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Association between Genotyping of Transforming Growth Factor Beta 1 with Oxidative Status in Type 2 Diabetic Nephropathy Complications

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Abstract

Objectives To assess the association between Transforming Growth Factor β1 gene polymorphism (T869C) and type 2 diabetes mellitus with and without nephropathy complications with endogenous antioxidant reduced glutathione levels in type 2 diabetic patients with/without nephropathy complications of Kerbala province, Iraq.

Methods A case-control study was performed at which 100 patients with diabetic nephropathy, 100 patients with only type 2 diabetic and another 100 apparently healthy individuals as control were recruited. Fasting blood glucose, HbA1c %, urea, creatinine and glutathione were measured by spectrophotometric methods using enzymatic procedures. Transforming growth factor β1 gene was genotyped for the T > C (T869C) SNP by PCR-ARMS technique.

Results The genotype and allele frequencies of TGFβ1 gene polymorphism in type 2 diabetes mellitus, type 2 diabetic nephropathy, and control were examined. The transforming growth factor β1 (T869C) C allele, TC and TC + CC genotypes were significantly higher in patients; the T allele and TT genotype were significantly higher in controls ($P \leq 0.001$). Glutathione give also a significant result in diabetic patients with and without nephropathy in when compared with controls.

Conclusion The observed data indicated that TGFβ1 (T869C) codon 10, allele C, and C allele-containing genotypes may be susceptible, and the T allele/TT genotype may be protective factors for type 2diabetic nephropathy complications. The results of glutathione showed that it may be one of the causes of presence high oxidants compounds, which is lead to the damage and destruction of mutations in the DNA of the cell.

Keywords TGF-β1, oxidative status, T2DMD, nephropathy complications, glutathione, PCR-ARMS

Introduction

Type 2 diabetes mellitus (T2DM) is a common, chronic, complex disorder of rapidly growing global importance. It accounts for 95% of diabetes worldwide and is characterized by concomitant defects in insulin secretion (from the β-cells in the pancreatic islets) and insulin action in fat muscle and liver.[1] Diabetes mellitus viewed as a wide spectrum of signs and symptoms which occur all in response to hyperglycemia that arises as a consequence to the disease pathology in which insulin secretion or action or both are defective. The fact that increased cellular resistance to insulin may be the causative factor to develop DM is considerable. Diabetes mellitus viewed as a wide spectrum of signs and symptoms which occur all in response to hyperglycemia that arises as a consequence to the disease pathology in which insulin secretion or action or both are defective, Figure 1.[2] The basic effect of insulin deficiency or increase tissue resistance to insulin is preventing the efficient up tack of glucose by all cells of body except brain. As a result the utilization of glucose by cells decrease and lead to increase blood glucose level and increase utilization of proteins and fats.[3,4]

The disease is associated with different types of complication that is attributed to morbidity and mortality.[5,6] These are the main cause of death in diabetic patients. It is affected by multiple genetic and environmental factors. Extensive efforts have been made to identification of the disease-affecting genes to get better understanding of the disease pathogenesis, find new targets for clinical therapy and allow prediction of the disease.[7] Apart from the conventional risk factors such as obesity, dyslipidemia, and arterial hypertension, hyperglycemia are the independent risk factors for the development of ischemic heart disease (IHD), and for long-term leads to vascular damage through several mechanisms.[8,9]

Various cytokines, chemokines, and growth factors have been shown to play important roles in fibrosis development. However, transforming growth factor-β (TGF-β) is considered to be the most potent and ubiquitous pro-fibrogenic mediator.[10] It has been well documented that TGF-β1 increases the production and deposition of various ECM proteins, including collagen I, III, IV, VII, and XVI, in various types of tissues and cells.[11,12] However, the signaling pathways or the molecular mechanisms underlying the stimulation of ECM protein production and deposition by TGF-β1 have not been completely elucidated.

The genetic polymorphisms of cytokines production were shown to affect the overall expression and secretion of cytokines both *in vitro* and sporadically in vivo systems. Associations among polymorphisms in cytokine genes and inflammation, allograft rejection, autoimmune, and infectious diseases have been reported.[13] In diabetes mellitus with/without various complications, the gene polymorphism of cytokines and various biomarkers may reflect or control the severity and progression of various immunological phenomena associated with the disease.[14]

Transforming growth factor-β1 (TGF-β1) belongs to a family of multifunctional growth factors, which have profound

