

Original Research Article

Oxidized-LDL, Toll-like receptor 4, Lipocalin2 and Omentin1: Role in diabetic nephropathy

ABSTRACT

Background: Diabetic nephropathy is the major micro-vascular complication of type 2 diabetes mellitus (T2DM) and is the main cause for end-stage kidney disease. In view of metabolic derangements of T2DM, we went further to investigate the role played by oxidized low density lipoprotein (ox-LDL), toll like receptor 4 (TLR4), lipocalin-2 (LCN2), and omentin-1 in DN. Patients and methods: 15 normo-albuminuria T2DM, 15 micro-albuminuria T2DM and 15 macro-albuminuria T2DM in addition to 15 healthy volunteer who served as control group were enrolled in this study. Demographic and clinical data were recorded. Plasma Ox-LDL, omentin- 1 and urinary LCN2 levels by immunoassay and TLR4 mRNA level with real time PCR were assessed. Results: *TLR4* gene expression, Plasma ox-LDL, urinary LCN2 levels were increased in T2DM cases as compared to their allied control group with the higher values were for macro-albuminuria T2DM cases. Meanwhile Plasma omentin-1 level was decreased in T2DM cases when compared to their allied control group with least values were for macro-albuminuria T2DM cases. Also there were positive correlations between TLR4 mRNA, ox-LDL, omentin 1 level, urinary LCN2 levels and serum creatinine, fasting blood glucose, urinary albumin/creatinine ratio. Conclusions: Ox-LDL, TLR4, LCN2 and omentin 1 may confer a relevant role in diabetic nephropathy development and progression.

Key words: Diabetic nephropathy; toll like receptor 4; oxidized low density lipoprotein; lipocalin2, omentin 1.

1. INTRODUCTION

Diabetes mellitus (DM) is the third serious chronic metabolic disease after cardiovascular disease and cancer which is characterized by hyperglycemia, the main precipitating factor for micro and macro-vascular complications [1]. Diabetic nephropathy (DN) is one of the micro-vascular diabetic complications and is the main cause of end-stage renal disease (ESRD). Metabolic abnormalities, systemic inflammation and oxidative stress associated with type 2 DM (T2DM) are the main player in the pathophysiological processes of DN and are facilitated by innate immune responses [2]. Renal infiltration with monocyte, macrophages and T lymphocytes in addition to intrinsic renal cells (endothelial, mesangial, glomerular, and tubular epithelial cells) are the main sources of cytokines mediating inflammation and renal injury in DM [3]. Diagnostic marker to detect DN at early stage is important as early intervention can slow the loss of kidney function and reduce adverse outcomes [4].

Toll-like receptors (TLRs) are transmembrane pattern recognition receptors that recognize conserved pathogen-associated molecular patterns to initiate an inflammatory response essential for host defence. TLRs also interact with endogenous ligands, such as oxidized- low density lipoprotein (ox-LDL), heat-

shock proteins [5]. Lipocalin 2 (LCN2) is a member of the lipocalin family covalently associated with matrix metalloproteinase 9 (MMP-9) and is expressed by renal tubular cells, hepatocytes and cells of the innate immune system such as polymorphonuclear neutrophils (PMN) and macrophages. LCN2 has many roles as MMP-9 transport and activation, induction of apoptosis, and regulation of the immune response [6]. Omentin-1 is an anti-inflammatory adipokine primarily expressed in stromal vascular cells and adipocytes of visceral adipose tissue. Omentin-1 has important roles in cellular energy homeostasis, increases insulin signal transduction, regulation of lipid metabolism, involved in stress responses, expression of heat shock proteins, T-cell differentiation and apoptosis [7,8]. As DN represents one of the most devastating outcomes during the progression of T2DM, early detection strategies to diagnose loss of renal function would be of essential to improve quality of life. Therefore, we investigated molecular mechanism through ox-LDL, TLR4 and LCN2 axis in addition to omentin1 level that may have role in pathogenesis and diagnosis of DN. Also we went further to determine urinary LCN2 to evaluate its role as an early noninvasive biomarker of renal damage during the course of DM.

