

Expression of Toll – Like Receptor (TLR4) Gene in Type 2 Diabetic Normal Albuminuria, Micro Albuminuria and Macro Albuminuria Patients in Najaf Governorate – Iraq

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ABSTRACT

TLR-4 receptor plays an important role in the pathogenesis of many inflammatory diseases like diabetes mellitus. The present study was designed to examine TLR-4 gene expression in Type 2 diabetes. Gene expression of TLR-4 the mean \pm SD of Δ Ct (normalization Ct values) of each study group: The mean \pm SD Ct value of TLR-4 cDNA amplification was (22.28 \pm 0.36) Normo Albuminuria group, Microalbuminuria group was 20.28 \pm 0.41). The Ct values of Macro Albuminuria group 21.51 \pm 0.31). There was a significant difference in the mean Ct values between different groups, ($p=0.0001$). The mean Ct values of control (Normo Albuminuria) group were higher than the Macro and this in turn was higher than that of Micro groups the mean \pm SD of Δ Ct (normalization Ct values) of each study group, for the Micro Albuminuria (3.04 \pm 2.5). For Macro Albuminuria group the mA significant difference was noticed between the study groups

($p=0.001$). Results of 2- Δ Ct revealed significantly higher results for the Micro Albuminuria group from the other two groups ($p=0.0001$). 2- Δ Ct for micro Albuminuria group (0.121). In the Macro Albuminuria group and Normo Albuminuria group a 2- Δ Ct were (0.051), (0.029) respectively. There is a significantly increased expression of TLR-4 gene in macro and Micro Albuminuria groups as compared to Normoalbuminuria group.

Keyword: Diabetes, nephropathy, TLR-4

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INTRODUCTION

The activated inbred immunity and inflammation attract to the pathogenesis of DN has been proved recently [1]. Inflammation plays an important role in the activation of fibroblast process then tissue fibrosis which most of DN substantial and distinguishing properties. So, inflammatory processing which is stimulated by the various physiological, metabolic, and hemodynamic agents' evidence to be critically contributed to the expansion and advancement of DN [2]. Moreover, enhancement for the contribution of inflammation to diabetic renal damage which comes from studies where the use of a drug that suppresses the immune response (immunosuppressive) strategies reduced the accumulation of renal macrophage causes reduction of the expansion of DN [3]. Advanced glycation products, hyperglycemia, and other components of diabetic milieu could drive activation of renal cells, which cause the expression of many compounds e.g. pro-inflammatory cytokines, chemokine, and adhesion molecules [4]. The cumulating of inflammatory cells in the nephritic considered a key driver in the creation of DN. For sure, obstruct the induction of inflammatory cells to the nephritic has been confirmed to protect renal damage of DN in lab animals. Pro-inflammatory cytokines which are generated by inflammatory cells immediately damage kidneys tissues [5]. During the last decade, the studies that inspected the correlations between inflammatory sign and diabetes, in the sequent studies, different inflammatory cytokines and acute stage proteins were

involved in the ever expanding roster of foreteller into the result of diabetes [6-8]. Increasing of the pro-inflammatory significance has been shown associated with insulin hormone resistance, fats accumulations, also the agents of syndrome metabolic so that predisposing type 2 diabetes mellitus and cardiovascular diseases [9-11]. According to several studies, proinflammatory markers (e.g. ICAM-1) related to microalbuminuria and development of DN [12], and a strong glycemic control will alter the amounts of inflammatory biomarkers [13]. Thus, it is thinkable that widespread inflammatory mechanisms might be contributing to the happening of diabetes and the development of diabetic elaboration. TLR4 is need to develop of immune-responses, and promotion of TLR4 mediates coding of pathways causes transcriptional expression of pro-inflammatory cytokines and chemokine, that may injury increasing of kidney failed in acute and chronic renal diseases, [14-16]. Recently, there were a lot of studies about the TLR4 expression in man kidney tissue, and TLR4 was built in expressed of glomeruli and tubules [17]. In glomeruli, TLR4 is present in endothelial cells of glomerular and podocytes, but it wasn't in mesangial cells [18-19]. Also, tubular TLR4 appears to drive ischemia and reperfusion damage which then will causes transplantation and cyclosporine of kidney, Almeida et al., 2018, investigated TLR4 expression, in some kidneys tissues, to detect in tubular-cells and tubule-interstitial infiltrates, and to a minor extent in glomerular cells. The TLR4 tubular-expression is considerably up-regulated in cortical part of