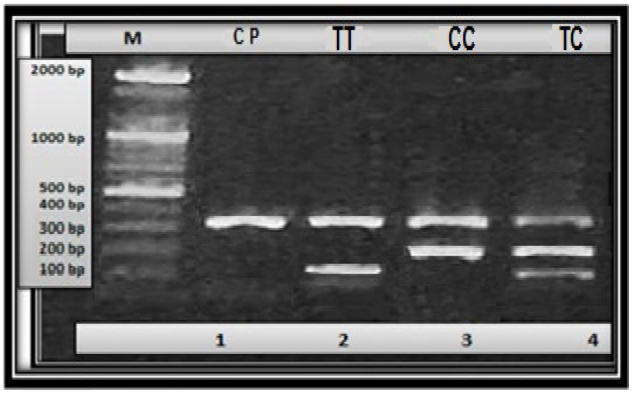

Fig. 1 **Result of gene polymorphism product on agarose gel electrophoresis.**

regulatory effects on many developmental and physiological processes.[17] The human TGF-β1 gene is located on chromosome 19q13.3–13.1 and more than 10 polymorphic loci are presently known, distributed in exons, introns, and the -5′flanking region.[18] The polymorphisms at codons 10 may be associated with higher or lower TGF-β1 synthesis *in vitro*.[19] Increases or decreases in the production of TGF-β1 have been linked to numerous diseases including atherosclerosis, and fibrotic diseases of the kidney, liver, and lung.[20] Polymorphism of TGF-β1 at codon 10 has been reported to be associated with higher or lower TGF-β1 synthesis.[21] In this sense, TGF-β1 polymorphisms may be associated with the susceptibility of T2DM. Single nucleotide polymorphism (SNP) at TGF-β1 has been linked with an increased likelihood of having diabetic nephropathy. It has been reported that the TGF-β1 gene participates in the development of renal hypertrophy and in the accumulation of extracellular matrix in diabetes.

The TGF-β1 is a pleiotropic cytokine and a key player in immune regulation and an important role in the activation of inflammation and the resolution of inflammatory responses in a variety of autoimmune diseases.[22,23] TGF-β1 also stimulates glucose uptake by enhancing the expression of glucose transporter 1 (GLUT1) in mesangial cells that leads to intracellular metabolic abnormalities in DM.[24] TGF-β1 regulates the production of almost every molecule of the extracellular matrix (ECM).[25] The central feature of T2DM is an alteration in the composition of the ECM, including thickening of the glomerular basement membrane (GBM) and expansion of the mesangial matrix.[26] In terms of above mentioned evidence, TGF-β1 expression may be associated with the risk of DM.

Diabetic nephropathy (DN) is a major long-term complication DM and is the leading cause of end-stage renal disease in many parts of the world. Factors known to be associated with development and progression of DN, including hyperglycemia, increased intra glomerular pressure, mesangial cell stretch, activation of renin–angiotensin system, and hypertension, have all been shown to induce TGF-β1 production in the kidney or in cultured mesangial or tubular cells.[5,8]

The aim of the presented work is to study the associations between transforming growth factor-beta 1 (TGF-β1) gene polymorphism located in the chromosome 19q13.1–13.3 by amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) and various biomarkers including endogenous non-enzymatic antioxidant reduced glutathione levels in sera of type 2 diabetic patients with/without nephropathy complications of Kerbala province : Iraq.

Materials and Methods

This case-control study was performed on 100 patients with diabetic nephropathy, 100 patients with only type 2 diabetes mellitus and another 100 samples obtained from apparently healthy individuals as control group. Five ml of whole blood was drawn from each of them, 4.0 ml was collected in a gel tube for serum separation and the remaining 1.0 ml was collected in EDTA tube and used for each of glycated hemoglobin, HbA1c % determination and genomic DNA extraction used for molecular analysis.

Fasting blood glucose, HbA1c %, urea, creatinine and reduced glutathione were determined by using spectrophotometric enzymatic procedures. Transforming growth factor β1 gene was genotyped for the T > C (T869C) single nucleotide polymorphism by PCR-ARMS technique by using genomic DNA extracted. All patients were admitted in Al-Hussein Teaching Hospital, Al-Hussein Medical City, Kerbala Health Directorates/Kerbala - Iraq. T2DM was selected according to the criteria of American Diabetes Association 2010.[27] The age of patients and control groups was matched and ranged between 45 – 65 years at disease onset, and the mean duration of diabetes was 9.13 ± 5.24 years. Informed consent was obtained from all the study subjects. All investigations were done in accordance with the Health and Human Ethical Clearance Committee guidelines for Clinical Researches. Local ethical committee approved the study protocol.

