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Diabetes Induced Renal Complications by Leukocyte Activation of Nuclear Factor κ-B and its Regulated Genes Expression

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Abstract

Type 2 diabetes mellitus (T2D) is a metabolic disorder characterized by inappropriate insulin function. Despite wide progress in genome studies, defects in gene expression for diabetes prognosis still incompletely identified. Prolonged hyperglycemia activates NF-κB, which is a main player in vascular dysfunctions of diabetes. Activated NF-κB, triggers expression of various genes that promote inflammation and cell adhesion process. Alteration of pro-inflammatory and profibrotic gene expression contribute to the irreversible functional and structural changes in the kidney resulting in diabetic nephropathy (DN). To identify the effect of some important NF-κB related genes on mediation of DN progression, we divided our candidate genes on the basis of their function exerted in bloodstream into three categories (Proinflammatory; NF-κB, IL-1B, IL-6, TNF-α and VEGF); (Profibrotic; FN, ICAM-1, VCAM-1) and (Proliferative; MAPK-1 and EGF). We analyzed their expression profile in leukocytes of patients and explored their correlation to diabetic kidney injury features. Our data revealed the overexpression of both proinflammatory and profibrotic genes in DN group when compared to T2D group and were associated positively with each other in DN group indicating their possible role in DN progression. In DN patients, increased expression of proinflammatory genes correlated positively with glycemic control and inflammatory markers indicating their role in DN progression. Our data revealed that the persistent activation NF-κB and its related genes observed in hyperglycemia might contribute to DN progression and might be a good diagnostic and therapeutic target for DN progression. Large-scale studies are needed to evaluate the potential of these molecules to serve as disease biomarkers.

Keywords: NF-κB, IL-1β, IL-6, VEGF, TNF-α, FN, ICAM-1, VCAM-1

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1. Introduction

Type 2 diabetes mellitus (T2D) has become one of the largest global healthcare problems of this century. Over 422 million adults of the global population have diabetes according to the World Health Organization [1]. All types of diabetes can lead to potential complications including nephropathy [2, 3]. Approximately 40% of T2D patients are predisposed to diabetic nephropathy (DN) despite good glucose control [4]. Due to multifactorial etiological metabolic and vascular factors in DN, it is still under study for the development of diagnostic and therapeutic strategies [5, 6]. The study of the transcriptome elucidated its implication in human disorders progression but still incompletely outlined [7-10]. Therefore, gene expression analysis could provide key-data to explain DN pathogenesis and could help in prevention of this complication. Further research is a need to explore more inflammatory and metabolically important genes that have been proposed to be involved in DN progression [6].

Chronic inflammation results in the release of a different proinflammatory and profibrotic cytokines from different leukocytes triggering inflammation and irreversible fibrosis process [11]. Activated leukocytes under the effect of advanced glycation end products in diabetic patients secrete many kinds of transcription factors that have a crucial role in inflammation, including nuclear factor kabba β (NF-kβ), tumor necrosis factor alpha (TNF-α), interleukin-1b (IL-1β) and interleukin-6 (IL-6) [13, 14]. Activation of the transcription factor nuclear factor-κB (NF-κB) has been suggested to participate in chronic disorders, such as diabetes and its complications. Upon activation, NF-κB, induces abnormal transcription various genes involved in vascular complications which function broadly in leukocyte recruitment, including inflammatory molecules and cell adhesion molecules [15, 16]. Leukocyte recruitment triggers mechanisms that induce remodeling of extracellular matrix that might lead to fibrotic tissue formation, thereby contributing to glomerulosclerosis [12].

Of the most cytokines that have altered gene expression after NF-κB activation are vascular adhesion molecules intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and vascular endothelial growth factor (VEGF) [17-20]. These genes participate in the impairment of insulin signaling in adipocytes ending in to vascular cell damage and inflammatory process [21-23]. Previous studies revealed that the gene-expression profile of peripheral blood cells significantly reflects the gene-expression profile of disease-affected tissues, and that changes in the former mirror changes in the micro- and macro-environment latter [24]. Due to restricted options to obtain human biopsy specimens from living T2D patients we used peripheral white blood cells as an easily accessible source of cells for gene expression profiling of organ-specific and systemic diseases [25]. Patterns of altered gene expression provide data for pathophysiological processes taking place in various sites throughout the human body. In this study, we evaluated the expression profiles of 10 candidate diabetes related genes in T2D and DN subjects compared to control subjects. The data obtained from our study would provide better understanding of the disease and its complications.

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2. Subjects and Methods

2.1. Patients and Study Design

One hundred and thirty peripheral blood samples were collected from 30 healthy control individuals with normal glucose metabolism and 50 T2D patients and 50 DN patient. A written informed consent was given to all subjects of the study before study performance. The medical records of the participants were evaluated for full history, clinical and laboratory variables. The protocol of this study was approved by the medical ethics committee of the Diabetes Research institute (31158/11/18).