kidney of diabetes nephropathy than that with renal tissue from patients who not-DN and control. Also, the tubular-TLR4 over-expression is associated to interstitial macrophage infiltration in the DN. Dasu, et al. 2010b investigated the up-arranging and promotion of toll-like receptors 4 and its ligand in the monocyte distribution of T2D lately diagnosed, complication a potential function for toll-like receptors 4 in between monocyte macrophage mobilization and tubule-interstitial inflammation in diabetes nephropathy. The obscurity of this situation in nephritic tissues of likely protein-uric but nondiabetic cases certain which the observed TLR-4 stimulation is not just a not specific result of hard proteinuria. Shigeoka, et al., 2007 documented that TLR4 has been much expressed in different type of renal cells, inclusive glomerular endothelial & tubular cells, per tubular and capillaries. In biopsies of human diabetes nephropathy and diabetic rats, expression of TLR4 was found to up-regulated in both the glomeruli and Tubulointerstitial and associated to elevated MyD88 expression, as well as activation of NF- κ B and infiltration of macrophage. In the lab animals, rising glucose induced the TLR4 in cells of rats. Diabetes promotes the TLR4 surface expression in peritoneal macrophages and renal macrophages, cancel of TLR4 showed that protective effect to Albuminuria, inflammation of renal, also the damage of podocytes, and consequently indicating that macrophages could be derived TLR4 which might mediate the nephritic injury in elementary DN. Recently, in the diabetes, systemic inflammation and kidney injury in the DN preliminary stage, the TLR-4 role has been proved. Right now, few studies have explained that rising TLR4 expression which promotes by glucose can prevent inflammation by down-regulating the TLR4 expression. In lab animals, hyperglycemia-induced TLR4 and TLR4 expression by the oxidize activation of PKC and NADPH, and knocking reduced of TLR2 and TLR4 important down-regulated the HG-induced NF- κ B activation. Furthermore, FFA augments the HG-induced pro-inflammatory affect macrophages by promoting the TLRs expression PPAR γ agonist can expand its anti-inflammatory effect by altering the TLR-2 and TLR-4 signaling in the mice dB/dB. Therefore, the role of TLR-4 in immunocytes (monocyte and macrophages) can provide a further indicator of systemic pro-inflammatory status in diabetes. Further investigation of the correlation between the TLR-4 proinflammatory mediators /mRNA expression and the multiple danger agents for DN in the biggest population is needed to estimate the possibility of pathogenic relation of TLR4 in the DN of human. In conditions of non-infectious inflammatory (e.g. ischemia-reperfusion injury, diabetes, and immune disease), the TLR4 reported being stimulated via reacting with endogenous ligand including HMGB1 and HSP. Dasu and his group identified that TLR4 endogenous ligand-activated by the state of diabetic, also, proved to mediate tubular inflammatory response by TLR4 in renal ischemia-reperfusion hurt. Circulating HMGB1 might involve the human DN expansion. In our work, we detected that a rise expression of TLR-4 and plasma grade of HMGB1 in T2DM patients that more supports the function of TLR4 activation in DN. Moreover, the growing roster of TLR4 endogenous ligand may also drive TLR4 activation in DN patients.

MATERIALS AND METHODS

Patients were selected from Center of hormones and diabetes in Al - Saddar Teaching Hospital

The inclusion criteria which have been taken for patients as following:

All patients were more than 5 years diagnosed with diabetes type 2.