DNA was extracted from each blood using a Mini kit with from whole blood by applying a protocol (Geneaid/Korea), according to the manufacture company, DNA concentration was quantified by using NanoDrop™ Spectrophotometer and hold in 1% gel agarose electrophoresis.

For special set of primers was designed using web-based software accessible from the website (http://primer1.soton.ac.uk/public-html/primer1.html). The ARMS-primers used for detection of mutation screening PIT1 were provided in Table 1.

Table 1. **Primer sequences with melting and annealing temperature**

SNP	Primer sequence (5′ to 3′)	Melting Temp	Annealing Temp.	Expected products
	Forward outer primer 5′-CCACACCA GCCCTGTTCGCG-3′	63°C		Common primer 220 bp
TGF-β1 (T/C)	Reverse outer primer 5′- TTGGACAGG ATCTGGCCGCG-3′	61°C	59°C	
	Forward inner primer (C allele) 5′-CGGGCTGCG GCTGCTTCC-3′*	60°C		157 bp
	Reverse inner primer (T allele) 5′-CCACAGCAGCG GTAGCAGCATCA-3′*	69°C		104 bp

*Underline letter represented inducer mismatch nucleotide's, bold letter represented the mutant and wild type neucleotides for C allele and T allele, respectively.

The polymerase chain reactions were optimized for 25 µl final volume using 50-100 ng genomic DNA, 200 µM each dNTP, 15 mM $MgCl_2$; 1 µl from 7 picomole of each primer (four different primers), 5X green *Taq* reaction buffer, 0.5 U of *Taq* DNA Polymerase (Promega) and completed with nuclease-free water.

The PCR product for TGF-β1 gene was first denatured for 2 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 1 minute. Then the product was annealed at 59°C for 1 minute and extension at 72°C for 1 min, and final extension at 72°C for 2 min. The amplification products were hold through 1.5% agarose gel stained with and ethidium bromide, the procedure involved all the four different primers in one reaction tube, the reaction tube have detect the mutant-type denoted by the letters (C) and the wild-type denoted as (T).

All of the statistical analysis was performed on a personal computer using the SPSS version 24 data are presented as the mean ± SD. Comparisons among different groups of patients were performed by one-way analysis of variance. The frequencies of the polymorphisms TT, TC and CC were expressed in numbers and percentages for wild-type, heterozygosity and recessive genotypes. χ^2 test was used to evaluate consistency of genotype distributions with Hardy-Weinberg equilibrium as the following formula:

$$p = f(\mathrm{AA}) + \frac{1}{2} f(\mathrm{AB}) = \text{frequency of A}$$

$$q = f(\mathrm{BB}) + \frac{1}{2} f(\mathrm{AB}) = \text{frequency of B}$$

$$p + q = f(\mathrm{AA}) + f(\mathrm{BB})\ f(\mathrm{AB}) = 1$$

$$q = 1 - p \text{ and } p = 1 - q$$

Results

The molecular basis that involved several genes polymorphisms related to type2 diabetes mellitus with and without nephropathy disease are considered to be a field of continuous studies including Iraqi studies.[28,29] Basically, the main objectives of this study which include 300 samples were to evaluate the role of transforming growth factor β1-T869C gene polymorphism (rs1982073) in the development of type 2 diabetic nephropathy complications and to verify the relationship between the SNP of TGFβ1 (T869C) with BMI, HbA1c %, renal biomarkers and glutathione. The clinical and biochemical characteristics of the recruited individuals were presented in Table 2.

The comparison of the clinical and biochemical properties revealed significant variations for all parameters except the age, gender and lipid profile.

The mean ± SD of blood urea in groups of control, T2DM without/with nephropathy complications were (27.86 ± 6.83, 26.82 ± 6.73, 116.44 ± 82.81) mg/dl respectively and was significantly difference and agreed with other study as shown in the Table 2. A highly significant result between the three groups regarding serum creatinine (P = 0.001) was also observed. The glycated hemoglobin value at baseline was associated with newly diagnosed diabetes, by using ANOVA test as shown in Table 2 which indicate a highly significant differences between T2DM with/without nephropathy complications (8.26 ± 1.12%, 11.70 ± 1.13%) as compared with control group (5.24 ± 0.34%, P = 0.001) respectively.

Table 2 indicated the mean ± SD of the three groups age studied (56.62 ± 6.55, 57.72 ± 7.13 and 59.29 ± 7.55) year for control, T2DM with/without nephropathy complications respectively, the age observed of T2DM with nephropathy complications was higher than T2DM without nephropathy complication as compared with control group.