2. PATIENTS AND METHODS

2.1. Participants and study design

All patients have given their informed consent, and the study was institutionally approved by the Research Ethical Committee of Faculty of Medicine, Tanta University, Egypt. This current study included forty five type 2 diabetic patients who were selected from the Outpatient Clinic, Internal Medicine Department, Tanta University Hospitals through the period from April 2015 to October 2015 and were diagnosed according to the 1999 World Health Organization (WHO) criteria [9]; 35 of them were receiving oral hypoglycaemic agents and 10 were receiving insulin. Diabetic cases were subdivided into three groups according to urinary albumin/creatinine ratio (UACR) which was measured by early morning spot urine sample into 3 groups [10]: normo-albuminuria T2DM cases (n=15; 10 female and 5 male) with urinary albumin to creatinine ratio (UACR) < 30 mg albumin/g creatinine; micro-albuminuria T2DM cases (n=15; 11 females and 4 males) with UACR = 30-299 mg/g creatinine and macro-albuminuria T2DM cases (n=15; 10 females and 5 males) with UACR more than 299 mg/g creatinine in addition to 15 age and sex matched apparently healthy individuals without any systemic diseases as diabetes, hypertension, cardiovascular disease, or renal insufficiency and they will be served as healthy controls (group I). Subjects with acute illness, liver dysfunction, heart failure, malignancy, history of cerebrovascular or cardiovascular disorders and other renal disorders were excluded from the study. All subjects enrolled in this study were subjected to full clinical history taking, clinical examination and calculation of BMI (weight in kilograms divided by the square of the height in meters (kg/m²).

2.2. Sampling

Overnight fasting blood samples were obtained from each participant on sterile EDTA treated and plain tubes for serum and plasma separation. Serum, plasma and EDTA treated blood were stored at -80°C till analysis. First morning mid-stream urine samples were collected, centrifuged and the supernatant was stored at -80°C for further analysis.

2.3. Biochemical and immunoassays

Serum lipid profile including triacylglycerol (TG) (TAG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were assayed by enzymatic-colorimetric method using commercial assay kits (Biodiagnostic., Egypt). Fasting blood glucose (FBS) was detected by oxidase method using commercial assay kit (Biodiagnostic., Egypt). Serum/urinary creatinine and urea levels were estimated using commercial kits (Diamond Diagnostic, Egypt). Glycated haemoglobin (HbA1c) was measured using quantitative colorimetric measurement of glycohemoglobin as percent of total hemoglobin using commercial kits (STANBIO, San Antonio, Texas, USA). Urinary albumin was measured using commercial kit (BioSystems Company, Spain). Enzyme-linked immunosorbent assay (ELISA) was used to detect plasma ox-LDL [M1] level (OxiSelect™, Cell Biolab Inc., STA-369, CA, USA), Urinary LCN2 level (R&D systems, Minneapolis, MN, USA) and plasma omentin 1 level (Avisera Bioscience, Inc., USA) using ELISA Reader (Star fax 2001)

2.4. Molecular assesement

RNA extraction, cDNA synthesis and real-time PCR for TLR4 gene expression

Total RNA was extracted from EDTA peripheral blood using Magna pure Compact Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the protocol provided by the manufacturer. Total RNA was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first strand using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Real-time PCR was carried out with cDNAs. PCR reactions were performed using Roche Light Cycler Fast Start DNA Master^{PLUS} SYBR Green I kits (Roche Diagnostics) following the manufacturer's instructions. TLR mRNA transcripts were quantified, relative to the housekeeping gene, glyceraldehyde-3-phosphatedehydrogenase (GAPDH) which was used as an internal control. Sequence specific primers were designed by Primer3 software: (<http://bioinfo.ut.ee/primer3/>) as follows: *TLR4* (NM_138557.2) forward, (5'-TGTCTCCCACTCCAGGTAAGT-3'), reverse, (5'-GATTGCTCAGACCTGGCAGTT-3'); GAPDH (No: NM_001289746.1): forward primer (5'-AGTGCCAGCCTCGTCTCATAG-3'), and reverse primer (5'-CGTTGAACTTGCCGTGGGTAG-3'). The final results were automatically calculated from the cross-point values of the target and the reference gene by Light Cycler 4 Relative Quantification Software (Roche Diagnostics).

