating markers between the compared groups, area under the curve (AUC) also was calculated.

RESULTS

The Demographic data, clinical characters and biochemical parameters of all subjects are presented in Table 1. No significant variations were found between the studied groups in age and sex (P>0.05). Regarding the clinical characteristics, there was a significant difference during the disease on comparing the patient groups with each other

(P<0.05). In addition, there was a significant difference regarding the studied biochemical parameters (FBG, HbA1c, serum creatinine, and urinary albumin) between the studied groups. Furthermore, there was significant up-regulation of serum miR-216a and Caspase-3 gene expressions in DN group compared to both T2DM and control groups. Parallel to that, there was significant down-regulation of serum LC3 gene expression in DN group compared to both T2DM and control groups.

Table 2: Demographic, clinical and biochemical parameters of the studied groups

Variables	DN group n = 20	T2DM group n = 20	Control group n = 20	p-value
Age (year)	55.5 (54 – 57)	58.5 (55 – 61)	57 (54 – 59)	P1> 0.05 P2> 0.05 P3> 0.05
Sex Male (n) (%) Female (n) (%)	 14 (70 %) 6 (30 %)	 11 (55 %) 9 (45 %)	 12 (60 %) 8 (40 %)	 P1> 0.05 P2> 0.05 P3> 0.05
Duration (year)	8.5 (6 - 10)b	6.5 (5 - 8)	-	P3< 0.05
FBG (mg/dl)	180.5a,b (174.25 – 186)	165 b (150 -172.25)	86.5 (78.25 – 93.75)	P1<0.001 P2< 0.001 P3<0.001
HbA1c (%)	8a,b (7.8 – 8.2)	7.6 b (7.4 – 7.9)	4.94 (4.76 - 5.2)	P1<0.001 P2< 0.001 P3<0.05
Serum Creatinine (mg/dl)	1.1a,b (1.02 – 1.2)	0.88 (0.655 - 0.9675)	0.83 (0.7 - 0.9)	P1< 0.05 P2>0.05 P3<0.05
Urinary albumin (mg/day)	162.5a,b (102 – 256)	19.35b (14.87-21.975)	8.35 (5.725 - 9.65)	P1<0.001 P2< 0.001 P3<0.001
eGFR	60a,b (54.5 – 69.5)	94.5 (77.25 – 103.75)	95.5 (91.5 – 98)	P1<0.001 P2>0.05 P3<0.001
MiR-216a (RQ)	32.99a,b (17.52 - 58.04)	7.43b (3.94-10.03)	1.03 (0.89-1.31)	P1<0.001 P2< 0.001 P3<0.001
Caspase-3 (RQ)	32.12a,b (15.54 - 49.19)	9.77b (5.17-13.53)	1.26 (0.91 - 1.61)	P1<0.001 P2< 0.001 P3<0.001
LC3 (RQ)	0.19a,b (0.13 - 0.51)	0.61b (0.44 - 0.77)	1.05 (0.99 - 1.46)	P1<0.001 P2< 0.001 P3<0.001

P1: Significance between DN group and control group.
P2: Significance between T2DM group and control group.
P3: Significance between DN group and T2DM group.
a: Significant difference from control group while b: Significant difference from T2DM group.

By studying the correlation between fold expression of miR-216a, caspase-3, LC3 and the other assessed parameters in DN group, there was a significant positive correlation between microalbuminuria and each of miR-216a and caspase-3 serum expression levels (r = 0.714 and 0.570, respectively at P<0.05) while, there was a significant negative correlation between serum LC3 gene expression and microalbuminuria (r = - 0.690 at P<0.01); Table 3 and Figures (1, 2, and 3), respectively.

Table 3: Correlation between fold expression of MiR-216a, Caspase-3, LC3 and the other studied variables in DN group

Variables	MiR-216a fold expression		Caspase-3 fold expression		LC3 fold expression	
	r	P value	r	P value	r	P value
Age (year)	0.1	0.676	0.247	0.294	-0.293	0.21
Duration (year)	0.008	0.975	0.028	0.907	- 0.349	0.132
FBG (mg/dl)	0.077	0.747	0.08	0.738	-0.633	0.003
HbA1c (%)	0.1	0.675	0.037	0.875	-0.544	0.013
Serum Creatinine (mg/dl)	0.18	0.461	0.21	0.373	- 0.097	0.686
Urinary albumin (mg/day)	0.714	0.002**	0.570	0.030*	-0.690	0.008**
eGFR	0.044	0.855	0.019	0.937	-0.462	0.04
MiR-216a (RQ)	-	-	0.205	0.387	- 0.002	0.995
Caspase-3 (RQ)	0.205	0.387	-	-	-0.17	0.474
LC3 (RQ)	- 0.002	0.995	-0.17	0.474	-	-