In present study, the incidence of body mass index among patients and control groups were 29.44 ± 1.78, 25.30 ± 1.68 and 24.78 ± 2.04 (kg/m^2) for each of T2DM with nephropathy, T2DM and in control group respectively, see Table 2.

Fasting blood glucose which is a routine parameter showed a significant result as indicated in Table 2 for the three groups studied (P = 0.001), the mean ± SD observed was 138.28 ± 24.16 and 127.18 ± 31.85 mg/dl for T2DM and T2DM with nephropathy complications respectively as compared with the control group 81.01 ± 6.63 mg/dl, while the levels of endogenous non-enzymatic antioxidant reduced glutathione (GSH) was decreased significantly and reached to 0.81 ± 0.04, 1.2 ± 0.08 ng/ml in T2DM without/with nephropathy complications as compared with the control group respectively 1.85 ± 0.11 ng/ml, (P = 0.047) see Table 2.

The genomic DNA was extracted and its concentration (62.31 ± 51.53 µg/ml) and purity (1.90 ± 0.065) were estimated by the measurement of A260/A280 ratio.

Table 2. **Clinical and biochemical characteristics of study subjects**

Parameter	Control subjects Mean ± SD N = 100	T2DM without Nephropathy Mean ± SD N = 100	T2DM with Nephropathy Mean ± SD N = 100	P value
Age, year	56.62 ± 6.55	57.72 ± 7.13	59.29 ± 7.55	0.1
BMI (kg/m^2)	24.78 ± 2.04	25.30 ± 1.68	29.44 ± 1.78	0.1
FBG (mg/dl)	81.01 ± 6.63	138.28 ± 24.16	127.18 ± 31.85	0.001*
HbA1c %	5.24 ± 0.34	11.07 ± 1.13	8.26 ± 1.12	0.001*
Urea (mg/dl)	27.86 ± 6.83	26.82 ± 6.73	116.44 ± 82.81	0.001*
Creatinine (mg/dl)	0.85 ± 0.29	0.81 ± 0.28	6.0 ± 3.19	0.001*
GSH (ng/ml)	1.85 ± 0.11	0.81 ± 0.04	1.2 ± 0.08	0.047*

P < 0.05 Statistically significant: N: Number; BMI: Body mass index; FBG: Fasting blood glucose; HbA1c: Glycated hemoglobin; GSH: Reduced glutathione.

Figure 1 indicate the genotype variation of T-ARMS PCR of TGF-β1 gene which determined by common primer fragment (220 bp) represented Lane 1, lane 2: two fragment of T allele (220 and 104 bp) and lane 3: two fragment of C allele (220 and 157 bp); and Lane 4: represented three fragment of heterozygosis T and C allele (220, 157 and 104 bp); M lane: marker DNA Ladder 100–2000 bp were applied on (2%) gel electrophoresis.

Genotype frequencies of TGFβ1 gene polymorphism were indicated to be not consistent with Hardy–Weinberg equilibrium in T2DM with nephropathy complications, T2DM and the control groups, Table 3.

The genotype and allele frequencies of TGF-β1 gene polymorphism in T2DM and control persons were examined under the co-dominant, dominant and recessive models. Genotype distribution and the allele frequency showed significant changes among the comparison of the T2DM with the control group, Table 4, while a non-significant association among recessive model was observed.

The genotype and allele frequencies of TGF-β1 gene polymorphism codon 10 in T2DM in with and without nephropathy were examined under the co-dominant, dominant, recessive and additive models.

Genotype distribution and the allele frequency showed highly significant changes among the comparison of T2DM with nephropathy complications under dominant and co-dominant models, Table 5.

To verify the involvement of the investigated SNP in directing the changes of the pathophysiology inT2DM with/without nephropathy complications, the data analyzed were with respect to the distribution of the genotypes. Genotypes of the TGFβ1 gene were considered, it is also may be find a significant changes of blood glutathione concentration in relevance to the distribution of the genotypes in patients with T2DM and T2DM with nephropathy complications. The analyses were carried out by using the ANOVA test of data among the various groups. When the co-dominant model was considered for T2DM patient, significant variation was obvious for FBG (P = 0.04), HbA1c (P = 0.005), and glutathione (P = 0.001) in patients with TT, TC, and CC genotypes as shown in Table 7, while Table 6 indicate that creatinine and urea are non-significant in the type 2 diabetic group (P = 0.23), (P = 0.497) respectively.