Statistical analysis

Statistical analysis was conducted as mean and standard deviation using Statistical Package for Social Sciences (SPSS), version 16.0 for Windows (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) was used for multiple comparisons to evaluate the statistical significance between experimental groups followed by post hoc test. The correlation study was calculated using Pearson's correlation. *P* value < 0.05 was considered significant.

3. RESULTS

3.1. Clinical and metabolic characteristics

Demographic variables and clinical characteristics of the studied groups are shown in Table 1. There was no significant difference in age, body mass index between the studied groups. Meanwhile, there were statistically significant increase in disease duration; **TG TAG**, TC, FBG levels and HbA1C percentage in diabetic cases when compared to their allied control group with higher values were for macro-albuminuria T2DM group. LDL-C and HDL-C levels showed statistically significant difference in diabetic cases when compared to their allied control group but with no difference between micro and macro albuminuria T2DM groups. Serum urea, creatinine and UACR were statistically significantly higher in T2DM cases when compared to their allied control group and normo-albuminuria T2DM groups with higher values were for macro-albuminuria T2DM group (**Table 1**).

Table 1. Demographic characteristics and routine biochemical findings in studied groups.[M2]

Parameters/Groups		Control group (n=15) ^a	Normo- albuminuria T2DM (n=15) ^b	Micro- albuminuria T2DM (n=15) ^c	Macro- albuminuria T2DM (n=15) ^d
Age(years)		53.75±5.58	52.35±3.88	51.6±4.17	51.65±4.19
Sex	Male (%)	6(40%)	5 (33.3%)	6(40%)	4(26.66%)
	Female (%)	9(60%)	10(66.6%)	9(60%)	11(73.3%)
Duration (years)		-	7.25±1.77 ^{cd}	12.97±2.02 ^{bd}	16.03±1.07 ^{bc}
BMI (kg/m ²)		25.3±1.49	25.75±1.59	24.9±1.58	25.35±1.53
FBS level(mg/dl)		88.15±3.7 ^{bcd}	149.15±8.36 ^{acd}	174.19±18.86 ^{abd}	283.15±37.6 ^{abc}
Hb A1c%		4.4±0.24 ^{bcd}	6.6±0.47 ^{acd}	7.9±0.24 ^{abd}	8.7±0.29 ^{abc}
Systolic blood pressure (mmHg)		110.6 ± 5.5 ^{bcd}	125.2 ± 3.2 ^{acd}	127.6 ± 4.1 ^{abd}	136.6 ± 2.6 ^{abc}
Diastolic blood pressure (mmHg)		71.8 ± 3.7 ^{bcd}	75.45± 1.57 ^{ad}	81.55± 2.1 ^{ad}	87.65± 1.8 ^{abc}
Serum urea level (mg/dl)		23.35±2.08 ^{bcd}	25.1±2.67 ^{acd}	45.25±14.42 ^{abd}	63.35±7.03 ^{abc}
Serum creatinine level (mg/dl)		0.746±0.143 ^{cd}	0.78±0.1 5.13 ^{cd}	0.838±0.26 ^{abd}	1.13±0.47 ^{abc}
UACR (mg/g)		18.75±2.97 ^{cd}	19.6±2.51 ^{cd}	133.57±63.5 ^{abd}	389.31±53.5 ^{abc}
TC level (mg/dl)		142.35±5.85 ^{bcd}	192.85±18.42 ^{acd}	277.42±13.77 ^{abd}	301.05±17.47 ^{abc}
TG TAG level (mg/dl)		137.75±8.65 ^{bcd}	173.5±23.79 ^{acd}	198.19±12.9 ^{abd}	229.37±38.59 ^{abc}
LDL-C level (mg/dl)		98.65±5.8 ^{bcd}	110.4±18.1 ^{acd}	162.76±9.94 ^{ab}	164.95±10.61 ^{ab}
HDL-C level (mg/dl)		66.83±1.357 ^{bcd}	62.5±6.98 ^{acd}	44.52±1.7 ^{ab}	44.09±1.9 ^{ab}

