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Association of TLR4 expression and genomic instability with renal function markers in diabetic nephropathy

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Abstract. ***Background.*** *Diabetic nephropathy is a serious side effect of both type 1 and 2 diabetes mellitus. Toll-like receptor 4 (TLR4), the first identified and most extensively studied member of the TLR family, has been implicated in the development of various renal diseases such as acute kidney injury, renal ischemia-reperfusion injury, and glomerulonephritis. The purpose of this study was to assess the expression of TLR4 in relation to inflammation in diabetic patients with and without renal failure, to discuss the role of these receptors in the development of diabetic nephropathy, and to highlight chromosomal, nuclear, and biochemical changes (urea and creatinine) in patients with renal failure and diabetes.* ***Materials and methods.*** *The dialysis unit housed 40 healthy controls, 40 patients with diabetic mellitus, 40 with nephropathy, and 40 with diabetic nephropathy. This study was conducted from October 2024 to January 2025. Blood samples (5 ml) were collected from patients and healthy individuals and distributed into tubes for gene expression, chromosomal aberration, and micronucleus frequency, the remaining — to evaluate the renal function.* ***Results.*** *Participants with nephropathy and diabetic nephropathy have significantly higher TLR4 gene expression in their blood than healthy individuals. Patients with diabetes, nephropathy, or diabetic nephropathy were found to have more micronuclei and chromosomal aberrations. Significantly increased serum urea and creatinine levels have also been observed in patients with nephropathy and diabetic nephropathy.* ***Conclusions.*** *TLR4 expression increases: the percentage is lowest in healthy people (3.927), higher in diabetic (18.31), and nephropathy patients (17.352), and highest in those with diabetic nephropathy (27.158). Chromosomal abnormalities are associated with diabetic nephropathy and impaired renal function.*

Keywords: *diabetic nephropathy; TLR4 gene expression; genetic biomarkers renal function; dialysis*

Introduction

Diabetic nephropathy (DN) is one of the most serious and prevalent side effects of DM. This is associated with higher rates of death and morbidity in patients [1, 2]. After 15 years of illness, less than half of patients acquire true nephropathy, while 20–30 % of individuals develop microalbuminuria [3]. Today, diabetes mellitus is a serious health problem that has gotten out of control. Globally, diabetes affects more than half a billion people, with type 2 diabetes being the most common. One of the top ten causes of death worldwide in 2019 was DM, which was estimated to be responsible for 6.7 million deaths worldwide in 2021 [4, 5]. Insulin is involved in an anabolic pathway. Hormones that have cardinal roles in glucose homeostasis, cell growth, and metabolism [6]. TLR4 is essential for chronic kidney disease (CKD) and infection-related renal disease disorders such as pyelonephritis caused by UTIs [7]. TLR4 plays a pivotal role in activating the inflammatory response via the NF-κB pathway, leading to intense production of inflammatory cytokines and free radicals. This chronic oxidative environment, resulting from TLR4 activation, contributes to DNA damage, manifested by abnormal sister chromatid exchange, micronuclei, and chromatid breaks. DNA can be degraded by reactive oxygen species (ROS), such as oxygen (O), OH-, and (HO) [8, 9]. Concurrently, this inflammatory process and oxidative stress lead to progressive deterioration of renal function, reflected in elevated urea and creatinine levels. Furthermore, renal failure increases the accumulation of free radicals and metabolic toxins, exacerbating oxidative stress and genetic damage and thereby forming

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a vicious cycle [10]. In addition, it is evident that diabetic patients frequently have an impaired DNA repair mechanism and diminished antioxidant capacity. Damage to mitochondrial DNA caused by free radicals produced in the mitochondria results in organelle malfunction [11].

The purpose of this study was to assess the expression of Toll-like receptors (TLR4) in relation to inflammation in diabetic patients with and without renal failure, discuss the role of these receptors in the development of diabetic nephropathy (DNP), and highlight chromosomal, nuclear, and biochemical changes (urea and creatinine) in patients with renal failure and diabetes.

Materials and methods

Ethical approval

This study was approved by the Ethical Committee of the Department of Biology Sciences, University of Thi-Qar (no. 66, date: 10/10/2024).

Study design and setting

In this prospective study, 2 ml of blood from patients admitted to the dialysis unit of Imam Hussein Hospital and the consulting laboratories was placed in an EDTA tube to extract gene expression and measure chromosomal aberrations and micronucleus frequency. Blood (3 ml) were placed in a gel tube to assess renal function. Out of the total 160 individuals, 40 healthy control group, 40 cases with diabetes mellitus, 40 cases with nephropathy, and 40 cases with diabetic nephropathy (Fig. 1).

