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Transcription factor 7 like 2 gene polymorphism and advanced glycation end products as risk factors for diabetic nephropathy

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Abstract

Diabetic nephropathy (DN) is the most serious and prevalent complication, being a major contributing factor to end-stage renal disease, that can lead to death in diabetic patients. The main pathologic changes of DN are the accumulation of advanced glycation end products and growth factors. Genetic variations may have an impact on the initiation and development of DN. Transcription factor 7 like 2 (TCF7L2) gene may contribute to the etiology of DN in combination with other genes. We aimed to determine whether the genotype distribution of single nucleotide polymorphism rs7903146 of TCF7L2 gene and level of advanced glycation end products (AGEs) could be risk factors for DN. The study included 54 diabetic patients, classified as 36 diabetic patients with nephropathy and 18 diabetic patients without nephropathy. The frequency of genotypes of SNP rs7903146 of TCF7L2 gene in diabetic patients without nephropathy was CC: 50%, CT: 33.3% and TT: 16.7%, while in diabetic patients with nephropathy was CC: 16.7%, CT: 69.4% and TT: 13.9%. In diabetic patients with nephropathy, risk allele carrier genotypes (CT+TT) represented 83.3% compared to 50% in diabetic patients without nephropathy. The frequency of allele distributions of SNP rs7903146 C/T of TCF7L2 gene in diabetic patients without nephropathy was C allele: 66.7% and T allele: 33.3%, while in diabetic patients with nephropathy was C allele: 51.4% and T allele: 48.6%. The T allele of SNP rs7903146 C/T of TCF7L2 gene was predominant in diabetic patients with nephropathy. Also, AGEs were significantly elevated in diabetic patients with nephropathy. In conclusion, the T allele of SNP rs7903146 C/T of TCF7L2 gene and risk allele carrier genotypes (CT+TT) were associated with the susceptibility to development of nephropathy in type 2 diabetes mellitus patients. AGEs could be a good biomarker in monitoring progression of DN disease.

Keywords: Diabetic nephropathy, Transcription factor 7 like 2, Advanced glycated end products.

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Introduction

Diabetic nephropathy (DN) is one of the typical microvascular complications of diabetes, which is a multifactorial disorder that occurs in more than one third of patients with long standing diabetes mellitus.[1] Detection of DN as early as possible is the best chance for delaying or

preventing progression to end stage renal disease. [2] The main pathologic changes of DN are the accumulation of advanced glycation end products (AGEs), results in proteinuria, hypertension, and constant decreased kidney function. Genetic variations may have an impact on the initiation and development of DN. Transcription factor 7 like 2 (TCF7L2) gene polymorphisms may contribute to the etiology of DN in combination with other genes.[3]

TCF7L2 gene was mapped on chromosome 10q25.3. TCF7L2 is a key component of the Wnt-signaling pathway and plays an important role in the regulation of insulin secretion by pancreatic beta cells and maintenance of glucose homeostasis. TCF7L2 is widely expressed in mature pancreatic beta cells as well as in peripheral and omental adipocytes.[4] Variation in TCF7L2 gene involving two single nucleotide polymorphisms (SNPs): rs12255372 (G/T) and rs7903146 (C/T). SNP rs7903146 C/T of TCF7L2 gene is more associated with type 2 diabetes mellitus (T2DM) and DN.[5] Regarding the SNP rs7903146 C/T of TCF7L2 gene, the mutant TT genotype and/or the T allele frequency were significantly associated with diabetic patients who developed nephropathy when compared to diabetic without nephropathy, indicating that T allele may be a susceptible factor for DN.[5.]

Some studies indicated that there is a relation between SNP rs7903146 C/T of TCF7L2 gene and DN, which related to the activin receptor like kinase 1 (ALK1)/Smad1 pathway.[3,5,6] Additionally, AGEs increase the expression of TCF7L2 via transforming growth factor-β (TGF-β), which helped the transfer TCF7L2 from the cytoplasm to the nucleus.[5] Subsequently, TCF7L2 is combined with the promoter of ALK1 to increase its expression. ALK1 enhanced the effect of TGF-β and promoted the phosphorylation of cellular Smad1, resulting in glomerular sclerosis.[5,6]

Advanced glycation end products are generated by non-enzymatic reaction of amino groups in lipids and proteins with reducing sugars. Initially Schiff's bases are formed (within hours) followed by their Amadori rearrangement products (within days) and finally they are irreversibly converted into AGEs (within weeks). Although this process occurs in normal metabolism, it is more widespread in some conditions such as oxidative stress, hyperlipidemia, and hyperglycemia.[7] AGEs accumulate in glomerular basement membrane, mesangial cells, and podocytes of the diabetic patients as the kidney is the major site of AGEs clearance. The receptors of AGEs (RAGE) are expressed by mesangial cells, tubular cells, and podocytes.[8] The AGEs-RAGE interaction is considered as a causative factor for DN through activating a series of intracellular signal-cascade pathways which lead to mesangial expansion, tubular inflammation, and glomerulosclerosis. AGEs are recognized as the early markers for renal injury in diabetic patients.[8] This work aimed to evaluate the association between genotype and allele distribution of SNP rs7903146 C/T of TCF7L2 gene and risk for occurrence of nephropathy in diabetic patients. This study also aimed to determine the correlation of AGEs level in diabetic patients with and without nephropathy.