Data presented as mean ± SD; n number of cases; BMI Body mass index ; FBS fasting blood sugar; HbA1c: Glycated hemoglobin; UACR urinary albumin/creatinine ratio ;TC total cholesterol; **TG TAG** triacylglycerol; LDL-C low-density lipoprotein cholesterol; HDL-C high-density lipoprotein cholesterol; . * Significant difference. **P Value** was calculated by one-way ANOVA test followed by Tukey's post hoc test. Identical superscript letters indicate nonsignificant differences while different superscript ones show statistically significant results. **P Value** was considered significant at 0.05.

3.2. Immune/ inflammatory status and tubular damage

This current study revealed statistically significant increase in plasma ox-LDL level and up-regulation of TLR4 expression in T2DM cases when compared to their allied control group with higher values were for macro-albuminuria T2DM group. Meanwhile, plasma omentin1 level was statistically significantly decreased in T2DM cases than their allied controls with lower values were for macro-albuminuria T2DM

group. This current study revealed statistically significant increase in urinary LCN2 level in T2DM cases than their allied control group with higher values were observed in macro-albuminuria T2DM group (Table 2).

Table 2. Biochemical and molecular findings of the studied groups.^[M3]

Parameters/Groups	Control group (n=15) ^a	Diabetic cases with normo-albuminuria (n=15) ^b	DN with micro-albuminuria (n=15) ^c	DN with macro-albuminuria (n=15) ^d
Urinary LCN2 level (pg/l)	34.35±3.28 ^{bcd}	46.35±6.47 ^{acd}	59.76±11.54 ^{abd}	74.73±2.68 ^{abc}
Plasma omentin-1 level (ng/ml)	26.05±1.47 ^{bcd}	22.67±.94 ^{acd}	19.25±.94 ^{abd}	15.37±1.73 ^{abc}
Plasma ox-LDL level (ng/ml)	19.65±2.2 ^{bcd}	33.9±5.5 ^{acd}	64.05±12.18 ^{abd}	117.78±31.11 ^{abc}
Relative TLR4 mRNA	0.37±0.02 ^{bcd}	0.83±0.07 ^{acd}	1.03±0.12 ^{abd}	1.3±0.17 ^{abc}

Data presented as means ±SD; n number of cases; Ox-LDL oxidized low-density lipoprotein; TLR4 toll like receptor4; LCN2 lipocalin 2. * Significant difference. P Value was calculated by one-way ANOVA test followed by Tukey's post hoc test. Identical superscript letters indicate nonsignificant differences while different superscript ones show statistically significant results. P Value was considered significant at 0.05.

3.3. Correlation matrix

All correlations are summarized in Tables 3. In T2DM cases, FBG, serum creatinine, disease duration, UACR were correlated positively with ox-LDL, TLR4 mRNA, LCN2 levels but negatively with plasma omentin-1 level. LCN2 level showed positive correlation with TLR4 mRNA, ox-LDL levels, meanwhile, it showed a negative correlation with omentin-1 level (Table 3).