When the T2DM patient data were analyzed under the dominant model, the same result is presented. There is also significant in FBG (P = 0.05), HbA1c (P = 0.004) and

Table 3. **Hardy–Weinberg equilibrium examination of TGFβ1 gene polymorphism in DN, T2DM and control individuals**

Study Group	Genotype			χ^2	*P* value	Frequency	
	TT	TC	CC			T	C
T2DM with Nephropathy	27% (0.27)	61% (0.61)	12% (0.12)	6.028		0.575	0.425
T2DM	49% (0.49)	44% (0.44)	7% (0.07)	0.488	0.001	0.71	0.29
Control	69% (0.69)	27% (0.27)	4% (0.04)	0.484		0.825	0.175

χ^2: Chi square; $P < 0.05$ Statistically significant; C: Cytosine; T: Thymine

Table 4. **Distribution of genotype and allele frequency of TGFβ1 gene polymorphism in T2DM and control groups**

TGF-β1 gene	Control *N* = 100	T2DM *N* = 100	Unadjusted OR (95% CI)	*P* value
Co-dominant				
TT (Reference)	69	49		
TC	27	44	2.29 (1.25–4.19)	0.006*
CC	4	7	2.46 (0.68–8.88)	0.16
Dominant				
TC + CC	31	51	2.31 (1.30–4.12)	0.004*
Recessive				
TT + TC (Reference)	96	93		
CC	4	7	1.80 (0.51–6.37)	0.35
Additive				
2 (CC) + TC	35	58		

*$P < 0.05$ Statistically significant; C: Cytosine; T: Thymine

Table 5. **Distribution of genotype and allele frequency of TGFβ1 gene polymorphism in T2DM without and with nephropathy complications**

TGF-β1 gene	Control $N = 100$	T2DM $N = 100$	Unadjusted OR (95 % CI)	*P* value
Co-dominant				
TT (Reference)	49	27		
TC	44	61	2.51 (1.36–4.62)	0.002*
CC	7	12	3.1 (1.09–8.83)	0.03*
Dominant				
TC + CC	51	73	2.59 (1.43–4.68)	0.001
Recessive				
TT + TC (Reference)	93	88		
CC	7	12	1.81 (0.68–4.81)	0.23
Additive				
2 (CC) + TC	58	85		

*$P < 0.05$ Statistically significant; C: Cytosine; T: Thymine

Table 6. **Biochemical characteristics of T2DM in relevance to the genotypes of TGFβ1 gene polymorphism analyzed under co-dominant model**

Clinical Characteristics	TT ($N = 58$) Mean ± SD	TC ($N = 35$) Mean ± SD	CC ($N = 7$) Mean ± SD	*P* value
BMI (kg/m^2)	25.15 ± 1.60	25.66 ± 1.81	24.71 ± 1.61	0.23
Age, year	54.76 ± 4.41	61.028 ± 5.83	59.42 ± 5.19	0.53
FBG (mg/dl)	133.14 ± 17.98	126.87 ± 27.27	123.59 ± 12.34	0.04*
HbA1c %	7.41 ± 1.70	8.69 ± 1.34	9.06 ± 1.30	0.005*
Urea, (mg/dl)	27.46 ± 6.83	25.74 ± 6.71	26.92 ± 6.09	0.497
Creatinine, (mg/dl)	0.86 ± 0.28	0.88 ± 0.26	0.68 ± 0.34	0.23
GSH (ng/ml)	0.71 ± 0.03	0.84 ± 0.08	0.67 ± 0.05	0.001*

*$P < 0.05$ Statistically significant, M: Male, F: Female, Y: year, BMI: Body mass index, FBG: Fasting blood glucose, HbA1c: Glycated hemoglobin, GSH: Reduced glutathione.

glutathione ($P = 0.003$), in the group of T2DM patients with the TC + CC genotypes when they were compared with those of the TT genotype, Table 6.

On the other hand there the kidney function test in no significant in this type 2 diabetic group such as serum creatinine ($P = 0.84$), urea ($P = 0.26$), Table 7.

On the other hand the kidney function test is highly significant in co-dominant group such as serum creatinine ($P = 0.002$), serum urea ($P = 0.001$) because this group present patients that with nephropathy complication and it the cause of abnormality of renal function tests, Table 8.

When type 2 diabetic with nephropathy complications data were analyzed under the dominant model, the same result is presented with the same significance in the group of patients with the TC + CC genotypes when they were compared with those of the TT genotype as showed in Table 8. On the other side the creatinine and urea in highly significant in this group such as creatinine ($P = 0.001$), urea ($P = 0,006$) because this group include patients with nephropathy complication and it the cause of abnormality of renal function test.