Molecular study

RNA isolation from blood

Total blood RNA was extracted using the TRIzol kit. Transcribed cDNA was synthesized using an RT PreMix kit (Bioneer, Korea). The total RNA concentration was determined using nanodrops. Integrity requirements were met by the extracted RNA at a ratio of approximately 2.0 (280/260A). Approximately 10 L of total RNA concentrate was used for cDNA synthesis [12].

Quantification of TLR4

The Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) and Accu Power® Greenstar qPCR PreMix reagent kit (Bioneer, Korea) were used for qRT-PCR analysis. According to Zhang et al., 2024 [13], the SYBR Green-based qRT-PCR PreMix reagent kit was designed to quantify PCR amplification copy numbers in relation to genomic DNA qRT-PCR standard curve copy numbers and to amplify cDNA for target genes (TLR4 gene) using its primers (F = 5'-ATATTGACAGGAAACCCCATCCA-3' and R = 5'-AGAGAGATTGAGTAGGGGCATTT-3') at 300bp and the GapdH Housekeeping gene (F = 5'-GAGCCACATCGCTCAGACAC-3' and R = 5'-CATGTAGTTGAGGTCAATGAAGG-3' at 150bp).The kit's Green dye is a DNA-binding dye that reacts with fresh copies of the target and housekeeping gene's amplification-specific section. Fluorescent signals were captured using an RT-PCR thermocycler.

The ΔCT method with a reference gene was used as the following equations:

$\Delta CT\ (test) = CT\ (target, test) - CT\ (ref, test)$.

$\Delta\Delta CT = \Delta CT\ (test) - \Delta CT\ (calibrator)$.

Fold change $= 2^{-\Delta\Delta CT}$.

Ratio (reference/target) $= 2^{CT\ (reference) - CT\ (target)}$.

Therefore, relative expression was divided by the expression value of the chosen calibrator for each expression ratio of the test sample.

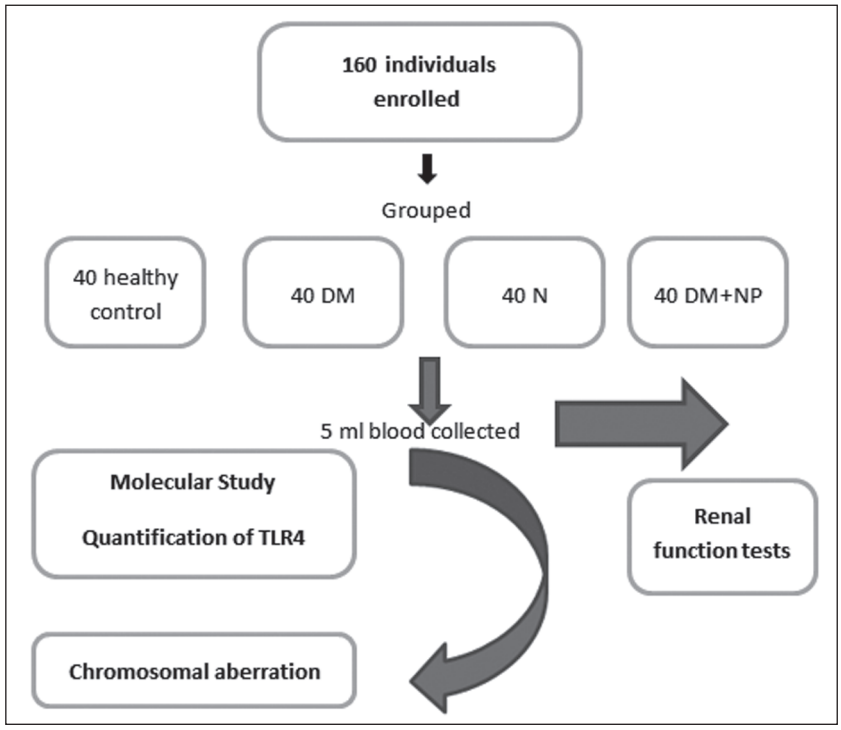

Figure 1. Flowchart of the study design

Cytogenetic examinations

Chromosomal aberration assay

Blood culture 16 drops of blood were added to each tube containing five milliliters of the (RPMI 1640) medium during the sterile process of blood transplantation in the research laboratory of the College of Science at the University of Thi-Qar. The contents of each tube were thoroughly mixed before being added to a previously prepared culture (0.3 ml of PHA), tightly sealed, and incubated for 72 h at 37 °C in an inclined position. The tubes were gently shaken at least twice every 24 h. The cells were prepared using the method described in [14].