Table 3. Correlations matrix of glycemic status, creatinine, urinary albumin creatinin ratio, lipocalin 2 in relation to plasma ox-LDL, peripheral blood TLR4 mRNA and plasma omentin-1 levels

		Fasting blood sugar (mg/dl)	Serum creatinine (mg/dl)	Urinary albumin creatinin ratio	Disease duration (years)	Urinary LCN2 level (pg/ml)
Plasma Ox-LDL level (ng/ml)	r	.454**	.491**	.932**	.806**	.844**
	p	.004	.003	.0032	.0051	.0041
Plasma omentin1 level (ng/ml)	r	-.699**	-.515**	-.861**	-.927**	-.848**
	p	.0041	.0002	.0001	.0003	.0004
Relative TLR4 mRNA level	r	.647**	.519**	.890**	.900**	.851**
	p	.0042	.0034	.0025	.0044	.00016
Urinary LCN2 (pg/ml)	r	.482**	.423**	.864**	.833**	-
	p	.0002	.00012	.0003	.00024	-

Values are Pearson rank correlation coefficients. Bold font marks significance at P < 0.05.

4. DISCUSSION

Diabetic nephropathy (DN) is one of the leading causes of chronic kidney diseases worldwide and most of the affected patients have T2DM. A considerable number of patients newly diagnosed with T2DM may already have developed nephropathy due to a preceding period of undiagnosed diabetes and impaired glucose tolerance [11]. According to the classification of American Diabetes Association, DN is divided

into three stages, incipient nephropathy (micro-albuminuria), clinical diabetic nephropathy (macro-albuminuria) and ESRD. To diagnose those DN patients in its early stage can effectively prevent or delay the progression to ESRD [12]. Renal biopsy is a useful way to diagnose DN but is an invasive method so we are in need to simple, accurate and non-invasive test for early diagnosis and/or monitoring DN progression [13]. Several mechanisms, including hyperglycemia, advanced glycation end products, oxidative stress, inflammation are the main contributors to DN pathogenesis through activation of nuclear factor κ B (NF- κ B) signaling which is the key regulator of inflammation and apoptosis [14].

TLRs have been implicated in diabetes-induced inflammation and vascular complications. TLRs recognize various damage-associated molecular patterns such as heat-shock proteins, fatty acids, ox-LDLs and AGEs. Activation of the immuno-inflammatory reactions through TLR4 was supposed to play a role in the pathogenesis of DN [15]. This current study revealed up-regulation of TLR4 in diabetic patients when compared to control with higher expression was in macro-albuminuria diabetic cases; also it was correlated positively to serum creatinin, disease duration, UACR. These results indicate that up-regulation of TLR4 expression might contribute to the pathogenesis, diagnosis and progression of DN. Lin et al., 2012 found that silencing of TLR4 gene expression with small interfering RNAs (siRNA) or anti-TLR4 antibodies attenuated high-glucose-induced NF- κ B activation [16]. Devaraj et al., 2010 reported up-regulation in TLR4 expression in type 1 diabetes mellitus patients (T1DM) with micro-vascular complications compared without micro-vascular complications T1DM and may contribute to increased inflammation and subsequent T1DM complications [17].

Ox-LDL causes endothelial injury, atherosclerosis, enhancing albumin excretion and can induce ischemia, stress and damage to cultured renal tubular cells with further development of advanced nephropathy [18]. The present study revealed increased s ox-LDL level in micro and macro albuminuria diabetic cases than control and normo-albuminuria diabetic cases with higher value were for macro-albuminuria cases. These findings in addition to the up-regulation of TLR4 expression may suggest a role played by ox-LDL in pathogenesis, progression of DN through activation TLR4. Moreover, ox-LDL was correlated positively with serum creatinine and disease duration revealing its role not only in pathogenesis but also in diagnosis of DN and its progression. Geng et al, 2010 reported up-regulation of TLR4 expression in response to ox-LDL and the inhibition of TLR4 expression by small interference RNA(siRNA) caused decrease in NF- κ B activity and monocyte chemoattractant protein 1 (MCP-1) and interleukin 8 (IL-8) secretion in response to ox-LDL so atherogenic effects of ox-LDL could be mediated in part via the TLR4 pathway [19]. Proximal tubules are particularly susceptible to injury associated with diabetes and tubular damage may occur before glomerular damage causing appearance of several tubular proteins in urine which may occurs before the appearance of microalbuminuria [20]. So that it is important to find new predictive biomarkers of tubular damage.