P <0.05(*), P <0.01(**)

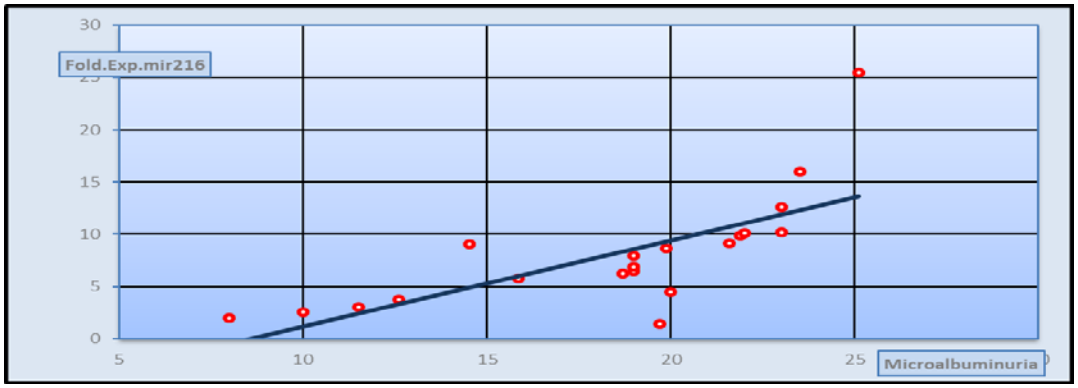

Figure 1: Correlation between fold expressions of miR-216a and microalbuminuria in DN group

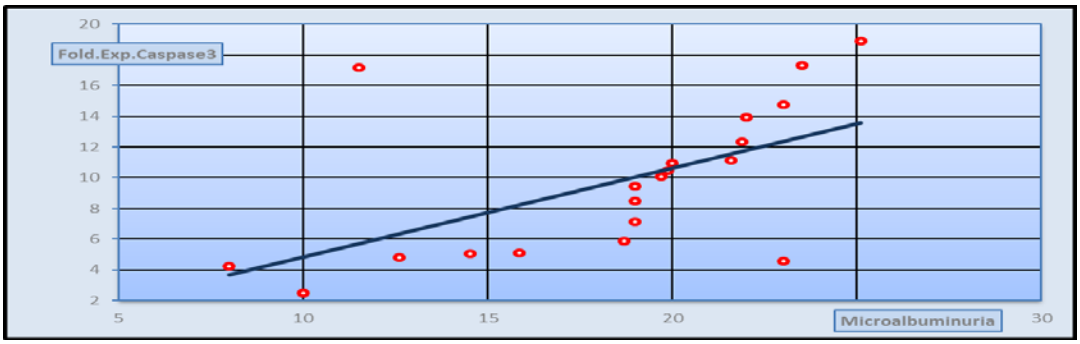

Figure 2: Correlation between fold expression of caspase-3 and microalbuminuria in DN group

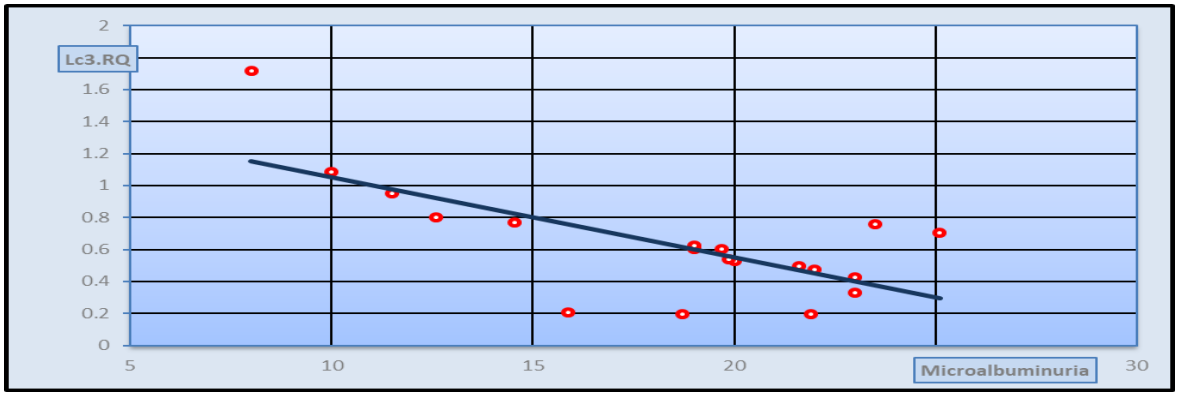

Figure 3: Correlation between fold expression of LC3 and microalbuminuria in DN group