Patients and Methods

The study included 54 patients of type 2 diabetes; their age ranged from 30 to 70 years. These patients were recruited from the Diabetic Outpatient Clinic of the Internal Medicine Department, Assiut University Hospital during the period from October 2020 to April 2021. The study patients were classified into two groups: diabetic patients without any microvascular or macrovascular complications (18 patients) and diabetic patients with nephropathy (36 patients).

Diagnosis of DN was defined by persistently albuminuria (albumin /creatinine ratio (ACR) > 30 mg albumin/g creatinine) and/or persistently reduction in the estimated glomerular filtration rate (eGFR) below 60 ml/min. While patients with ACR < 30 mg albumin/g creatinine and/or eGFR above 60 ml/min had no nephropathy [9].

Patients with kidney diseases other than diabetic nephropathy, macrovascular complications (cardiovascular, cerebrovascular), type 2 diabetic patients with retinopathy and/or neuropathy, type 2 diabetic patients on insulin

therapy, type 1 DM, and secondary DM were excluded from the study.

All study individuals were subjected to full history taking including age, residence, duration of DM, family history of DM, family history of DN. Clinical examination included measurement of blood pressure, and weight and height for calculation of body mass index (BMI).

A venous blood sample (8 ml) was withdrawn from each patient and divided into two portions. One portion (4 ml) was collected into two tubes (2 ml in each tube) of Ethylene-Diamine Tetra Acetic Acid (EDTA). One tube was used for complete blood count (CBC) and Glycated Hemoglobin (HbA1c) assay and the second one stored at −20°C for detection of polymorphism of TCF7L2 genes.

The second portion, (4 ml) was collected into a plain vacutainer tube, allowed to clot and serum separated, divided into aliquots, One aliquot for routine investigations and the second for determination of AGEs. CBC was done by using a hematology analyzer (ADVIA 2120i, Siemens Healthineers, USA), according to the manufacturer's instructions. Other routine investigations including urea, creatinine, uric acid, blood glucose and HbA1c, and insulin were done by using a chemistry analyzer (ADVIA 1800 Chemistry Auto-Analyzers, Siemens Healthineers, USA), according to the manufacturer's instructions.

Insulin Resistance (IR) was measured by Homeostasis Model Assessment (HOMA-IR).

HOMA-IR= Fasting insulin (mU/l) × fasting glucose (mmol/l)/22.5

Estimated GFR using the Cockcroft-Gault equation was calculated as follows:[10]

eGFR = ((140-age) × (weight in kg))/ serum creatinine (mg/dl×72) × (0.85 if female)

AGEs were determined by enzyme linked immunosorbent assay (ELISA) commercial kits (Catalog No: 10417, Glory Science, USA), according to the manufacturer's instructions. The optical density of the final ELISA product was measured at a wavelength of 450 nm by using a microtiter reader (Stat Fax 2100, Awareness Technology Inc., USA). The concentration of AGEs in the samples was then determined by comparing the optical density of the samples to a standard curve.

Detection of SNP rs7903146 of TCF7L2 gene polymorphism by Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP):

For performing the PCR-RFLP, genomic DNA was first isolated. Blood samples collected on EDTA were used for separation of genomic DNA using commercial kits (Catalog number: K0721, GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific, Baltics), according to the manufacturer's instructions.

Genotyping of SNP rs7903146 of TCF7L2 gene: The SNP rs7903146 of TCF7L2 gene was genotyped by amplifying 180 bp intron 3 regions of TCF7L2 gene by using commercial kits (Catalog number: K1081, Dream Taq Green PCR Kit, Thermo Fisher Scientific, Baltics), according to the manufacturer's instructions. The used primers were forward Primer: 5` ACA ATT AGA GAG CTA AGC ACT TTT TAG GTA 3`, and reverse Primer: 5` GTG AAG TGC CCA AGC TTC TC 3.

The total volume of the PCR amplification reaction was 25µL, including 2.5 µL 10 X PCR loading buffer, 2.0 µL dNTP mix, 1.0 µL forward primer, 1.0 µL reverse primer, 2.0µL Taq DNA polymerase, 2.0µL genomic DNA template, 2.0 µL MgCl2 solution, and 12.5 µL sterilized double-distilled water.

Non-specific amplification was prevented using touchdown thermal cycling. The program included first denaturation at 95°C for 3 minutes and 10 cycles composed of denaturation at 95°C for 30 seconds, annealing at 65°C with 1°C decrease per cycle for 30 seconds, and extension at 72°C for 45 seconds, and accomplished with 24 additional cycles when annealing temperature reached 60°C. Following amplification, the 188-bp PCR products were checked in 1% agarose gel electrophoresis.