Patients age was more than or equal to 30 years at diagnosis and equal or less than 65 years.

Patients were randomly collected in relation to gender.

Patients shouldn't receive any drugs at presentation phase.

The patients were referred from different provinces of Iraq.

The clinical findings for each patient were obtained, as showed in questionnaire forma Appendix 1, The following data will be recorded: age, gender, onset of diabetes, duration of diabetes, body mass index, blood pressure, General urine examination (GUE), urea, creatinine, albumin, mean glycosylated hemoglobin (HbA1c) more than 10%, and C-reactive protein are determined in all the enrolled subjects by standard laboratory techniques.

A case-control study was designed according to the following equation: (Menashe, Rosenberg, & Chen, 2008) $N = 2 (Z\alpha + Z\beta)^2 p^* q^* / (P1-P2)^2$

The diagnoses of cases were in the diabetes laboratory of Al-Sadder educated hospital and based on the following tests:

HBA1c

Albumen-creatinine ratio

General urine exam

Estimated glomerular filtration rate (GFR rate)

After diagnoses possesses the number of all patients were 400 that divided in to three studied groups for further investigation:

Patients with normal Albuminuria (No =145)

Patients with microalbuminuria (No=162)

Patients with macroalbuminuria (No=93)

The study protocol was approved by Kufa Medical College Ethical Committee

Five ml of venous blood samples have been collected using a 5 ml disposable syringe in EDTA tube from 400 patients of all studied groups (Normo, Micro, and Macro Albuminuria). Laboratory work for preservation blood samples was accomplished in Alfuratalausat unit for Cancer Researches. All blood collection tubes were gently shaking for mixing immediately after collected, incubated for 5 min. and preserved in ice bag for not more than one hour before preservation processing with TRIzol reagent and divided into two fractions:

Total RNA Isolation with TRIzol

The total RNA Isolation is achieved by five steps depends on Guy's protocol for total RNA isolation, unless stated, the protocol is carried out at room temperature. Total RNA was reversely transcribed to complementary DNA (cDNA) using Wiz Script™ RT FD mix Kit. The procedure was carried out in a reaction volume of 20 μ l according to the manufacturer's instructions; the total RNA volume to be reversely transcribed was (20 μ l). The amplification was done in the ABI/Prism 7900HT version 0.1.1. (Applied Biosystems) using TaqMan®

Universal PCR Master Mix, WISBIO for blood samples. The sequence of the primers and probes used in the study

was summarized in table (1)

Table (1)

Primer	Sequence (5' →3' direction)
TLR4 Chromosome 9	
Forward	ATCATTGGTGTGTCCGGTCCT
Reverse	CAGTCCTCATCCTGGCTTGA
GAPDH (endogenous gene)	
Forward	GAAATCCCATCACCATCTTCCAGG
Reverse	GAGCCCCAGCCTTCTCCATG

RESULTS

Table (2) shows the house keeping gene and the Ct value of GAPDH, which used in the present work. The range of

Ct value for GAPDH in the Micro Albuminuria group the mean ± SD was (17.24±0.36), for the Macro Albuminuria group the mean ± SD was (17.22±0.37)

Table (2)

Group	Means Ct of GAPDH	2 ^{-Ct}	2 ^{-Ct} of Studied group/2 ^{-Ct} of Control group	Fold of gene expression
1)Normoalbuminuria(Control)	17.21	65.9 E-7	65.9 E7/65.9 E7	1.000
2)Microalbuminuria	17.24	64.6E-7	64.6 E-7/65.9 E-7	0.98
3)Macroalbuminuria	17.22	65.5 E-7	65.5 E7/65.9 E7	0.99