Micronuclei examination

Al-Sabti (1986) determined the number of micronuclei [15]. After drying the blood smear on a glass slide, methanol was used to fix it for ten minutes, and Giemsa stain was used to dye it for 30 min. The prepared slides were examined at 40×. The number of micronuclei was calculated in 1000 cells [16].

Biochemical tests

Using a ready-made analysis kit from the French-origin Biolabo, the concentrations of a number of biochemical indicators in the groups under study were determined, including the assessment of creatinine and urea concentrations in blood serum [17]. Chromatography and absorption measurements are the foundations of this technique. An optical spectrometer was used for optical purposes [18].

Statistical analysis

The Statistical Package for the Social Sciences (SPSS, IBM version 20.0) was used to analyze the data using one-way ANOVA. The findings are displayed as mean ± standard error (SE). Statistical significance was set than 0.05, it was deemed statistically significant [19].

Results

Molecular study

The distribution of TLR4 expression between patient and healthy groups, as shown in Fig. 2. It was noted that the gene expression (TLR4) in nephropathy and diabetic nephropathy was the most prevalent among all groups and showed a significant increase ($P \leq 0.05$) compared to other groups, with the percentage in healthy people was (3.927), diabetic patients (18.31), and nephropathy (17.352), while diabetic nephropathy was (27.158), which was the most prevalent among the groups, and the healthy group was the least prevalent.

Cytogenetic examinations

Chromosomal aberration

In Table 1, it is noted that there is an increase in the rate of chromosomal aberrations in patients with diabetes mellitus and diabetic nephropathy compared to the healthy group.

Micronucleus frequency

In Table 2, it is noted that there is an increase in micronucleus frequency in patients with diabetes mellitus and diabetic nephropathy compared to the healthy group.

Renal function parameters

Urea levels

As can be seen in Table 3, urea had significantly higher values ($P \leq 0.05$) in diabetic nephropathy patients and nephropathy patients compared to diabetic patients and the healthy group. In addition, the overall means of the samples confirmed that the nephropathy and diabetic nephropathy groups had significantly higher urea levels than the healthy and diabetic groups, indicating that these conditions have an effect on kidney function.

Creatinine level

The two groups differed significantly ($P < 0.05$), as shown in Table 4. Nephropathy patients had the highest significant creatinine levels ($P \leq 0.05$) among the other groups, while the control group had the lowest. Nephropathy and diabetic nephropathy patients had significantly higher creatinine levels than control and DM patients. This suggests that nephropathy affects kidney function.

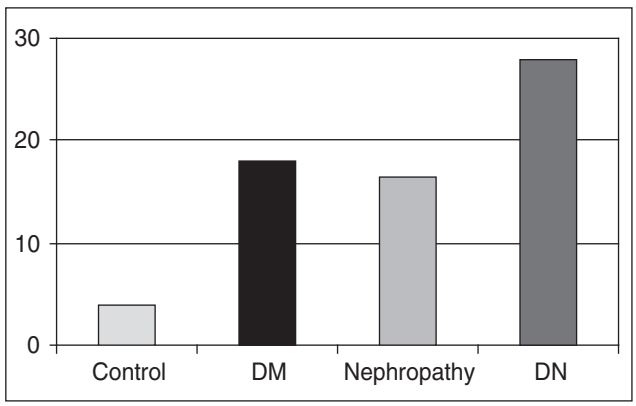

Figure 2. Relative mRNA expression of TLR4

Discussion

TLR4 gene expression is significantly higher in patients with nephropathy and diabetic nephropathy compared with the other groups and healthy controls. These results are consistent with those of earlier investigations [20]. TLR4 protein levels in the glomeruli and tubule interstitium were considerable higher in patients with macroalbuminuria and overt DN than in healthy disease-free controls.

This form of nephropathy may be facilitated by increased TLR4 activation. TLR4 has emerged as a critical mediator in the pathogenesis of diabetic nephropathy (DN) and its expression is significantly upregulated in renal tissues. Chronic hyperglycemia plays a pivotal role in this process by promoting the accumulation of advanced glycation end-products (AGEs) and enhancing oxidative stress, both of which serve as potent inducers of TLR4 expression through the activation of the NF-κB signaling cascade. Cel-

Table 1. Chromosomal aberration rates (CA/100 cells) in the blood of healthy control, DM, nephropathy and diabetic nephropathy patients

Groups	Mean ± SD
Control	0.02 ± 0.00 b
Diabetes mellitus	0.09 ± 0.04 a
Nephropathy	0.08 ± 0.02 a
Diabetic nephropathy	0.10 ± 0.02 a
LSD	0.01

Note (here and in Table 2): SD — standard deviation.