After amplification, the PCR product was digested with RsaI restriction enzyme. This restriction enzyme was derived from *Rhodopseudomonas sphaeroides.* The enzyme digestion reaction was with a total volume of 20µL, includithe2.0 µL 10 X buffer, 1 µL of RsaI

restriction enzyme for rs7903146 at a concentration of 10 U/µL, 10 µL PCR products, 7.0 µL double distilled water. The 188-bp PCR products were incubated overnight at 37°C with RsaI restriction enzyme (RsaI, Thermo Fisher Scientific, Catalog number: ER1121, Baltics). DNA fragments were analyzed using 3% agarose gel electrophoresis. The RsaI enzyme cuts the restriction site 5'GT▼AC3' at the C allele of the SNP and yielded two fragments, 159 and 29 bp, but the T allele (mutant) was not digested, remaining the intact PCR product. So, the digestion of homozygous wild type (C/C) rs7903146 produced two fragments, 159 bp and 29 bp, whereas digestion of homozygous mutant type (T/T) rs7903146 was not cleaved and its PCR products remained at the original size (188 bp). Whereas digestion of heterozygous (C/T) rs7903146 produced three fragments with 188 bp, 159 bp and 29 bp.

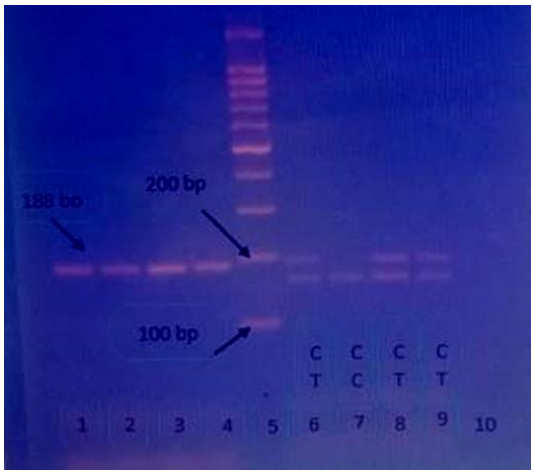

Figure 1. Electrophoresis image of the PCR products. Lane 1,2,3 and 4 are PCR products without RsaI digestion. Lane 5 is a 100 bp ladder. Lane 6,7,8 and 9 are PCR products after digestion with RsaI. Lane 10 is a negative control.

Statistical Methods

Data were analyzed by using the Statistical Package for the Social Science (SPSS, version 20, IBM, and Armonk, New York). The Shapiro test was used to determine compliance of the data to normal distribution. Based on this test, it was found that age, BMI, uric acid, eGFR, cholesterol, HDL-c, LDL-c were normally distributed quantitative data while the other quantitative data in the current study were abnormally distributed. Quantitative data with abnormal distribution were compared by Mann-Whitney U test. Quantitative data with normal distribution were compared by the Student t test. The Chi square test was implemented on Nominal data. Spearman correlation was used to determine the correlation of AGEs with other variables in the study. The receiver operator characteristics (ROC) curve was used to assess the diagnostic accuracy of AGEs in prediction of nephropathy in type 2 diabetic patients. Level of confidence was kept at 95% and hence, *p* value was considered significant if < 0.05.

Results

We found that diabetic patients with nephropathy had significantly higher mean age compared to diabetic patients without nephropathy (*p*= 0.02). The duration of DM and the percentage of family history of DM and DN were significantly higher in diabetic patients with nephropathy compared to diabetic patients without nephropathy (*p* <0.001 for each) (Table 1).

Table 1. Demographic data among the studied groups.

Studied parameters	Diabetic without nephropathy (n= 18)	Diabetic with nephropathy (n= 36)	*p* value
Age (years)			
Range	30-60	44-70	0.02
Mean ±SD	50.22 ± 7.73	55.44 ± 7.61	

Table 1. Continued.

Studied parameters	Diabetic without nephropathy (n= 18)	Diabetic with nephropathy (n= 36)	*p* value
Duration of DM (years)			
Range	1-9	1-30	< 0.001
Mean ±SD	5.67 ± 2.54	14.50 ± 7.77	
BMI (kg/m2)			
Range	25.90-37.20	23.40-39	NS
Mean ±SD	31.22 ± 2.89	31.91 ± 4.44	
Smoking			
Yes	13 (72.2%)	32 (88.9%)	NS
No	5 (27.8%)	4 (11.1%)	
Family history of DM			
Yes	12 (66.7%)	36 (100%)	< 0.001
No	6 (33.3%)	0	
Family history of DN			
Yes	2 (11.1%)	29 (80.6%)	< 0.001
No	16 (88.9%)	7 (19.4%)	
SBP (mmHg)			
Range	110-140	100-165	NS
Mean ±SD	128.89 ± 9.63	131.53 ± 13.87	
DBP (mmHg)			
Range	70-95	75-100	NS
Mean ±SD	82.22 ± 7.52	85.97 ± 9.69	

Data are expressed as frequency (percentage), mean ± standard deviation (±SD), and range. $p > 0.05$ is not significant (NS). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; DM: diabetes mellitus; DN: diabetic nephropathy.