Table (4) represents the output data of ROC curve (figure 4) for each of serum miR-216a, caspase-3 and LC3 gene expressions. Regarding cut off value, it was (12.63, 14.75 and 0.173) for miR-216a, caspase-3 and LC3, respectively. The specificity and sensitivity of miR-216a, caspase-3 and LC3 were (95% and 85%), (92.5% and 80%) and (100% and 50%) with AUC of (0.919, 0.923 and 0.834) and efficiency of (91.7%, 88.3% and 83.3%), respectively. In addition, multi-ROC analysis showed that both miR-216a and caspase-3 revealed sensitivity, specificity and efficiency of 100%, severally at cut off value of 4.798 for caspase-3.

Table 4: The discriminative power of serum miR-216a, Caspase-3 and LC3 between DN patients and T2DM patients

The studied biochemical parameter	Cutoff	AUC	%Specificity	%Sensitivity	%Efficiency
RQ of serum miR-216a	12.63	0.919	95	85	91.7
RQ of serum caspase-3	14.75	0.923	92.5	80.0	88.3
RQ of serum LC3	0.173	0.834	100.0	50.0	83.3
Multi-ROC: RQ of Serum miR-216a at 12.63					
RQ of serum caspase-3	4.798	1	100.0	100.0	100.0

DISCUSSION:

Diabetic nephropathy has become a major cause of renal failure and even death worldwide; however, there is no proven biomarker that can detect its early stages. Detection of microalbuminuria was considered the ideal screening test for DN early stages; however, it has some drawbacks. One of the remarkable drawbacks is that micro albuminuria develops when advanced changes have already set in [25].

The role of micro RNAs in DN pathogenesis was highlighted [26]. MiR-216a was found to be expressed in the pancreatic tissue and was used in early detection of pancreatic ductal adenocarcinoma as it was notably down regulated in pancreatic cancer through down-regulation of JAK2 mRNA levels [27, 28].

The expression of miR-216a in pancreatic tissue makes us ask ourselves if it is possibly related with DM. In the present work, we studied the relation of serum miR-216a gene expression with DN and T2DM patients without complications and found that it is significantly upregulated in DN group than T2DM and control groups at $p<0.001$. Also, there was a significant positive correlation between serum miR-216a gene expression and microalbuminuria in DN group at $p<0.01$.These results indicate an efficient role of miR-216a in the pathogenesis of DN and this point of view was in accordance with Liu et al. [29] who explained the relation between miR-216a and DN by the role of Aktkinase as a mediator between both. They reported that miR-216a plays a role in the activation of Aktkinase, a regulatory component of diabetic nephropathy, which in turn down-regulates phosphatase and tensin homologue (PTEN). Surprisingly, overexpression of PTEN was observed in early stages of DN [29; 30].

In the current study, we assessed serum caspase-3 gene expression in the studied groups as one of the executioner caspases in apoptotic pathway [18]. We found significant up-regulation of serum caspase-3 gene expression in DN group compared to T2DM and control groups at $p<0.001$. Moreover, there was a significant positive correlation between serum caspase-3 gene expression and micro albuminuria in DN group ($p<0.05$) reflecting the connection between renal affection and apoptosis. These findings were consistent with Habib [31] who reported that apoptosis contributes to the development of DN. Hyperglycemia , in tubular cells, triggers free radicals and oxidative stress generation that consequently, causes apoptosis [32] through activation of a network of intracellular signaling pathways including the phosphatidylinositol 3 kinase (PI3k)/(AKT) signaling pathway [33]. In the same line, Bălăşescu et al. [34] explained this relation by the activation of MAPk pathway by diabetes that in turn leads to a loss of normal intracellular signaling. This leads to renal remodeling through accumulation of extracellular matrix, expansion of basement membrane of glomeruli, glomerulosclerosis, tubular cell hypertrophy, fibrosis and hence apoptosis.