2.2. Diagnostic Criteria

Diagnosis of diabetes based on World Health Organization (WHO) criteria [1] at baseline for a fasting glucose was ≥126 mg/dl or a 2 h postprandial blood glucose ≥ 200 mg/dl. DN was diagnosed based on urine ACR suggested by American Diabetes Association (ADA) 2019 [26].

2.3. Exclusion Criteria

Exclusion criteria included diabetic patients with type-1 DM, diabetic women in pregnancy and diabetic patients with chronic liver diseases. Positive hepatitis serology and smokers are all excluded from this study. Also, individuals who suffer from UTI, heart diseases, coronary artery diseases, and chronic renal diseases other than diabetic nephropathy were excluded from the study based on history, physical examination, and urinalysis.

2.4. Sample Collection and Biochemical Investigations

Morning urine samples were collected, the urine was centrifuged at 3000 r.p.m. Routine urine analysis was performed by Uri-Trak® 120 semi-automated urine analyzer. Urinary albumin was measured by immunoturbidimetric method, in Cobas auto analyzer. Urinary creatinine was analyzed by Aeroset autoanalyzer. Estimated glomerular filtration rate(e-GFR) by modification of diet in renal disease (MDRD) equation [27]. Blood samples were obtained by venipuncture after an overnight fast. WBC counts were performed in blood samples using an automatic blood counter (XE-5000; Sysmex Corp, Kobe, Japan). Biochemical parameters were performed using routine clinical assays in the hospital laboratory using a colorimetric method kit (Spnireact-Spain) [28]. HbA1c was analyzed using Nycocard kit (Alere, Norway) [29].

2.5. RNA Preparation and Reverse Transcription

Total RNA was isolated was extracted from WBCs using PureLink™ RNA Mini Kit according to the manufacturer's instructions. The quantity and quality of the isolated RNA were determined by using a NanoDrop ND-1000 spectrophotometer. Quality and quantity of RNA were checked by denaturing gel electrophoresis. The intensity of the 18S and 28S rRNA bands was examined on a 1% formaldehyde agarose gel. Only total RNA extracts with an OD260/OD280 ratio of about 2 were processed for RT-PCR. Approximately 2µg of total RNA

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was were subjected to reverse transcription using SuperScript III (Invitrogen Corp.) and random hexamers in a final reaction volume of 20 µl according to the manufacturer's instructions. cDNA was stored until using as template for RT-PCR.

2.6. Real-Time RT-PCR Assay

RT-PCR reaction mixtures were prepared and run on ViaSure Real Time PCR System using quantiteque SYBR Green qPCR Master Mix. The reaction was performed under the following conditions: an initial denaturation step at 95°C for 15 min, followed by 40 cycles of 94°C for 30 sec, annealing temperature (Table 3.1) for 30 sec, 72°C for 30 sec and then fluorescence was measured. The primers were purchased from Invitrogen and the sequences of the primers are listed in Table 2.1 [30-40]. Quantification of expressed gene as relative mRNA level compared with healthy control levels, was calculated after normalization to GAPDH according to Livak method ($2^{-\Delta\Delta Ct}$) [41].

2.7. Statistical Analysis

Data were expressed as means ± SEM. Statistical analysis was carried out with SPSS 18.0 (SPSS Inc., Chicago, USA). p value < 0.05 was considered statistically significant. Statistical analysis of the differences between mean values from control subjects and T2D and DN were determined by One-way ANOVA test. Pearson and spearman correlation test were used for correlation analysis.

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3. Results

3.1. Demographic Characteristics

Demographic characteristics are shown in Table 3.1. This study included a balanced distribution of the studied subjects in gender with control group including 15 females and 15 males, and T2D group including 25 females and 25 males. Among 50 patients with DN there were 25 females and 25 males. DN patients were markedly older and had longer diabetic duration than T2D patients. Both groups had a significantly higher body mass index BMI than control with no significant difference between them. As compared to control and T2D group, DN patients had significantly higher SBP and DBP.

3.2. Clinical Characteristics

The biochemical characteristics of the studied groups are summarized in Table 3.2. No significant difference in fasting and post-prandial blood glucose levels, was found between DN and T2D but still higher than control. The patients diagnosed as having DN had significantly higher HbA1c, higher triglyceride concentration, urea, creatinine and ACR as well as lower HDL-cholesterol and e-GFR than T2D patients. DN patients had markedly lower serum albumin ($P<0.05$), than had the patients who were diagnosed with T2D. As shown in Table 3.2, this study demonstrated a marked difference in the total leukocyte count between DN group (with glycemic metabolic deterioration) and both T2D and control groups. No significant ($P>0.05$) difference was found between groups for VLDL.

3.3. RNA Integrity

Conventional RNA quality assay (formaldehyde gel assay) indicated that extracted RNA is intact in samples before cDNA synthesis. Visual assessment of the 28S:18S rRNA bands on agarose gels is somewhat subjective for such integrity. Figure 3.1 illustrates detection of 28S and 18S band on gel.