Serum urea, creatinine and uric acid showed statistically significant elevations in diabetic patients with nephropathy compared to diabetic patients without nephropathy (p= 0.01, p=0.04 and p=0.02, respectively) (Table 2). eGFR was significantly lower in diabetic patients with nephropathy compared to diabetic patients without nephropathy (p<0.001) (Table 2).

Table 2. Kidney function tests among studied groups.

Studied parameters	Diabetic without nephropathy (n= 18)	Diabetic with nephropathy (n= 36)	*p* value
S. urea (mmol/l)			
Range	2.40-5.90	2.60-34	0.01
Mean ± SE	3.70 ± 0.99	8.23 ± 1.24	
S. creatinine (µmol/l)			
Range	56-97	51-624	0.04
Mean ± SE	71.38 ± 2.71	137.52 ± 22.51	
S. uric acid (mg/dl)			
Range	2.40-6	2.90-9.10	0.02
Mean ± SD	4.90 ± 0.95	5.79 ± 1.47	
eGFR (ml/min)			
Range	91.50-126.50	16-135	< 0.001
Mean ± SD	99.77 ± 15.47	81.60 ± 27.37	

Data are expressed as mean ± standard deviation (±SD), range. eGFR: estimated glomerular filtration rate. Significance was set at p< 0.05.

AGEs and HOMA-IR were significantly higher in diabetic patients with nephropathy compared to diabetic patients without nephropathy (p= 0.03 and p=0.02, respectively) (Table 3).

Table 3. Glycemic profile and advanced glycated end products among studied groups.

Studied parameters	Diabetic without nephropathy (n= 18)	Diabetic with nephropathy (n= 36)	*p* value
Fasting serum glucose (mmol/l)			
Range	4.60-22.80	5.40-25.90	NS
Mean ± SD	11.03 ± 4.31	11.23 ± 5.53	
Glycosylated hemoglobin HbA1c (%)			
Range	5.30-10.20	5.90-13.10	0.06
Mean ± SD	8.05 ± 1.37	9.14 ± 2.19	
S. AGEs (pg/ml)			
Range	30-135	65-5980	0.03
Mean ± SE	54.44 ± 5.68	1622.89 ± 270.48	
Fasting Serum insulin (mU/L)			
Range	3.40-43.67	4.39-204.10	0.02
Mean ± SE	17.37 ± 2.65	39.29 ± 6.41	
HOMA-IR			
Range	0.92-26.39	2.93-108.80	0.02
Mean ± SE	9.24 ± 01.7	20.18 ± 4.03	

Data expressed as range and mean ± standard deviation (±SD) or standard error (SE). $p > 0.05$ is not significant (NS).
AGEs: advanced glycation end products; HbA1C: Glycosylated hemoglobin; HOMA-IR: hemostasis model assessment-insulin resistance.

Genotyping and allele distribution of TCF7L2

In diabetic patients without nephropathy, CC genotype represented 50% with C allele represented 66.7% while in diabetic patients with nephropathy, CT genotype represented 69.4% () with T allele represented 48.6%. In diabetic patients with nephropathy, risk allele carrier genotypes (CT+TT) represented 83.3% compared to 50% in diabetic patients without nephropathy (Table 4, Figure 2, and Figure 3).

In addition, T allele in diabetic patients with nephropathy represented 48.6% compared to 33.3% in diabetic patients without nephropathy (Table 4). Genotype risk assessment for DN, CT genotype displayed considered risk for DN by 6.25 times (95% CI 1.59-24.45) more than CC genotype. While risk allele carrier genotypes CT+TT displayed considered risk for DN by 5.00 times (95% CI 1.39- 17.87) more than CC genotype. Allele risk assessment for DN indicated that T allele displayed considered risk for DN by 1.892 times (95% CI 1.23-4.351) more than C allele.

Table 4. Genotype and allele distribution of rs7903146 SNP of TCF7L2 gene among studied groups.

Studied parameters	Diabetic without nephropathy (n= 18)	Diabetic with nephropathy (n= 36)	*p* value	OR (95% CI)
Genotyping				
CC	9 (50%)	6 (16.7%)	-	Reference
CT	6 (33.3%)	25 (69.4%)	0.008	6.25 (1.59 -24.45)
TT	3 (16.7%)	5 (13.9%)	NS	2.50 (0.42-14.60)

Table 4. Continued.