In the present study, we assessed serum LC3 gene expression, a sensitive marker of autophagy, as it is ubiquitin-like conjugation system implicated in autophagosome formation and vesicle nucleation [7, 35]. We found a significant down-regulation of serum LC3 gene expression in DN patients compared to T2DM and control groups at $p<0.001$. Besides, there was a significant negative correlation between serum LC3 gene expression and microalbuminuria in DN group ($p<0.01$). Hence, these results shed the light on a substantial role of autophagy in repairing and maintaining kidney function. These results were in agreement with Ding and Choi (36) and Matboli et al. [37] who stated that the impairment of autophagy is nested with DN pathogenesis. They claimed that targeting the autophagic pathway to activate and restore autophagy activity might be Reno-protective. Wang and Choi [38] reported that autophagy regulates considerable critical aspects of normal and disease cases in the kidney. In addition, several pre-clinical studies indicated autophagy impairment at the early stage of diabetic kidney disease in experimental animals. Moreover, evidence of impaired autophagy was bserved in kidney biopsy samples from patients with T2DM exhibiting accumulation of p62 protein in proximal tubular cells suggesting that autophagy impairment also occurs in human T2DM [39, 2013].The link between autophagy impairment and DN can be understood by studying two researches done by Zoncu et al. [40] and Gödel et al. [41] . Zoncu et al. [40] reported that the mechanistic target of rapamycin (mTOR) is the classical nutrient-sensing pathway controlling gautophagic activity through its association with two protein complexes, mTORC1 and mTORC2. In general, mTORC1 is a negative regulator of autophagy by inhibiting the activity of Ulk1 complex through direct phosphorylation. Normally, nutrient starvation stimulates autophagy primarily through inhibition of mTORC1. Unfortunately, mTORC1 activity was found to be enhanced in type 2 DN in both human and experimental animals [41].

In the current study, there was a significant decrease of LC3 serum gene expression and a significant increase of caspase-3 serum gene expression in T2DM patients than control group and the same was observed in DN patients compared to T2DM patients at $p<0.001$ together with a significant increase of FBG and related HbA1cin T2DM patients compared to control group and the same was noticed in DN patients compared to T2DM patients at $p<0.001$. All these results elucidate that autophagy impairment is related to poor glycemic control and accompanied by stimulation of apoptosis. Tanaka et al. [42] agreed that hyperglycemic conditions might impair autophagic machinery. Recently, Yang et al. [43] reported that defects in autophagy contribute in the etiology of numerous diseases, including diabetes mellitus, cancer, neurodegeneration, infectious diseases and aging. The mechanism underlying the relation of DM with autophagy and apoptosis was discussed by Bachar-Wikstrom et al. [44] who explained it by endoplasmic reticulum (ER) stress. Glucotoxicity, lipotoxicity, chronic inflammation and increased oxidative stress that occur as a result of DM; all can induce improper protein folding and thereby accumulation of these proteins could cause β-cell ER stress and apoptosis while autophagy is impaired. They found that induction of autophagy alleviates stress and prevents apoptosis improving the condition. This contradiction between autophagy and apoptosis was found to be caused by Bcl-2, which is involved in the crosstalk between both mechanisms, as it is essential for vesicle nucleation in autophagy pathway [35]. Moreover, it is shown to prevent the release of cytochrome C from mitochondria and hence prevents the activation of caspases preventing apoptosis [14].

In order to evaluate the diagnostic performance of serum miR-216a, Caspase-3 and LC3 gene expressions, ROC curves were carried out for each of them and we found that LC3 (specificity 100%) is the most specific but the least sensitive (sensitivity 50%), while miR-216a and caspase-3 have analogical specificity and sensitivity (95%, 92.5% and 85%, 80%), respectively, at the best cut off values; (12.63) for miR-216a, (14.75) for caspase-3 and (0.173) for LC3.

Consequently, we assessed the diagnostic performance of both serum miR-216a and Caspase-3 gene expressions and manifested that those markers together give 100% specificity and 100% sensitivity at the cut off values (12.63 and 4.798), respectively. Hence, assessing both of them is more efficient in identifying type 2 diabetic nephropathy patients than assessing each of them separately.

CONCLUSION:

Overall, miR-216a has a potential role in the pathogenesis of diabetic nephropathy revealed by increased its serum gene expression. Moreover, autophagy and apoptosis are believed to be two contradicted mechanisms involved in the pathogenesis of diabetic nephropathy taking into consideration altered serum gene expression of both LC3 and caspase-3. On top of that and according to our study population, estimation of serum gene expression of miR-216a and caspase-3 together is more worthy in diagnosing type 2 diabetic nephropathy patients than evaluating each of those three biochemical markers individually.

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