3.4. RTq-PCR Relative Expression

Real-time PCR of peripheral WBCs from 30 healthy subjects 50 T2D and 50 DN subjects were performed on our 10 selected genes. Information of primers for the selected genes and GAPDH was shown in (Table 2.1). All selected genes have a known function in glucose metabolism and fibrosis therefore, they were classified into 3 categories according to their function (proinflammatory, profibrotic and proliferative genes). All groups of genes under study showed differential expression between groups. The fold changes of gene expressions, as determined by real-time PCR, are shown in Table 3.3. Genes that play a significant role in inflammation including (NFK-β, IL-6, IL-1β, TNF-α and VEGF*)* showed a significant ($P<0.05$) tendency toward increase in relative expression in DN group when compared to T2D subjects Table 3.3.

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Genes belonged profibrotic activity including FN, ICAM-1 and VCAM-1 also showed differential expression between groups. They showed a significant increase in DN group than in T2D group however is not as high as such increase in expression of inflammatory genes. Table 3.3 summarizes the differential expression of all target genes in both groups. On contrast, we could not find any statistically significant variation in the transcript levels of MAPK-1 and EGF genes as proliferative genes in any of the T2D and DN group studied which is still have normal expression as control (Table 3.3). Differential pattern of expression for all selected genes was shown in Table 3.3

3.5. Intercorrelation between Gene Expression

There was a significant ($P<0.05$) positive intercorrelation between the proinflammatory genes with in both T2D and DN group expression (Table 3.4 & Table 3.5 respectively). The profibrotic category of genes also showed a significantly positive intercorrelation with in the DN group whereas all the 4 genes in this category did not have any significant correlation with each other or with other genes in T2D group as shown in Table 3.4 suggesting their important impact on the DN pathogenesis. MAPK-1-1 and EGF genes as a proliferative gene did not show any correlation with other gene in both groups under study. In addition, there was a strong positive correlation between the proinflammatory genes expression and the profibrotic genes (except FN) expression in DN group indicating the strength of the relationship between these two categories of genes and the glycemic control of patients (Table 3.5).

3.6. Correlations between Proinflammatory Gene Expression and Biochemical Variables

In patients with T2D, the expression proinflammatory set of genes have a positive correlations WBCs count as an inflammatory marker as well as with the glycemic control markers (FBG, 2hPPBG and HbA1c). In addition, there was also a positive correlation between the expression proinflammatory set of genes and cholesterol of T2D patients. Data are shown in Table 3.6. In patients with DN, there were positive correlations between the relative expression level of all members of proinflammatory genes and all the inflammatory markers (CRP, fibrinogen and WBCs count). A good positive correlation was observed between all proinflammatory genes expression in DN group glycated hemoglobin as a glycemic control biomarker (Table3.7). On the contrary, all lipid profile biomarkers did not significantly correlate with changes in inflammatory gene expression except for LDL had significant positive correlation with all set of inflammatory genes as shown in Table 3.7. Moreover, all of serum creatinine, e-GFR and ACR were found to have a correlation with NFk-B, IL-6, IL-1β, TNF- α and VEGF in DN group.

3.7. Correlations between Profibrotic Gene Expression and Biochemical Variables

No correlation was found between both inflammatory and glycemic control markers and profibrotic genes under study in T2D group except for NFK-B gene expression which showed a marked positive correlation with both FBG and HbA1c (Table 3.6). No correlation

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was found between this set of genes and lipid markers in T2D group except for NFKB which had a positive correlation with cholesterol. Data are shown in Table 3.6. On the other hand, in DN group all inflammatory biomarkers showed a significant positive correlation with all genes categorized as profibrotic genes except for FN expression which had only a positive correlation with WBCs count. HbA1c showed positive correlation with all profibrotic genes in DN group. No correlation was found with FBG or 2hppbg in DN group. A significant positive correlation was found between serum levels of LDL and ICAM-1 in DN group as shown in Table 3.7.

On contrast, there was a strong positive correlation between all profibrotic genes and all kidney deterioration markers (creatine and ACR) and negative correlation with e-GFR. These data suggest the important role of our selected genes in later stages of DN development.

3.8. Correlations between Proliferative Gene Expression and Biochemical Variables

On the contrary, no correlation ($P > 0.05$) was found between MAPK-1 and EGF gene and all the studied biomarkers as well as demographic markers in both T2D and DN groups. This result suggests the absence of their clinical significance in DN progression. Data were shown in (Tables 3.6 & 3.7)

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4. Discussion

Understanding molecular and genetic mechanisms of microalbuminuria may help early prognosis of diabetic patients under risk of nephropathy [41]. Despite recent advances in DN diagnosis, the risk for its development still depends on genetic components of diabetic patients [42]. Due to the risks associated with renal biopsies from DN patients, gene expression analysis in kidney tissue is limited [43]. The pro-inflammatory cytokines which are related to renal tubulointerstitial injury serve in disease strength and are involved in DN progression but are incompletely outlined. Therefore, further research is necessary to identify more genes [44]. Herein, we investigated the expression patterns of candidate genes in leukocytes of patients and explored their possible associations with DN parameters and risk factors. Subjects with T2D showed significant alterations in the expression of all candidate genes as shown in (Table 3.3). For the purpose of analysis, genes were clustered functionally into proinflammatory (NF-KB, IL-1β, IL-6, TNF-α, VEGF), profibrotic (FN, ICAM-1, VCAM-1) and proliferative (MAPK-1, EGF) genes. Based on the fact that our candidate genes are involved in endothelial injury and extracellular mass synthesis, our suggested genes may be therapeutic or diagnostic target for diabetes complications [45-54].