Studied parameters	Diabetic without nephropathy (n= 18)	Diabetic with nephropathy (n= 36)	*p* value	OR (95% CI)
Risk allele carrier genotypes (CT+TT)	9 (50%)	30 (83.3)	0.003	5.00 (1.39 – 17.87)
Non risk allele carrier genotype (CC)	9 (50%)	6 (16.7%)	-	Reference
Allele				
C	24/36 (66.7%)	37/72 (51.4%)	-	Reference
T	12/36 (33.3%)	35/72 (48.6%)	0.03	1.892 (1.23-4.351)

Data expressed as frequency (percentage). $p > 0.05$ is not significant (NS). TCF7L2: transcription factor 7 like 2.

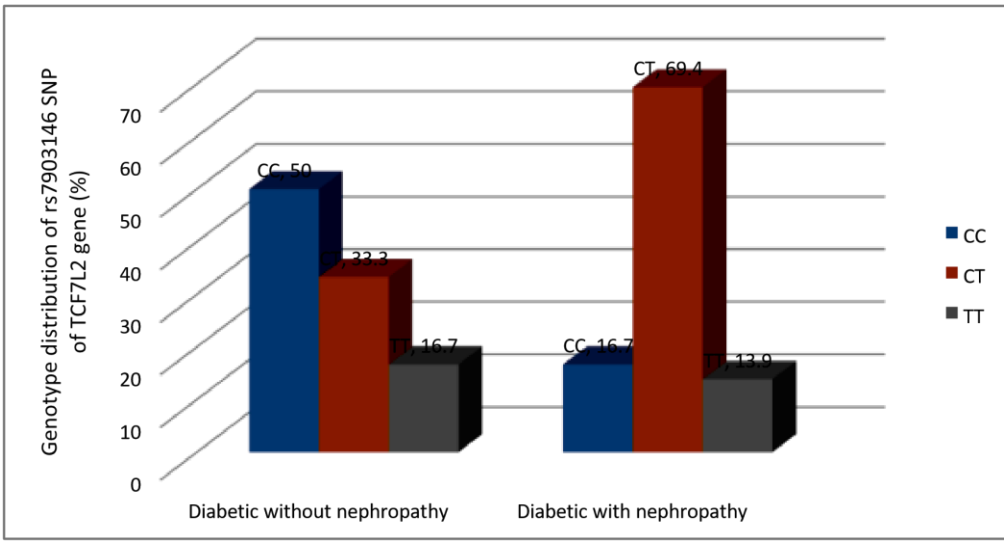

Figure 2. Genotype distribution of rs7903146 SNP of TCF7L2 gene among studied groups.

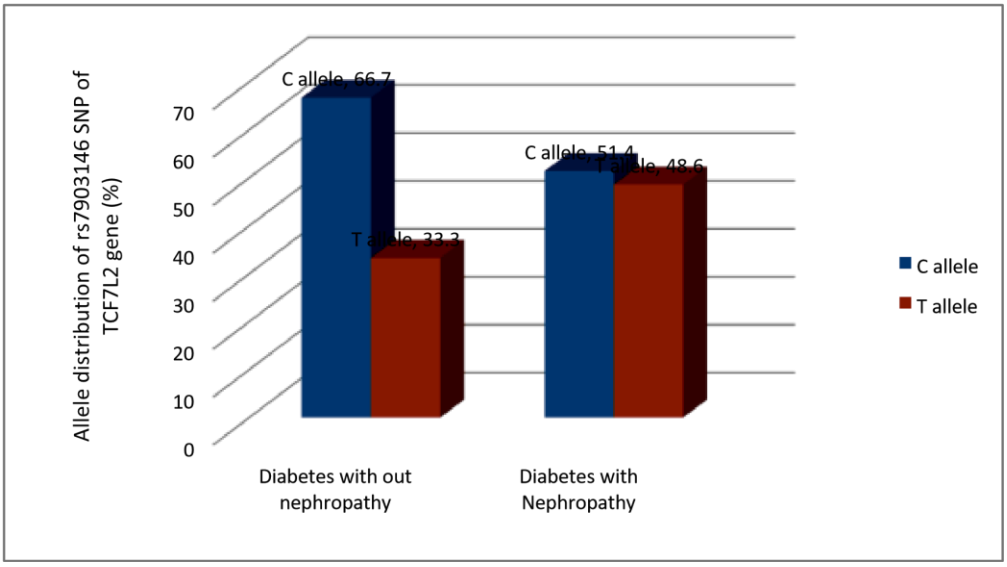

Figure 3. Allele distribution of rs7903146 SNP of TCF7L2 gene among studied groups.

eGFR was significantly lower in risk allele carrier genotypes (CT+TT) compared to non-risk allele carrier genotype (CC) (p=0.003). ACR, AGEs and HOMA-IR were significantly higher in risk allele carrier genotypes (CT+TT) compared to non-risk allele carrier genotype (CC) (p= 0.03, p=0.01 and p=0.01, respectively) (Table 5).

Table 5. Association between different genotypes of rs7903146 SNP of TCF7L2 gene and laboratory parameters in diabetic patients with nephropathy.