DISCUSSIONS

In Normo Albuminuria group it was a mean ± SD (17.21±0.44). A significant difference was found in between these groups regarding the mean Ct value of GAPDH, (p=0.016: p<0.05) with an LSD value of (0.29). The inherent assumption within the housekeeping genes that used in studies of molecular is which their expression left over constant within the tissues. The GAPDH is one of the foremost usually used genes of house-keeping within the companion of the expression of genetic information, studied the gene expression of 1, 718 by using qRT-PCR. The GAPDH was applied as a gene reference in seventy-two types of normal tissues of a human. They found GAPDH that used is a kind of are liable strategy for the standardization in qRT-PCR when clinical studies applied the 2-ct value of first group was 65.9 E-7. For second group, it was 65.5 E-7 and for the rd

group it was 64.6E-7. The folds expression ratio of the gene was 1 in control (Normo Albuminuria), 0.99 to the Macro Albuminuria and 0.98 to Micro Albuminuria group, there are a lot of studies had been end one about the total change in GAPDH expression utilizing the values of 2^{-Ct} and the ratio of 2^{-Ct} compared with control (Normo Albuminuria) group, table (4). Each quantitative PCR reactions was run in triplicate for each sample, in each run, samples from all groups were run in addition on template and non-primer controls. This was important to make the statistical calculation of each group and in order to specify the calibrator. Plots of each run were recorded including the amplification plots and dissociation curves, in figures (1-4) show the amplification plots and dissociation curves for GAPDH and TLR-4.