Our data revealed that DN patients expressed exclusively higher levels of total mRNA of both proinflammatory and profibrotic genes (NF-κB, IL-1β, IL-6, TNF-α, VEGF & FN, ICAM-1, VCAM-1) compared to T2D and have steady levels of total mRNA of the proliferative genes (MAPK1 and EGF) in both T2D and DN groups. Proinflammatory cytokines including NF-κB and its dependent cytokines mainly TNF-α were shown to have a direct toxic effects on renal cells, alters endothelial permeability and induces albuminuria during nephropathy progression [44-47]. Persistent leukocyte activation is established in and type 2 diabetes mellitus patients [48]. leukocyte activation has been linked to microvascular diabetic complications in response to overactivation of the inflammatory cascade and increased tissue damage [-49]. According to this criterion, the expression of our candidate genes within leukocytes was examined in our study.

In our study, it was revealed that DN group exerted a significantly higher relative expression of NF-κB as well as IL-6 and IL-1β compared to T2D patients. One of the most highly induced NF-κB-dependent cytokines is IL-6. In agreement with a previous study which reported that IL-6 and IL-1β were upregulated in T2D patients PMNC compared to control subjects [50]. Also, it was found that TNF-α gene was overexpressed in both T2D and DN group with a significant difference in expression in DN group supporting the concept of the presence of inflammation state is associated with long-term predisposition to hyperglycemia. Previous studies reported TNF inflammatory cascade induces insulin resistance, with subsequent diabetes and other comorbidities associated [51, 52].

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A significant increase of the expression level of VEGF in DN patients compared to T2D patients was also observes in our results. VEGF is related to the fenestration of glomerular endothelium and increased permeability in the glomerulus, thus facilitating glomerular filtration [53]. In addition, hyperglycemia was shown to upregulate VEGF mRNA expression in podocytes [54]. Therefore, our data suggest that VEGF overexpression enhance capillary formation under long term hyperglycemic conditions. Totally the strongly expressed inflammatory genes in both DN and T2D suggest that their activation may be an early event in DN development. Moreover, our results are in accordance with previous study which detected high concentrations of inflammatory markers in T2D subjects and the persistence of inflammation and high markers during DM progression, is related to the development of complications [55-60].

Regarding correlation between inflammatory genes expression and glycemic condition and renal impairment our study revealed a significant positive correlation between inflammatory genes and markers of inflammation as well as glycemic control markers in DN group of patients as shown in Table 3.5. In addition, there was also a significant positive correlation between inflammatory genes expression and kidney injury parameters (urea, creatinine, s.albumin, e-GFR and ACR). Interestingly, there was also a markedly positive correlation between inflammatory genes expression and LDLc and VLDLc in patients with DN indicating that there may be a direct link between inflammation and disturbance in lipid metabolism. This might indicate an important pathway in the pathogenesis of DN. On contrast, there was no correlation between inflammatory genes expression and inflammatory markers in T2D group except for WBCs count suggesting the delayed effect of inflammation on microvascular complications.

Surprisingly, there was a positive correlation between the genes of inflammation and cholesterol in T2D group confirming the suggested link between long term inflammation and stepwise disturbance in lipid metabolism. In addition, there was a positive intercorrelation was found between all set of inflammatory genes (NF-κB, IL-1β, IL-6, TNF-α, VEGF) in both groups under study confirming their dependent role in inflammation. The significant intercorrelation between the proinflammatory genes expression with in both DN and T2D group confirm their synergistic effect in the development of DN.

There are a multitude of profibrotic growth factors implicated as pathogenic mediators in DN, including FN, ICAM-1 and VCAM-1 [**61-**67]. Interestingly, in our study, FN has increased relative expression level in leukocytes of DN. DN patients exhibited also elevated levels of ICAM-1 and of *VCAM-1* when compared to their expression in T2D group which still has normal expression as control. There is an evidence that elevated ICAM-1 and of *VCAM-1* levels are involved in the development of DN-associated glomerulosclerosis and tubulointerstitial fibrosis [50,51, 68-70]. In our study, there was not a significant correlation between profibrotic genes expression levels in T2D group and the biochemical parameters. On the same line there was no intercorrelation between them in T2D group. On the other hand, these set of genes presented significantly positive correlations with each other in DN group of patients confirming their late role in diabetic complication. They also exerted a positive

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correlation with parameters and risk factors of ESRD like fibrinogen, HbA1c, LDLc and ACR in DN group. These correlations may provide an explanation for their possible implication in DN pathogenesis. A probable reason for DN progression in diabetic patients is that the high levels of these profibrotic mediators appear primarily at the local level, through the advance of T2D [71-73].