Discussion

The data observed of the two patients groups presented the gender distribution between male and female. Type 2 diabetes mellitus with and without nephropathy complications indicated that the male is higher than female, epigenetic mechanisms, nutritional factors and sedentary lifestyle affect risk and complications differently in both sexes. Furthermore, sex hormones have a great impact on energy metabolism, body composition, vascular function, and inflammatory responses. Both biological and psychosocial factors are responsible for sex and gender differences in diabetes risk and outcome. Overall, psychosocial stress appears to have greater impact on woman rather than on men.[30] In addition, women have greater increases of cardiovascular risk, myocardial infarction, and stroke mortality than men, compared with non-diabetic subjects.[31] However, when dialysis therapy is initiated, mortality is comparable in both males and females.[32]

The high BMI increased the incidence of some diseases, such as diabetes mellitus, hypertension, and lipid

Table 7. **Biochemical characteristics of T2DM in relevance to the genotypes of TGFβ1 gene polymorphism analyzed under dominant model**

Clinical Characteristics	TT ($N = 58$) Mean ± SD	TC + CC ($N = 42$) Mean ± SD	*P* value
BMI (kg/m²)	25.15 ± 1.60	25.50 ± 1.79	0.31
Age, year	54.76 ± 4.41	57.50 ± 5.70	0.41
FBG, (mg/dl)	133.14 ± 17.98	122.64 ± 20.83	0.05*
HbA1c %	7.41 ± 1.70	8.83 ± 1.40	0.004*
Urea (mg/dl)	27.46 ± 6.83	25.94 ± 6.56	0.26
Creatinine (mg/dl)	0.86 ± 0.28	0.84 ± 0.28	0.84
GSH (ng/ml)	0.71 ± 0.03	0.91 ± 0.07	0.003*

P < 0.05 Statistically significant, M: Male, F: Female, BMI: Body mass index, FBG: Fasting blood glucose, HbA1c: Glycated hemoglobin A1c, GSH: Reduced glutathione.

Table 8. **Biochemical characteristics of type 2 diabetes mellitus with nephropathy in relevance to the genotypes of TGFβ1 gene polymorphism analyzed under co-dominant model**

Clinical Characteristics	TT ($N = 27$) Mean ± SD	TC ($N = 61$) Mean ± SD	CC ($N = 12$) Mean ± SD	*P* value
BMI (kg/m²)	26.930 ± 3.060	26.007 ± 2.053	25.504 ± 1.53	0.13
Age, year	56.75 ± 8.22	59.18 ± 6.66	61.00 ± 3.83	0.99
FBG (mg/dl)	136.26 ± 38.06	126.96 ± 30.47	107.859 ± 5.816	0.03*
HbA1c %	7.70 ± 1.09	8.32 ± 1.25	9.04 ± 1.20	0.006*
Urea (mg/dl)	136.26 ± 38.06	126.96 ± 30.47	57.54 ± 6.02	0.001*
Creatinine, (mg/dl)	7.46 ± 2.91	5.78 ± 3.34	3.82 ± 0.52	0.002*
GSH (ng/ml)	0.82 ± 0.04	0.71 ± 0.04	0.93 ± 0.08	0.002*

*$P < 0.05$ statistically significant, M: male, F: female, BMI: body mass index, FBG: fasting blood glucose, HbA1c: glycated hemoglobin A1c, GSH: reduced glutathione.

Table 9. **Biochemical characteristics of diabetic nephropathy in relevance to the genotypes of TGFβ1 gene polymorphism analyzed under dominant model**

Clinical Characteristics	TT ($N = 58$) Mean ± SD	TC + CC ($N = 42$) Mean ± SD	*P* Value
BMI (kg/m²)	25.15 ± 1.60	25.50 ± 1.79	0.31
Age, year	56.76 ± 4.41	58.50 ± 5.70	0.41
FBG (mg/dl)	133.14 ± 17.98	122.64 ± 20.83	0.05*
HbA1c %	7.41 ± 1.70	8.83 ± 1.40	0.004*
Urea (mg/dl)	27.46 ± 6.83	25.94 ± 6.56	0.006*
Creatinine (mg/dl)	0.86 ± 0.28	0.84 ± 0.28	0.001*
GSH (ng/ml)	0.82 ± 0.04	0.90 ± 0.03	0.004*

*$P < 0.05$ Statistically significant, M: Male, F: Female, BMI: Body mass index, FBG: Fasting blood glucose, HbA1c: Glycated hemoglobin A1c, GSH: Reduced glutathione.

abnormalities.[33] Also changes of BMI values observed are indicated to be non-significant among the three groups of genotypes for patients and controls, but it is very difficult to speculate and obtain correct decision, since changes are not stratified.