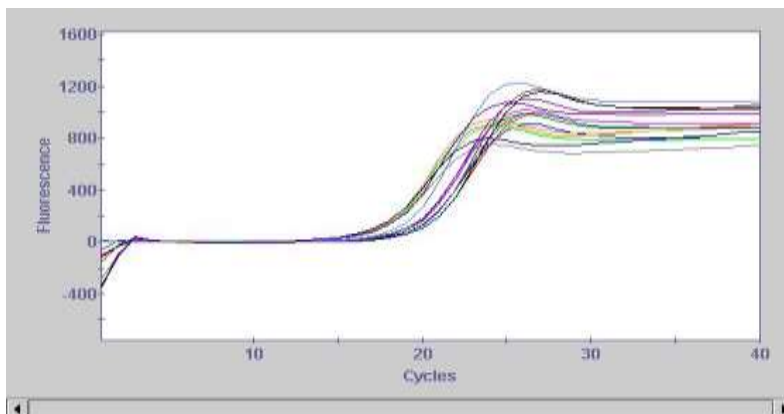


Figure (1): Plots of GAPDH amplification by using Qpcr

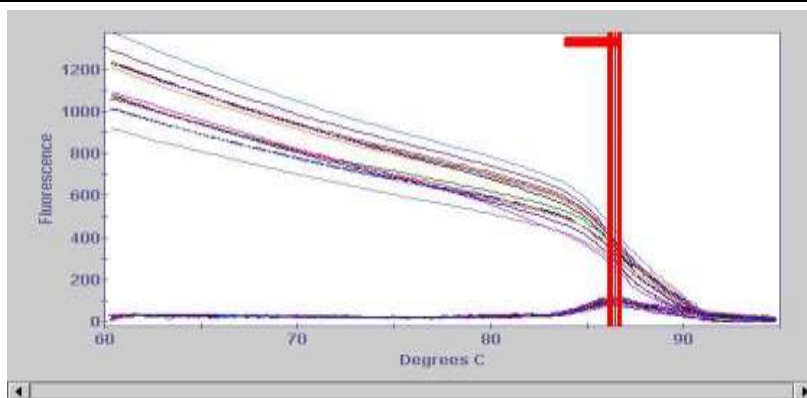


Figure (2): GAPDH dissociation curves by Qpcr

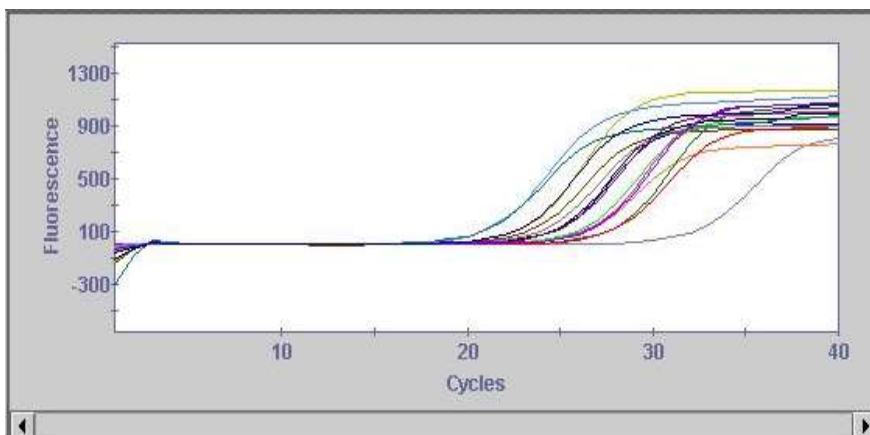


Figure (3): TLR4 amplification plots by Qpcr

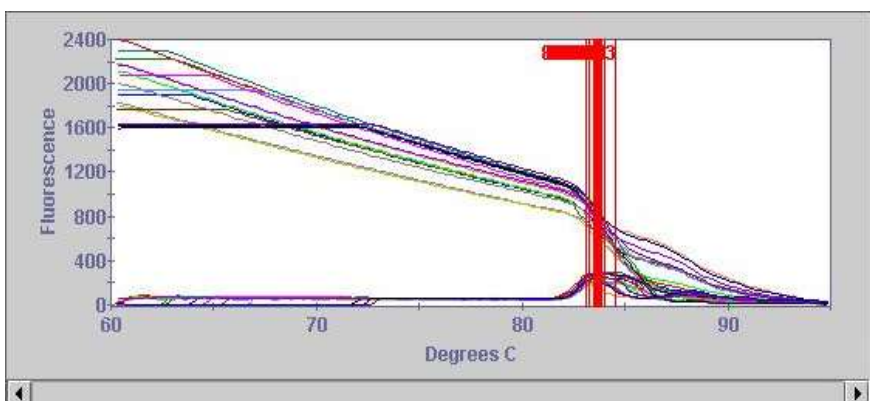


Figure (4): TLR4 dissociation curves by qPCR samples included all studied groups

The samples were included in all studied groups, the range of Ct values were 16.0-17.0.

This photograph was taken directly from Rout agene (smart cycler) qPCR machine

The samples included in all the studied groups. Melting temperature ranged from 86°C to 87°C, No primer dimer could be seen. The photo graph was taken directly from Rout agene (smart cycler) qPCR machine.

Samples included all studied groups; this photograph was taken during the work directly from Rout agene (smart cycler) qPCR machine

Melting temperature ranged from 83°C to 84°C, No primer dimer could be seen.

The photograph was taken directly from Rout agene (smart cycler) qPCR machine

Normalization of Ct (cycle threshold) values

Quantitative RT-PCR assay in the present study analyzed them RNA expression of TLR-4 and compared its expression between Normo Albuminuria control group, Micro Albuminuria group and Macro Albuminuria group. The calculation of gene expression fold change was made using relative quantification (Stamova et al., 2009). This depends on normalization of Ct values calculating the Ct which is the variation between the mean values of C to replica of TLR-4cDNA amplification of each single case and that of the GAPDH.