The proliferative genes MAPK and EGF did not show considerable fold change with no change between gene category in DN group and T2D group. From these data, we observed that the main tendency of proinflammatory genes expression to be the most increased suggesting uncontrolled inflammation and predisposition to fibrosis. These data support the association of profibrotic category of genes with an overall state of kidney fibrosis thus may explain the development of deteriorated kidney function in DN patients.

Conclusion

DN progression is conducted mainly by amplification of inflammatory response mediated by activated leukocytes which are infiltrated to the kidney causing tissue damage ending up to fibrosis development under the effect of liberated pro-inflammatory and profibrotic cytokines. Our results revealed significantly overexpressed NF-κB, IL-6, IL-1β, TNF-α and VEGF genes; and upregulated expression of FN, ICAM-1 and VCAM-1 in DN patients. The expression of MAPK and EGF genes remains unaffected in all studied groups, which is contrary to the expected result, as MAPK is reported to contribute in inflammatory response. Furthermore, the significant correlation between these two sets of genes (proinflammatory and profibrotic) suggests the vital role of the proinflammatory genes in DN development under long-term hyperglycemia. In addition, both inflammatory and profibrotic genes expression profiling of blood leukocytes may have a potential to be used as a diagnostic tool to evaluate the status of inflammation in diabetic and the possibility of fibrosis development in kidney. However, the present study has some drawbacks including the small number of study samples and the exclusion of the hypoglycemic drugs effect on gene expression and the lack of generalizing results on different populations and animal model-based experiments Further studies with large sample size are preferred to reach more precise conclusion.

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Disclosure Statement

The authors report no conflict of interest.

Abbreviations

T2D= type 2 diabetes mellitus without nephropathy.
DN=diabetic nephropathy.
M= male, F= female.
BMI= body mass index.
SBP= Systolic blood pressure.
DBP=Diastolic blood pressure.
FBS= fasting blood glucose.
2hPPBG= 2 hour post prandial blood glucose.
HbA1c= Glycosylated hemoglobin.
S.Cr = serum creatinine.
TC =total cholesterol.
TGs=Triglyceride.
HDL =High density lipoprotein-cholesterol.
LDL =Low density lipoprotein-cholesterol.
VLDL= Very low-density lipoprotein.
e-GFR=estimated glomerular filtration rate.
ACR= albumin creatinine ratio.

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Table 2.1. Primer sequence and annealing temperature**.**

Gene	Name	Primer Sequence	Annealing Temp	Amplicon size(bp)	Reference
GAPDH	**Glyceraldehyde-3-phosphate dehydrogenase**	5'-CCACTCCTCCACCTTTGAC-3' 3'-ACCCTGTTGCTGTAGCCA-5'	60 °C	102	[30]
NF-κB	**Nuclear factor kabba B**	5'-GCAGCACTACTTCTTGACCACC-3' 3'-TCTGCTCCTGAGCATTGACGTC-5'	58 °C	103	[31]
IL-1β	**Interleukin beta -1**	5'- GCAAGGGCTTCAGGCAGGCCGCG -3' 3'- GGTCATTCTCCTGGAAGGTCTGTGGGC -5'	60 °C	96	[32]
IL-6	**Interleukin-6**	5'- GGTACATCCTCGACGGCATCT-3' 3'- GTGCCTCTTTGCTGCTTTCAC-5	57 °C	81	[33]
TNF-α	**Tumor necrosis factor alpha**	5'- CTCTTCTGCCTGCTGCACTTTG-3' 3'-ATGGGCTACAGGCTTGTCACTC-5'	59°C	135	[34]
VEGF	**Vascular endothelial growth factor**	5'- CTACCTCCACCATGCCAGT-3' 3'-GCAGTAGCTGCGCTGATAGA-5'	58°C	101	[35]
ICAM-1	**Intercellular adhesion molecule 1**	5'- GGCCGGCCAGCTTATACAC-3' 3'-TAGACACTTGAGCTCGGGCA-5'	60 °C	166	[36]
VCAM-1	**Vascular cell adhesion molecule 1**	5'- TCAGATTGGAGACTCAGTCATGT-3' 3'-ACTCCTCACCTTCCCGCTC-5'	59°C	109	[36]
FN	**Fibronectin**	5'- CCATCGCAAACCGCTGCCAT 3'-AACACTTCTCAGCTATGGGCTT-5'	60°C	153	[37]
MAPK-1	**Mitogen-Activated Protein Kinase-1**	5'– CCTAAGGAAAAGCTCAAAGA-3' 3'-AAAGTGGATAAGCCAAGAC-5'	60°C	179	[38]
EGF	**Epidermal growth factor**	5'- GTGCAGCTTCAGGACCACAA-3' 3'-AAATGCATGTGTCGAATATCTTGAG-5'	57°C	67	[39]

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Table 3.1 Demographic characteristics of the study subjects.