Studied parameters	Non risk allele carrier genotype (CC) (n= 6)	Risk allele carrier genotypes (CT + TT) (n= 30)	*p* value
S. urea (mmol/l)			
Range	3.0-19	2.6-34	NS
Mean ± SE	7.5 ± 2.46	8.38 ± 1.42	
S. creatinine (µmol/l))			
Range	52-335	51-624	NS
Mean ± SE	120.5 ± 43.43	140.91 ± 25.81	
S. uric acid (mg/dl)			
Range	4.6-8.90	2.9-9.10	NS
Mean ± SD	6.03 ± 1.54	5.74 ± 1.48	
eGFR (ml/min)			
Range	80.6-130	16-135	0.003
Mean ± SE	105.48 ± 8.72	67.72 ± 4.79	
Albumin/Creatinine ratio (mg Albumin/gm Creatinine)			
Range	36-1378	37.5-4200	0.03
Mean ± SE	503.31 ± 250.21	601.18 ± 184.85	
Fasting serum Glucose (mmol/l)			
Range	6.10-22.60	5.4-25.90	NS
Mean ± SD	11.45 ± 6.98	11.19 ± 5.33	
Glycosylated hemoglobin HbA1C (%)			
Range	7.5-12.40	5.90-13.1	NS
Mean ± SD	10.26 ± 2.08	8.91 ± 2.17	
S. AGEs (pg/ml)			
Range	80-1450	65-5980	0.01
Mean ± SE	330.83 ±223.9	1028.16±318.48	
Fasting serum Insulin (mU/L)			
Range	4.39-48.12	8.44-204.10	NS
Mean ± SE	26.34 ± 7.34	41.88 ± 7.5	
HOMA-IR			
Range	4.4-14.74	2.93-108.80	0.01
Mean ± SE	9.7 ± 1.76	22.18 ± 4.74	

Data expressed as range and mean ± standard deviation (±SD) or standard error (SE). $p > 0.05$ is not significant (NS).

TT genotypes and risk allele carrier genotypes (CT+TT) had significant higher frequency in diabetic nephropathy patients with <10 years duration of DM compared to diabetic nephropathy patients with >10 years duration of DM (p=0.01, p=0.03, respectively). Also, T allele was significantly higher in diabetic nephropathy patients with <10 years duration of DM compared to diabetic nephropathy patients with >10 years duration of DM (p=0.01). CC and CT genotypes had significantly higher frequency in diabetic nephropathy patients with >10 years duration of DM compared to diabetic nephropathy patients with <10 years duration of DM. Also, C allele was significantly higher in diabetic nephropathy patients with >10 years duration of DM compared to diabetic nephropathy patients with <10 years duration of DM.

Genotype risk assessment for occurrence of nephropathy in <10 years duration of DM, TT genotype displayed considered risk by 5.00 times (95% CI 1.866-18.861) more than CC genotype. While risk allele carrier genotypes (CT+TT) displayed considered risk by 1.429 times (95% CI 1.13- 1.806) more than CC genotype. Allele risk assessment for occurrence of nephropathy in < 10 years duration of DM, T allele displayed considered risk by 5.5 times (95% CI 1.594- 18.979) more than C allele (Table 6).

Table 6. Genotype and allele distribution of SNP rs7903146 C/T of TCF7L2 gene among diabetic nephropathy patients according to duration of diabetes mellitus (DM).

Studied parameters	Diabetic nephropathy (n =36)		*p* value	OR (95% CI)
	Duration of DM < 10 years (n= 9)	Duration of DM ≥ 10 years (n= 27)		
Genotype				
CC	0	6 (22.2%)	<0.001	Reference
CT	4 (44.4%)	21 (77.3%)		1.19 (1.003-1.413)
TT	5 (55.6%)	0	0.01	5.0 (1.866- 18.861)
*Risk allele carrier genotypes (CT+TT)	9 (100%)	21 (77.8%)	0.03	1.429 (1.13-1.806)
*Non risk allele carrier genotype (CC)	0	6 (22.2%)		Reference
Allele				
C	4/18 (22.2%)	33/54 (61.1%)	0.01	Reference
T	14/18 (77.8%)	21/54 (38.9%)		5.5 (1.594-18.979)

Data are expressed as frequency (percentage). Significance was set at p<0.05.

AGEs in the diabetic group had positive correlations with urea (r = 0.33, p = 0.01), creatinine (r = 0.31, p = 0.02), ACR (r = 0.52, p<0.001), HbA1c (r = 0.40, p < 0.001) and fasting insulin (r = 0.45, p < 0.001) and negative correlation with eGFR (r = - 0.31, p = 0.02).

AGEs in diabetic patients with nephropathy had a positive correlation with ACR (r = 0.46, p< 0.005), HbA1c (r = 0.38, p< 0.02) and fasting insulin (r = 0.39, p <0.01) (Table 7).

Table 7. Correlations between AGEs and other variables in diabetic group and in diabetic nephropathy patients.