Several facts may account for the association between TGF-β1 gene polymorphism with the risk of T2DM. TGF-β1 plays a role of both pro-inflammation and anti-inflammation in many pathophysiological conditions. TGF-β1 inhibits and reverses the activation of macrophages and down regulates central effector mechanisms of the innate immunity.[34] The innate immune system modulates the effects of many factors, such as genes, fetal programming, nutrition, and age on the later development of metabolic sequel associated with insulin resistance.[35] On the other hand, TGF-β1 can also positively regulate immune responses. TGF-β1 could possibly prevent or slow down the autoimmune-mediated destruction of pancreatic Langerhans islets, leading to an absolute lack of insulin production.[36] In this sense, the activation of the innate immune system and the development of a systemic low-grade chronic inflammation are closely involved in the development of T2DM. In terms of above-mentioned evidence, TGF-β1 is closely associated with the susceptibility of T2DM. Second,

the ability of an individual to produce high or low levels of TGF-β1 may be genetically predetermined. Gene polymorphisms can influence cytokine production or function; they may contribute to genetic predisposition to the disease.[34] The C allele was repeatedly associated with increased TGF-β1 production, resulting from a leucine to proline substitution in the signal amino-acid sequence of the protein, which indicated that certain allele/genotype may affect the risk of T2DM.

The data of the presented study found that codon 10 TC genotype increased genotype at the TGF-β1 and may be increase the risk of T2DM, which was consistent with the notion that C allele was linked to an increased production of TGF-β1.

The inflammatory and anti-inflammatory activities of TGF-β1 and its signaling pathway is often inactivated by mutation or altered expression of its component during pancreatic disease progression such as T2DM.[37] SNP in codons 10 of TGF-β1 alter the amino acid sequence and also affect TGFβ1 levels.[38] TGFβ1 (C) allele secretes almost twice as much as (T) allele. TGF-β1 over-expression is one of the most constant molecular features of pathological tissue fibrosis leading to organ failure.[39] Depending on these studies, it is expected that the documented polymorphism in codon 10 in the present work as shown in Table 5**.** The increasing percentage of TC and CC genotypes may be resulted in elevation in TGF-β1 secretion level and consequently to increased incidence of T2DM. Therefore, it is expected from this study that T allele is protective while C allele is susceptible for occurrence of T2DM.[38]

One of the major growth factors involved in extracellular matrix accumulation in fibrotic disorders, including type 2 diabetic nephropathy (DN), is TGF-β1 which represents important regulator of tissue fibrosis that plays a pivotal role in the pathogenesis of nephropathy complications.[40] Involvement of TGF-β1 in DN has been indicated by prior findings that protein and mRNA production of TGF-β1 were significantly enhanced in the renal tissues of patients with DN.[41] TGF-β1 expression is especially increased in mesangial cells of diabetic glomeruli**,** but increased TGF-β1 expression in glomerular endothelial cells has been also reported.[42] Inhibition of TGF-β1 results in prevention of fibrosis under experimental diabetic conditions. Elevated concentration of TGF-β1 could induce renal hypertrophy and promote excessive accumulation of extracellular matrix.[43] These pathological changes contribute to the initiation and progression of nephropathy complications in T2DM.

The association results between the TGF-β1 T869C polymorphism and T2DM with nephropathy risk showed contradictory results on TGF-β1 T869C (codon 10) polymorphism. They confirmed that the C allele and the C allele-containing genotypes (TC and CC) were associated with increased risk of nephropathy complicated as compared with T allele, and TT genotype in all studied population. Although the exact mechanism underlying the effect of TGF-β1 T869C polymorphism on T2DN susceptibility was not well known, multiple studies conducted in various populations suggested that the T869C polymorphism was associated with altered TGF-β1 protein expression.[44]

Elevated concentration of TGF-β1 could induce renal hypertrophy and promote excessive accumulation of extracellular matrix,[45] and exactly these pathological changes contribute to the initiation and progression of nephropathy complications.

Evidences from in vivo and *in vitro* studies have indicated that increased concentration of glucose could stimulate TGFβ1 expression both in cultured renal cells and in the kidney which suggested that TGF-β1 might play an important role in the etiology of T2DN in T2DM.[41]

To verify the involvement of the investigated SNP in directing the changes of the pathophysiology in type 2 diabetic with and without nephropathy patients, data were analyzed with respect to the distribution of TGFβ1genotypes.