Table (3)

groups	Means Ct of TLR4	Means Ct of GAPDH	ΔCt	2-ΔCt	2-ΔCt of Studied group/2-ΔCt of Control group	Fold of gene expression
1)Normo Albuminuria	22.28±0.36	17.21	5.07 ± 2.43	0.029	0.029/0.029	1.0
2) Micro Albuminuria	21.28±0.41	17.24	3.04±2.5	0.121	0.029/0.121	4.192
3) Macro Albuminuria	22.01±0.31	17.22	4.79±3.18	0.051	0.029/0.036	1.76
P value =	0.0001					

Table (3) shows the mean ± SD of Ct (normalization Ct values) of each study group

The mean ± SD of Ct value of TLR-4cDNA amplification was (22.28 ± 0.36) in Normo Albuminuria group, while Micro Albuminuria group was ± SD (20.28±0.41). The Ct values of Macro Albuminuria group with a mean ± SD

was (21.51±0.31). Results are shown in Table (4-8), the Ct mean values of the control (Normo Albuminuria) were higher than Macro Albuminuria and this in turn was higher than that of Micro Albuminuria group.

Table (4)

Groups	Means Ct of TLR4	Means Ct of GAPDH	Mean ΔCt Target (ctTLR4 - ctGAPDH)	Mean ΔCt Calibrator (ctTLR4 - ctGAPDH)	ΔΔCt	2-ΔΔCt	2-ΔΔCt of Studied group / 2-ΔΔCt of Control group	Fold of gene expression
1)Normo Albuminuria	22.28	17.21	5.07	6.2	-1.13	2.188	2.188/2.188	1.0
2)Micro Albuminuria	20.28	17.24	3.04	6.2	-3.16	8.938	8.932/2.188	4.08
3)Macro Albuminuria	21.51	17.22	4.29	6.2	--1.91	2.657	3.758/2.188	1.717

Table (4) show the mean ± SD of Ct (normalization Ct values) of each study group

CONCLUSION

For the Micro Albuminuria (3.04 ± 2.5), for Macro Albuminuria group the mean ± SD (4.79±3.18) and the Normo Albuminuria group Ct a mean ± SD was (5.07±2.43), for the gene expression folds in relation to the housekeeping genes the result of 2- Δ -ct of each group was measured in relation theorem Albuminuria (control). As showed in Tables (4-9), the results of 2- Δ -ct revealed significantly higher results for the Micro Albuminuria group from the other two group, ($p = 0.0001$) 2- Δ Ct for Micro Albuminuria group (0.121). In the Macro Albuminuria group and Normo Albuminuria group a 2- Δ Ct were (0.051), (0.029) respectively. The gene expression fold of in Micro Albuminuria group was more than 4 time higher than that of Normo Albuminuria, and for the Macro Albuminuria was 0.76 time higher than the Normo Albuminuria group. These results indicate significantly increase expression of TLR-4 gene in macro and Micro Albuminuria group compared to control group. Histological, The most prevalent, severe and threatening complications of DM is Diabetic Nephropathy (DN) which correlated within verse outcomes of cardiovascular disorder, renal failure, and premature mortality. In inflammatory diseases, the participation of TLR-4 mediated rout have been proposed, therefore, TLR-4 mediates activation of

natural immunity controls not solely host defense against pathogen show ever also immune disorders. An evident from these results that the Micro Albuminuria group is associated with the highest copy number of m RNA so fTLR-4 gene reflecting its higher expression, which an inevitable result of accumulation of the glycation of plasma proteins like albumin, fibrinogen, globulins, and sclera protein to create different kinds of AGEs many previous studies have confirmed a high level of liquid body substance TLR-4 concentration in diabetic patients compared to healthy folks. Isaza-Correa et al, 2014, Liu et al., 2001 augmented TLR4 expression discovered in monocyte from diabetes style 2 patients (Jhengetal, 2015) reported that the inviter-TLR could energizing in renal system according to hyperglycemia (increasing of glucose in the bloodstream), which are unregulated in polygenic disorder. Reanimation the likelihood that a similar mechanisms charge able for initiating protein production at the early-stage of diabetic nephropathy. On the other hand, the TLR4 expression was also found to be associated well with this verity of Albuminuria in T2DM, suggesting its possible role in the pathogens is and progression of diabetic nephropathy and may by a good marker for diagnostic DN. This may reflect an increase depression of TLR4 by accumulation of TLR4-positive NK cells, mast cells, dendrite cells, monocyte,