Table 2. Micronucleus frequency in the blood of healthy, DM, nephropathy and diabetic nephropathy patients

Groups	Mean	SD	SE
Healthy	3.9500 c	2.80978	0.44427
Diabetes mellitus	10.075 b	3.88546	0.61435
Nephropathy	11.025 ab	3.40051	0.53767
Diabetic nephropathy	15.050 a	2.41735	0.38222
LSD	4.975		

Table 3. Serum urea levels (mg/dL) in control group, diabetes mellitus, nephropathy and diabetic nephropathy patients

Control	Diabetes mellitus	Nephropathy	Diabetic nephropathy	LSD
29.11 ± 1.21 aD	49.60 ± 1.67 cC	112.00 ± 6.95 cB	120.27 ± 9.72 cA	5.21

Notes (here and in Table 4): different capital letters denote significant differences (P ≤ 0.05) between groups; different lowercase letters indicate significant differences (P ≤ 0.05) between the age groups of the same group.

Table 4. Serum creatinine levels (mg/dl) in control group, diabetes mellitus, nephropathy, and diabetic nephropathy patients across different age categories

Control	Diabetes mellitus	Nephropathy	Diabetic nephropathy	LSD
3.36 ± 0.40	3.57 ± 0.25 aB	9.94 ± 0.61 bA	8.50 ± 1.09 aA	1.012

lular injury within the diabetic kidney leads to the release of damage-associated molecular patterns (DAMPs), including high-mobility group box 1 (HMGB1), fibrinogen, and heat shock proteins, which are known ligands for TLR4. These ligands further amplify inflammatory signaling independent of microbial infection. Innate immunity may play a role in the onset and progression of diabetic nephropathy (DN), even though DN has long been thought to be a nonimmune disease [20, 21]. Aly et al. (2020) found that TLR4 expression is higher in diabetic patients with renal failure than in non-diabetic individuals. This was associated with lower HOMA-IS values, which indicate insulin sensitivity, and higher HOMA-IR values, which indicate insulin resistance. Meanwhile, the serum levels of inflammatory cytokines such as TNF-α, IL-6, and IFN- are greater in diabetic individuals with end-stage renal disease (ESRD) [22]. These correlations show how TLR4 contributes to the development of inflammation and insulin resistance, which can cause kidney disease to worsen and eventually result in end-stage renal disease (ESRD) and renal failure [23]. Yang et al. (2014) argued that TLR4 activation may not be a primary causal event, but rather a downstream effect of the broader metabolic disturbances characteristic of diabetes, including oxidative stress, chronic hyperglycemia, and the accumulation of advanced glycation end-products (AGEs). According to this view, upregulation of TLR4 reflects an already activated inflammatory milieu rather than an initiating factor. Such interpretations suggest that TLR4 may serve as an amplifier of renal injury rather than a root cause [24, 25].

Diabetic nephropathy (DN) is influenced by various genetic factors. The development of DN is significantly influenced by genetic and structural variations, as well as epigenetic modifications. In addition to the nuclear genome, mitochondrial DNA (mtDNA) is essential for controlling DN development [26]. Several studies have demonstrated the presence of chromosomal damage and micronuclei in patients with diabetes and diabetic nephropathy. Micronuclei and chromosomal aberrations were more common in diabetic nephropathy and diabetes patients than in the control group in the current study. These results are in line with those of previous studies [27, 28]. Chronically high levels of inflammation are associated with poor blood lipid profile, insulin resistance, obesity, and insufficient blood glucose management. Chronic inflammation increases oxidative stress, which can lead to oxidative DNA damage and further enhance genomic instability by increasing reactive oxygen and nitrogen species (RONS) and decreasing antioxidant defense. Damaged chromosomes can cause mutations, impair cellular proliferation, and cause malfunction in cells, tissues, and organs [29, 30]. These effects may be sufficient to exacerbate chromosomal damage that occurs during mitosis, resulting in the production of micronuclei [31]. Quintero et al. (2018) analyzed chromosomal stability in patients with type 1 and type 2 diabetes compared to the control group and observed a significant increase in nuclear abnormalities compared to the healthy control group [32]. A defect in the genetic material (DNA) that results in a break in the double strand causes chromosomal abnormalities. The primary defect may be in the single or double strands of the DNA. Cross-links may occur between DNA molecules, such as between pentose sugars and the phosphate group in the i-DNA strand [33]. These errors can be identified and corrected by using DNA repair systems. However, chromosomal aberrations or genetic mutations can occur if they are not repaired or are repaired incorrectly. Most published investigations using the CBMN cytome test demonstrated that patients had lower blood glucose control, greater medication use, more chromosomal abnormalities than controls, and worse DNA stability as the disease advanced [34, 35]. The frequency of chromosomal damage in PBMCs from patients with type 2 diabetes was examined by Müllner et al. and compared to controls without the disease. Those with an MNi frequency over the 50th percentile, however, may have noticeably higher fasting plasma glucose and glycated hemoglobin levels [36, 37].