Studied parameters	Diabetic group (*p* value)	Diabetic patients with nephropathy (*p* value)
Age (years)	0.04 (NS)	0.03 (NS)
Duration of DM (years)	0.07 (NS)	0.05 (NS)
BMI (kg/m2)	0.25 (NS)	0.26 (NS)
Urea (mmol/l)	0.33 (0.01)	0.26 (NS)
Creatinine (µmol/l)	0.31 (0.02)	0.25 (NS)
Uric acid (mg/dl)	0.18 (NS)	0.11 (NS)
eGFR (ml/min)	-0.31 (0.02)	-0.23 (NS)
Albumin/creatinine ratio (mg Albumin/gm creatinine)	0.52 (< 0.001)	0.46 (0.005)
FSG (mmol/l)	-0.21 (NS)	-0.13 (NS)
HbA1c (%)	0.40 (< 0.001)	0.38 (0.02)
Insulin (mU/L)	0.45 (< 0.001)	0.39 (0.01)
HOMA-IR	0.26 (NS)	0.21 (NS)

Data are expressed as r (strength of correlation) and p (significance of correlation). $p > 0.05$ is not significant (NS).

To determine the optimum diagnostic cut-off value and evaluate the sensitivity of AGEs for diagnosis and prediction of DN, the ROC analysis was performed. The ROC curve was constructed to study their diagnostic performance, in such a way that the higher AUC corresponds to a better diagnostic test.

At a cut-off value of > 65 pg/mL, AGEs were 94.4 % accurate, 97.2 % sensitive and 89 % specific for diagnosis of DN, with AUC of 0.97, positive predictive value (PPV) of 94.6 % and negative predictive value (NPV) of 94.1 % (p<0.001) (Table 8 and Figure 4).

Table 8. Diagnostic performance of AGEs in prediction and diagnosis of nephropathy in diabetic patients.

	AGEs (Pg/ml)
Cutoff point	> 65
Area under curve	0.97
Accuracy	94.4%
Sensitivity	97.2%
Specificity	89%
Positive predictive value	94.6%
Negative predictive value	94.1%
p value	< 0.001

$p \leq 0.05$ is significant.

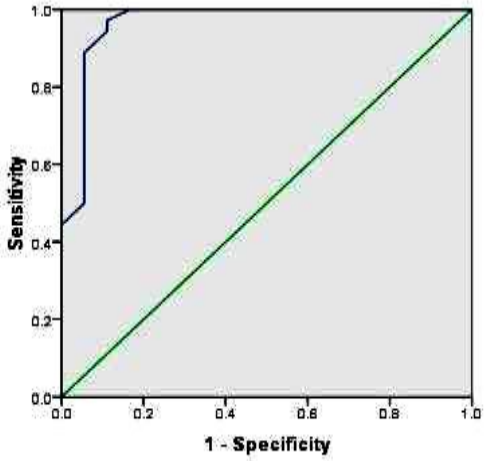

Figure 4. Receiver operator characteristics (ROC) curve of AGEs as predictors and diagnostic of nephropathy in diabetic patients.

Discussion

TCF7L2 gene is the strongest predisposing gene for initiation and development of DN, so we aimed to study the association of genotype and allele distributions of SNP rs7903146 of the TCF7L2 gene and DN. In the present study, serum urea and creatinine were statistically significantly elevated in diabetic patients with nephropathy compared to diabetic patients without nephropathy. Prolonged hyperglycemia causes irreversible damage to nephrons of kidney leading to diminishing of GFR and reduce infiltration capacity of the kidney, so we considered that blood urea and serum creatinine were the prognostic markers and predictors for renal damage in diabetic patients.[6.]

In the present study, eGFR was statistically significantly lower in diabetic patients with nephropathy compared to diabetic patients without nephropathy. Our results were consistent with those of Rabia Ali et al., 2021,[6] who reported that higher levels of ACR and lower levels of eGFR were found in diabetic patients with nephropathy than diabetic patients without nephropathy. This may be explained by thickening of glomerular basement membrane and diffuse or nodular mesangial expansion. These structural changes correlated with the level of ACR and GFR in T2DM.[11]

HBA1c did not differ in diabetic patients with nephropathy compared to diabetic patients without nephropathy. Our result agreed with that of Rabia Ali et al., 2021,[6] who reported no significant difference in HBA1c between diabetic patients without nephropathy and diabetic patients with nephropathy. Poor control of DM in both groups leading to chronic hyperglycemia which induces oxidative stress that impaired endothelial function and podocytes, leading to impaired glomerular filtration barrier, glomerular hyperfiltration and increased albumin excretion.[12]

The current study revealed that AGEs levels were significantly higher in diabetic patients with nephropathy compared to diabetic patients without nephropathy. The AGEs-RAGE interaction was considered as a causative factor for DN through activating a series of intracellular signal-cascade pathways which might induce the generation of further signaling factors, such as vascular endothelial growth factor, fibronectin, TGF-β, and nuclear factor kappa β. Those signaling factors lead to mesangial expansion, tubular inflammation, and glomerulosclerosis.[13] Also, our results revealed that AGEs levels were significantly higher in diabetic patient with macroalbuminuria compared to diabetic patient with microalbuminuria. Also, we found positive correlation between AGEs and ACR This was consistent with that of Wadén et al., 2019,[14] who reported that the lowest AGEs concentration was seen in diabetic participants with normal ACR and in those with microalbuminuria, where the highest concentration was observed in diabetic participants with macroalbuminuria and end-stage renal disease.