LCN2 is one of proteins that expressed abundantly in ischemic or nephrotoxic kidney injury and serum lipocalin-2 has been described as a sensitive and specific biomarker for early identification of acute kidney

injury as a result of cardiac surgery [21]. LCN2 binds to its receptor and transports small lipophilic substances, such as retinoids, arachidonic acid, steroids, iron, and fatty acids in addition it has roles in induction of apoptosis, the inhibition of bacterial growth, regulation of iron metabolism, and insulin resistance. LCN2 is induced by inflammatory stimuli through the activation of the NF- κ B pathway [22]. This current study revealed increased urinary LCN2 level in T2DM cases than controls with more increase were observed in macro-albuminuria T2DM. Increased levels of urinary LCN2 were already found in diabetic patients without early signs of glomerular damage in the form of normo-albuminuria, demonstrating usefulness of urinary LCN2 as a marker of early DN. LCN 2 was correlated positively to serum creatinine, disease duration, UACR. The significant increase in macro-albuminuria T2DM cases may indicate that urinary LCN2 level may be a useful biomarker for reflecting the severity of renal damage caused by diabetic disease. These results may be due to impaired tubular reabsorption as a result of specific saturation of the endocytic pathway during heavy proteinuria or proximal tubules dysfunction [23].

These results indicate that LCN2 might contribute to early diagnosis of DN and its progression. These results were in agreement with those obtained previously [24, 25]. Wu et al., 2015 reported that high glucose caused activation of TLR4/NF- κ B p65/ LCN2 signaling pathways and promoted the downstream fibrosis and inflammatory reaction in cultured mesangial cells (MCs) [26]. Flo et al., 2004 demonstrate that injection of mice with the TLR4 ligand lipopolysaccharide induces increased expression of LCN2 in a TLR4-dependent manner [27]. So that from the results obtained from the present study; oxidized lipids through acting as ligand and up-regulator for TLR4 expression with subsequent increased LCN2 production can cause renal damage in diabetic patients.

Omentin-1 is an anti-inflammatory adipokine with important role in cellular energy homeostasis, vascular tone regulation, stress responses, T-cell differentiation and apoptosis [28]. Tan et al., 2010 reported that omentin-1 can attenuate C-reactive protein (CRP) and TNF- α -induced NF- κ B activation in human endothelial cells suggesting that omentin-1 might be an anti-inflammatory adipokine in humans [29]. However, little information is available with respect to the associations of serum omentin levels and diabetic microvascular complications. This current study revealed decreased omentin 1 level in diabetic cases than their allied control group with lowest levels were observed in macro-albuminuria T2DM cases, also it was correlated negatively with serum creatinine, disease duration, UACR. These results indicate that omentin 1 might contribute to the pathogenesis, diagnosis and progression of DN. Tekce et al., 2014 suggested that DM is associated with low omentin 1 levels in chronic kidney disease population [30]. Zhang et al., 2014 suggested that decreased omentin-1 level may contribute to the development of insulin resistance and T2DM [31].

5. CONCLUSION

The synergistic increase of ox-LDL, TLR4, LCN2 and decreased omentin-1 level may mediate DN pathogenesis and progression in addition to the novel proposed role of LCN2 as a non-invasive biomarker for early detection of DN. The results of this study may open the door for support further diagnostic and

therapeutic approaches that ensure modification of metabolic and immune-modulatory status to prevent premature micro-vascular complications in diabetic patients.

9. REFERENCES

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