Variable	Group I Control	Group II T2D	Group III DN	P value
Number	30	50	50	---
Gender (M:F)	15:15	25:25	25:25	---
Age (years)	49.07 ±3.02	51.82.2±2.73	65.47±3.12	[a]P< 0.05
DM Duration (yrs)	0 ± 0	8.54 ± 4.03	14.60 ±4.34	[a]P< 0.05
BMI (Kg/m^2)	24.07±2.65	30.05 ± 1.76	36.01±1.97	P> 0.05
SBP (mmHg)	117.97 ±0.96	121.96 ± 1.76	138.42 ± 3.85	[a]P< 0.05
DBP (mmHg)	78.96 ±2.76	79.65 ± 3.76	88.98 ± 6.86	[a]P< 0.05

Data are presented as mean± SEM.

P> 0.05= Significant difference if compared DN versus T2D.

[a]p<0.05 = Significant difference if compared DN versus T2D and control.

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Table 3.2: Demonstrating the P value of difference in the levels of biochemical markers in the patients

Variable	Group I Control	Group II T2D	Group III DN	P value
Glycemic Control Markers				
FBG (mg/dl)	78.0 ± 8.05	196.48 ± 7.73	205.57±9.68	[a]P < 0.05
2hPPBG (mg/dl)	102.16 ± 13.11	264.15 ±16.55	251.33±21.07	[a]P < 0.05
HbA1c %	5.07 ± 1.03	7.13 ± 1.32	10.74 ± 2.09	[b]P < 0.05
Inflammatory Markers				
WBCs count	6574 ± 98.65	7719 ± 65.05	15755 ±107.03	bP < 0.05
CPR	1.88 ±0 .57	7.8±1.6	60.33±5.76	P > 0.05
Fibrinogen	300 ± 25.76	354±10.76	576.7 ± 34.32	bP < 0.05
Kidney Profile				
S.urea (mg /dl)	28.02±3.05	36.08±6.34	145.0±16.66	[b]P < 0.05
S.Cr (mg /dl)	0.88±0.26	0.79±0.24	6.07±0.45	[b]P < 0.05
S.Albumin (g/dl)	4.97±0.67	4.56±0.18	2.13± 0.43	[b]P < 0.05
e-GFR (ml/min/1.73 m2)	127.96 ±9.0	119.99± 6.76	40.65 ±7.02	[b]P < 0.05
ACR (mg/g)	9.45 ± 2.06	13.2 ± 0.72	456.78± 32.6	[b]P < 0.05
Lipid Profile				
TC (mg/dl)	145.25±8.42	177.86±10.37	266.29±12.67	[b]P < 0.05
TGs (mg/dl)	98.0±6.33	107.82±9.04	188.75±18.02	[b]P < 0.05
HDL (mg/dl)	70.25±7.48	65±8.07	25.8±4.32	[b]P < 0.05
LDL (mg/dl)	81.5±7.0	106.29±9.06	156.91±9.48	[b]P < 0.05
VLDL (mg/dl)	27.78±7.06	24.06±5.0	30.07±5.22	p> 0.05

Data are expressed as mean ± SEM. Group comparison was done by a nova test.

[a]P<0.05= Significant difference if compared DN and T2D with control.
[b]P<0.05= Significant difference if DN compared with T2D patients and control.

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Table 3.3: Differential expression of the three categories of genes under study in both T2D and DN

Proinflammatory Genes	**Fold Change in T2D**	**Fold Change in DN**	**P value**
NF-κB	2.14±0.97	6.32±2.41	<0.05
IL-1β	4.23±2.01	10.07± 3.60	<0.05
IL-6	4±1.33	12.43±4.11	<0.05
TNF-α	3.03±1.23	7.23±1.80	<0.05
Profibrotic Genes (ECM component)	**Fold Change in T2D**	**Fold Change in DN**	**P value**
FN	1.21±0.36	2.98 ±0.87	<0.05
ICAM-1	1.30±0.32	5±2.43	<0.05
VCAM-1	1.06±0.26	4.41±1.23	<0.05
Proliferation Genes	**Fold Change in T2D**	**Fold Change in DN**	**P value**
MAPK-1	1.2±0.45	1.32±0.98	>0.05
EGF	1.03±0.36	1.54±1.09	>0.05

Fold was the ratio of both DN and T2D patient to control in each group. The differences in all listed genes between DN and T2D patients and controls were <0.05.

P<0.05 significant difference if compared DN versus T2D.

P> 0.05= No significant difference if compared DN versus T2D.

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Table 3.4: Intercorrelation between genes in T2D group