Patients with nephropathy and diabetic nephropathy exhibit elevated serum urea and creatinine levels. Patients with nephropathy and diabetic nephropathy had significantly elevated urea and creatinine levels, and these findings are in line with those of other researchers [38–40] due to impaired renal function. This elevation is primarily attributed to a reduction in the glomerular filtration rate (GFR), which leads to accumulation of nitrogenous waste products in the bloodstream. Urea, a by-product of protein metabolism, and creatinine, derived from muscle metabolism, are key biomarkers commonly used to assess renal function and monitor

the progression of kidney impairment [41, 42]. According to previous research, blood urea and serum creatinine measurements are readily accessible tests that can evaluate the early detection and prevention of diabetic nephropathy [43]. This investigation supports the findings of Al-Musawi et al., who reported that elevated blood glucose levels in uncontrolled diabetics are linked to increased urea and creatinine levels, which are often linked to the degree of renal damage [44]. Increased urea production, decreased urea elimination, or a combination of the two can result in an excess of urea in the plasma; the highest concentrations occur when chronic renal disease and the corresponding marked decline in glomerular filtration rate cause decreased urea removal in the urine [45]. A high blood creatinine level induced by OTA is a serious indicator of renal damage and impairment [46]. Given the critical role the kidney plays in converting citrulline to arginine, Cao et al., hypothesized that elevated serum citrulline levels in individuals with diabetic nephropathy may be linked to worsening of this function [47]. Additionally, citrulline participates in the citrulline-NO cycle, which produces nitric oxide (NO). It is well recognized that nitric oxide plays a significant role in controlling kidney morphology and function [47].

Conclusions

Elevated TLR4 expression increases, with the percentage in healthy people is least (3.927), diabetic patients (18.31), and nephropathy (17.352), while diabetic nephropathy is the highest (27.158). Chromosomal aberration rates (CA/100 cells) in the blood of healthy control (0.02) are lower than in diabetic nephropathy patients (0.1). Diabetic nephropathy causes impaired renal function.

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Зв'язок експресії TLR4 та геномної нестабільності з маркерами функції нирок при діабетичній нефропатії

Резюме. Актуальність. Діабетична нефропатія є серйозним побічним ефектом цукрового діабету 1-го та 2-го типу. Toll-подібний рецептор 4 (TLR4), перший ідентифікований та найбільш ретельно вивчений член родини TLR, був пов'язаний із розвитком різних захворювань нирок, як-от гостре пошкодження нирок, ішемічно-реперфузійне ураження нирок та гломерулонефрит. **Мета:** оцінити експресію TLR4 у зв'язку із запаленням у пацієнтів із діабетом із нирковою недостатністю та без неї, обговорити роль цих рецепторів у розвитку діабетичної нефропатії та виділити хромосомні, ядерні та біохімічні зміни (сечовина та креатинін) у пацієнтів із нирковою недостатністю та діабетом. **Матеріали та методи.** У відділенні діалізу перебувало 40 здорових осіб контрольної групи, 40 пацієнтів із цукровим діабетом, 40 із нефропатією та 40 із діабетичною нефропатією. Це дослідження проводилося з жовтня 2024 року по січень 2025 року. Зразки крові (5 мл) були зібрані в пацієнтів та здорових осіб і розподілені по пробірках для визначення експресії генів, хромосомних аберацій та частоти мікроядер, решта — для оцінки функції нирок. **Результати.** В учасників із нефропатією та діабетичною нефропатією експресія гена TLR4 у крові значно вища, ніж у здорових осіб. У пацієнтів із діабетом, нефропатією або діабетичною нефропатією було виявлено більше мікроядер та хромосомних аберацій. Значно підвищений рівень сечовини й креатиніну в сироватці крові також спостерігався у хворих із нефропатією та діабетичною нефропатією. **Висновки.** Експресія TLR4 зростає, причому відсоток у здорових людей найменший (3,927), вищий — у пацієнтів із діабетом (18,31) та нефропатією (17,352), а при діабетичній нефропатії — найвищий (27,158). Хромосомні аномалії пов'язані з діабетичною нефропатією та порушенням функції нирок.

Ключові слова: діабетична нефропатія; експресія гена TLR4; генетичні біомаркери функції нирок; діаліз