TGF-beta increases reactive oxygen species production and decreases the concentration of glutathione (GSH), the most abundant intracellular free thiol and an important antioxidant, which mediates many of the fibrogenic effects of TGF-beta in various types of cells. A decreased GSH concentration is also observed in human fibrotic diseases and in experimental fibrosis models. Although the biological significance of GSH depletion in the development of fibrosis remains obscure, GSH and N-acetylcysteine, a precursor of GSH, have been used in clinics for the treatment of fibrotic diseases.[46,47]

In this study, we showed that TGF-β1 decreased the intracellular GSH content in sera of T2DM without and with nephropathy complications. It has been reported that TGF-β decreases glutathione (GSH), the most abundant intracellular non-protein thiol and important antioxidant in epithelial and

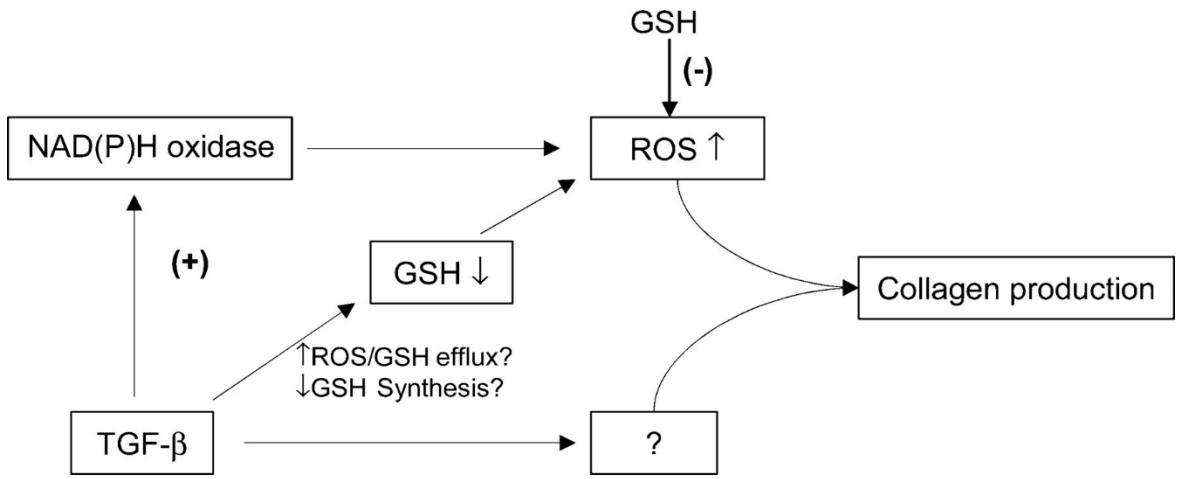

Fig. 2 **Schematic diagram of relationship between GSH depletion, ROS, and TGF-β-stimulated collagen production.**

endothelial cells.[48–50] The decrease in GSH content preceded the increase in collagen synthesis, suggesting that GSH depletion may play a role in TGF-β-stimulated collagen production.

The data of the presented work indicate significant changes of blood glutathione concentration in relevance to the distribution of the genotypes in patients of T2DM with/without nephropathy. The analyses were carried out by using the ANOVA test of data among the various groups. Then the co-dominant model was considered for diabetic nephropathy patient, significant variation was obvious for FBG, HbA1c, glutathione, in patients with TT, TC, and CC genotypes as shown in Table 8**.** The significant result presented diabetic with and with endogenous antioxidant glutathione it may be caused by different causes, such as the life style, environment pollution different studies that showed patients with diabetic have elevated ranges of oxidative stress**,** and that mean elevated with free radicals which is danger and accelerate the presence of diabetic complications by DNA damage that cause different SNP and mutations such as that we studied.[50]

The significant result presented between diabetic with and without nephropathy with endogenous antioxidant glutathione it may be caused by different causes, such as our life style, environment pollution, and that mean elevated with free radicals which is danger and accelerate the presence of diabetic complications by DNA damage that cause different SNP and mutations such as that we studied**.**

Conclusion

Tetra primer-ARMS PCR technique which applied in this tudy was effective assay and time saving for genotyping (T/C) of TGFβ1 gene. The results obtained were concluded that**:**

1. TGFβ1 gene polymorphism (T869C) is associated with Type 2 diabetic nephropathy patients.
2. TGFβ1 gene polymorphism (T869C) is highly associated with glutathione, FBG, HbA1Ic, urea and creatinine in T2DM with nephropathy complications.
3. The reduced glutathione, FBG, HbA1c, urea and creatinine show a significant result with T2DN.
4. ARMS sequencers primer enjoyable as useful gene for detection of patient have TGFβ1 polymorphism.

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