Gene	NF-κB		IL1-β		IL-6		TNF-α		VEGF		ICAM-1		VCAM-1		FN		MAPK-1		EGF	
	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r
NF-κB	---	---	<0.05*	0.56	<0.05*	0.50	<0.05*	0.68	<0.05*	0.73	>0.05	0.19	>0.05	0.31	>0.05	0.22	>0.05	0.21	>0.05	0.23
IL1-β	<0.05*	0.56	---	---	<0.05*	0.62	<0.05*	0.72	<0.05*	0.58	>0.05	0.32	>0.05	0.22	>0.05	0.37	>0.05	0.29	>0.05	0.43
IL-6	<0.05*	0.50	<0.05*	0.62	---	---	<0.05*	0.56	<0.05*	0.71	>0.05	0.42	>0.05	0.38	>0.05	0.23	>0.05	0.30	>0.05	0.32
TNF-α	<0.05*	0.68	<0.05*	0.72	<0.05*	0.56	---	---	<0.05*	0.77	>0.05	0.32	>0.05	0.26	>0.05	0.34	>0.05	0.20	>0.05	0.30
VEGF	<0.05*	0.73	<0.05*	0.58	<0.05*	0.71	<0.05*	0.77	---	---	>0.05	0.32	>0.05	0.30	>0.05	0.38	>0.05	0.18	>0.05	0.12
ICAM-1	>0.05	0.19	>0.05	0.32	>0.05	0.42	>0.05	0.32	>0.05	0.32	---	---	>0.05	0.23	>0.05	0.36	>0.05	0.43	>0.05	0.24
VCAM-1	>0.05	0.31	>0.05	0.22	>0.05	0.38	>0.05	0.26	>0.05	0.30	>0.05	0.23	---	---	>0.05	0.40	>0.05	0.39	>0.05	0.17
FN	>0.05	0.22	>0.05	0.37	>0.05	0.23	>0.05	0.34	>0.05	0.38	>0.05	0.36	>0.05	0.40	---	---	>0.05	0.13	>0.05	0.43
MAPK-1	>0.05	0.21	>0.05	0.29	>0.05	0.30	>0.05	0.20	>0.05	0.18	>0.05	0.43	>0.05	0.39	>0.05	0.13	---	---	>0.05	0.36
EGF	>0.05	0.23	>0.05	0.43	>0.05	0.32	>0.05	0.30	>0.05	0.12	>0.05	0.24	>0.05	0.17	>0.05	0.43	>0.05	0.36	---	---

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Table 3.5: Intercorrelation between genes in DN group

Gene	NF-κB		IL1-β		IL-6		TNF-α		VEGF		ICAM-1		VCAM-1		FN		MAPK-1		EGF	
	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r
NF-κB	---	---	<0.05*	0.65	<0.05*	0.70	<0.05*	0.55	<0.05*	0.53	<0.05*	0.61	<0.05*	0.67	>0.05	0.34	>0.05	0.22	>0.05	0.23
IL1-β	<0.05*	0.65	---	---	<0.05*	0.65	<0.05*	0.58	<0.05*	0.62	<0.05*	0.58	<0.05*	0.64	>0.05	0.23	>0.05	0.12	>0.05	0.32
IL-6	<0.05*	0.70	<0.05*	0.65	---	---	<0.05*	0.73	<0.05*	0.56	<0.05*	0.63	<0.05*	0.51	>0.05	0.18	>0.05	0.43	>0.05	0.21
TNF-α	<0.05*	0.55	<0.05*	0.58	<0.05*	0.73	---	---	>0.05	0.66	<0.05*	0.53	<0.05*	0.44	>0.05	0.25	>0.05	0.14	>0.05	0.34
VEGF	<0.05*	0.53	<0.05*	0.62	<0.05*	0.56	>0.05	0.66	---	---	<0.05*	0.60	<0.05*	0.48	>0.05	0.41	>0.05	0.27	>0.05	0.32
ICAM-1	<0.05*	0.61	<0.05*	0.58	<0.05*	0.63	<0.05*	0.53	<0.05*	0.60	---	---	<0.05*	0.49	<0.05*	0.65	>0.05	0.45	>0.05	0.17
VCAM-1	<0.05*	0.67	<0.05*	0.64	<0.05*	0.51	>0.05	0.44	>0.05	0.48	<0.05*	0.49	---	---	<0.05*	0.67	>0.05	0.37	>0.05	0.25
FN	>0.05	0.34	>0.05	0.23	>0.05	0.18	>0.05	0.25	>0.05	0.41	<0.05*	0.65	<0.05*	0.67	---	---	>0.05	0.31	>0.05	0.27
MAPK-1	>0.05	0.22	>0.05	0.12	>0.05	0.43	>0.05	0.14	>0.05	0.27	>0.05	0.45	>0.05	0.37	>0.05	0.31	---	---	>0.05	0.30
EGF	>0.05	0.23	>0.05	0.32	>0.05	0.21	>0.05	0.34	>0.05	0.32	>0.05	0.17	>0.05	0.25	>0.05	0.27	>0.05	0.30	---	---

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Table 3.6: Correlation between gene expression and clinical markers in T2D group