macrophage ages, granulocytes and lymphocytes, nevertheless, the TLR4 up regulation mechanism in DN still obscure. TLR4 is unparalleled in the ability to promote both of the TRIF-and the My D88-subordinaterout, leads to activation of an early-step NF-KB & late-step NF-KB and stimulation of inflammatory cytokines rote in production. In DN, the NF-KB activation role in stimulating inflammation has been certified in vivo and in-vitro. In-vitro, NF-KB was promoting directly by albumin, HG, and AGE-BSA, also in directly by activating of growth factors and cytokines in a diabetic environment in tubular cells, glomerular & macrophage cells. On calculating relative expressionofTLR-4geneinallstudied groups applying the 2- $\Delta\Delta$ CT results, a calibrator was used, which is the highest value of the expression of TLR-4 among all the values of control group. As shown in table (4), the mean \pm SD of 2- $\Delta\Delta$ CT values of Micro Albuminuria group was (8.938) that for Macro Albuminuria were (2.657). The mean for Normo Albuminuria was (2.188). There was a significant difference within these groups regarding the mean 2- $\Delta\Delta$ CT (p=0.0001). Based on a large number of previous studies, TLR-4 expression or TLR-4 serum level are increased in diabetic patients as compared with healthy people (Rosa Ramirez & Ravi Krishna Dasu, 2012). This difference has also been confirmed among diabetic complications patients. But we are focusing on this study on DN only, although, in some study TLR4 has not been suggested to play a role in DN. However, several another investigations reported an avidness that agree with our result, which revealed that the TLR-4 gene expression was significantly higher in DN patients than other DM patients, more than four times as it is in Normo Albuminuria group, while, that gene expression fold number was 0.7 times in Macro Albuminuria group higher than that in the Normo Albuminuria. Table (4-11). Our result is agree with several other studies, (Fathy, Soliman, Ragheb, & Al Ashram, 2016), who reported that TLR4 are in dependent predictors of the occurrence of micro Albuminuria in T2DM patients. TLR4-expression is stimulating cells to increase their number in the glomeruli and within the Tubulointerstitial macro Albuminuria (inflammation of the kidney) with micro-albuminuria. Of note, macrophage which accumulation in kidney has been discovered to be related to the renal fibrosis and Albuminuria and in type 2DN animal models. Verzola et al, 2014, mentioned that the microalbuminuria in kidney, DN and TLR4-MRNA could ever express fourfold tenfold in tubules and glomeruli compared with control. However, Leemans, et al, 2005b mentioned to that the obscurity of detectable kidney TLR4 over-expression in DN is abating looked foras TLR4has been involved in ischemic TLR4 mouse-mutant models, While Chaetal., 2013 mentioned to that to nephritic and Kimetal., 2013 reported that in renal damages, and more recently, in STZ-induced diabetic kidney of rats (Maetal.,2014), the study of DN biopsies from patients which suffering from advanced DN and chronic (more than 5.7 years) with diffuse or nodular glomeruli sclerosis (extra cellular matrix accumulation), rare faction of the capillary, and tubular leanness, which may explain their failed to find any increase in overall TLR4 expression. In this current study the relation

normo albuminuria patients and TLR4 gene expressions in comparison with micro Albuminuria were significantly elevated in Micro Albuminuria than in normoalbuminuria participants, although the TLR-4 levels were slightly higher in macroalbuminuria as compare with normoalbuminuria patients. There was a significant statistical differences between the studied groups, p<0.05 suggesting the importance of detecting high expression of TLR-4 gene as a marker for. Pulskenetal, 2010, they suggested that TLR4 signaling may be hence of renal fibrosis by modulating the tendency of renal cells to TGF.

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