Gene	Inflammatory Markers						Glycemic control				Lipid profile	
	CRP		Fibrinogen		WBCs count		FBG		HbA1c		TC	
	r	p	r	p	r	p	r	p	r	p	r	p
Proinflammatory Genes												
NF-κB	0.81	<0.05*	0.63	<0.05*	0.70	<0.05*	0.61	<0.05*	0.74	<0.05*	0.55	<0.05*
IL1-β	0.73	<0.05*	0.56	<0.05*	0.65	<0.05*	0.7	>0.05	0.47	>0.05	0.46	>0.05
IL-6	0.63	<0.05*	0.66	<0.05*	0.59	<0.05*	0.35	>0.05	0.86	>0.05	0.22	>0.05
TNF-α	0.59	<0.05*	0.65	<0.05*	0.48	<0.05*	0.55	>0.05	0.43	>0.05	0.27	>0.05
VEGF	0.40	>0.05	0.74	<0.05*	0.62	<0.05*	0.30	>0.05	0.31	>0.05	0.32	>0.05
Profibrotic Genes												
ICAM-1	0.4	>0.05	0.17	>0.05	0.51	>0.05	0.17	>0.05	0.18	>0.05	0.62	>0.05
VCAM-1	0.29	>0.05	0.43	>0.05	0.50	>0.05	0.53	>0.05	0.43	>0.05	0.55	>0.05
FN	0.34	>0.05	0.31	>0.05	0.49	>0.05	0.41	>0.05	0.23	>0.05	0.49	>0.05
Proliferation Genes												
MAPK-1	0.33	>0.05	0.23	>0.05	0.15	>0.05	0.3	>0.05	0.55	>0.05	0.37	>0.05
EGF	043	>0.05	0.26	>0.05	0.35	>0.05	0.26	>0.05	0.43	>0.05	0.26	>0.05

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Table 3.7: Correlation between gene expression and clinical markers in DN group

Gene	Inflammatory Markers						Glycemic Markers		Lipid Markers		Kidney Profile Markers					
	CRP		Fibrinogen		WBCs count		HbA1c		LDL		Creatinine		ACR		eGFR	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Proinflammatory Genes																
NFk-B	0.8	<0.05*	0.7	<0.05*	0.56	<0.05*	0.91	<0.05*	0.67	<0.05*	0.50	<0.05*	0.65	<0.05*	-0.61	<0.05*
IL1β	0.7	<0.05*	0.55	<0.05*	0.54	<0.05*	0.85	<0.05*	0.91	<0.05*	0.47	<0.05*	0.52	<0.05*	-0.5	<0.05*
IL-6	0.63	<0.05*	0.51	<0.05*	0.93	<0.05*	0.64	<0.05*	0.85	<0.05*	0.51	<0.05*	0.59	<0.05*	-0.65	<0.05*
TNF-α	0.73	<0.05*	0.65	<0.05*	0.65	<0.05*	0.58	<0.05*	0.64	<0.05*	0.88	<0.05*	0.48	<0.05*	-0.76	<0.05*
VEGF	0.64	<0.05*	0.54	<0.05*	0.71	<0.05*	0.48	<0.05*	0.58	<0.05*	0.59	<0.05*	0.54	<0.05*	-0.61	<0.05*
Profibrotic Genes																
ICAM-1	0.74	<0.05*	0.48	<0.05*	0.65	<0.05*	0.50	<0.05*	0.48	<0.05*	0.45	<0.05*	0.56	<0.05*	-0.48	<0.05*
VCAM-1	0.52	<0.05*	0.52	<0.05*	0.7	<0.05*	0.70	<0.05*	0.50	>0.05	0.44	<0.05*	0.70	<0.05*	-0.60	<0.05*
FN	0.26	>0.05	0.14	>0.05	0.54	<0.05*	0.91	<0.05*	0.30	>0.05	0.40	<0.05*	0.67	<0.05*	-0.56	<0.05*
Proliferation Genes																
MAPK-1	0.31	>0.05	0.15	>0.05	0.37	>0.05	0.15	>0.05	0.41	>0.05	0.34	>0.05	0.28	>0.05	0.47	>0.05
EGF	0.43	>0.05	0.23	>0.05	0.34	>0.05	0.28	>0.05	0.17	>0.05	0.24	>0.05	0.32	>0.05	0.32	>0.05

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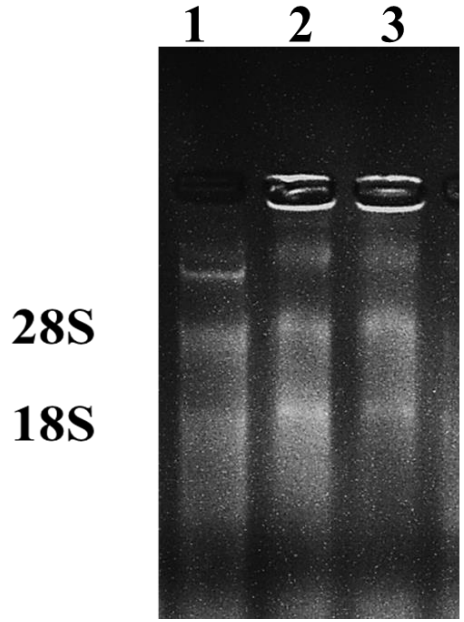

Figure 3.1: Samples of leukocyte total RNA were assessed using Formaldehyde/ Formamide denaturant. **Lane 1:** RNA from control subject; **Lane 2:** RNA sample from T2D subject. **Lanes 3:** RNA sample from DN subject. The 28S and 18S ribosomal RNA bands are visible in the gel photo implying that the RNA samples are intact.