The extracellular matrix of podocytes and renal capillaries are negatively charged and able to prevent loss of serum albumin into the glomerular filtrate owing to charge-charge repulsion. Glycation and accumulation of AGEs in the extracellular matrix components of renal capillaries alter the electrostatic interactions that increased vascular permeability to albumin lead to micro and macroalbuminuria.[15]

In the present study, there was a strong negative correlation between AGEs and eGFR and positive correlation between AGEs with urea and creatinine in the diabetic group. Our results agreed with those of Koska et al., 2022, who reported that higher AGEs levels were associated with lower eGFR and presence of micro- and macroalbuminuria.[16] AGEs disrupt the balance between synthesis and degradation of glomerular basement extracellular matrix components particularly collagen. The cross linking of AGEs with collagen in the basement membrane were contributed to membrane thickening, impaired filtration and eventually loss of glomerular function and decreased clearance of urea and creatinine which lead to increased urea and creatinine in blood.[17]

Our result showed that there was a positive correlation between HbA1c and AGEs. DM was associated with hyperglycemia and with accelerated rate of glycation between the free amino group at N-terminal valine of β-chain of HbA1c and glucose and spontaneously there was glycation between reducing sugars and long-lived proteins, nucleic acid, and lipoprotein.[7] SNP rs7903146 C/T of TCF7L2 gene is more associated with T2DM which mediated by decreased insulin secretion associated or not with defects in insulin processing, reduced effects of glucagon-like peptide-1, increased hepatic glucose production and insulin resistance.[18]

In our study, comparing genotype distributions of SNP rs7903146 of TCF7L2 gene in diabetic patients without nephropathy and diabetic patients with nephropathy, showed that the CC genotype frequency was significantly higher in diabetic patients without nephropathy. While CT genotype frequency was significantly higher in diabetic patients with nephropathy compared to diabetic patients without nephropathy. The frequency of risk allele carrier genotypes (CT+TT) was significantly higher in diabetic patients with nephropathy, while the frequency of non-risk allele carrier genotype CC was significantly higher in diabetic patients without nephropathy compared to diabetic patients with nephropathy.

Our study showed that subjects carrying one risk allele (heterozygous genotype, CT) have about 6.25-fold risk of developing DN compared to those without any risk allele (homozygous non-carrier, CC). Subjects carrying both risk alleles (homozygous carrier, TT) have about 2.5-fold risk of developing DN compared to those without any risk allele (homozygous non-carrier, CC). Our study showed that the odds ratio for heterozygous genotype (CT) was higher than that reported by Zhuang et al., 2018, this may be due to genetic variation among different ethnicities and sample size among the two studies.[19]

Our result showed that the frequency of C allele was significantly higher in diabetic patients without nephropathy, while T allele frequency was significantly higher in diabetic patients with nephropathy compared to each other. The risk of DN was higher in risk allele (T) than non-risk allele (C) with an odds ratio of 1.892. Our results agreed with those of Rabia Ali et al., 2021, who showed that the frequency of the T allele was significantly higher in patients with DN compared to patients without DN.[6]

There was a relation between SNP rs7903146 C/T of TCF7L2 gene and DN, which related to the ALK1/Smad1 pathway, as the AGEs increased the expression of TCF7L2 via TGF-β1, which helped transfer TCF7L2 from the cytoplasm to the nucleus.[5] Subsequently, TCF7L2 combined with the promoter of ALK1 to increase its expression. ALK1 enhanced the effect of TGF-β and promoted the phosphorylation of cellular Smad1, resulting in glomerular sclerosis.[3]

The results of our study showed that the CC and CT genotype had significantly higher frequency in diabetic nephropathy patients with >10 years duration of DM compared to diabetic nephropathy patients with <10 years duration of DM. The TT genotypes had significantly higher frequency in diabetic nephropathy

patients with <10 years duration of DM compared to diabetic nephropathy patients with >10 years duration of DM. The frequency of the risk allele carrier genotypes (CT+TT) was significantly higher in diabetic nephropathy patients with <10 years duration of DM, while the frequency of non-carrier genotype (CC) was significantly higher in diabetic nephropathy patients with >10 years duration of DM compared to each other. Our results agreed with those of Rabia Ali et al., 2021, who reported that in the DN group, 71% of TT homozygous genotype had an early onset of nephropathy [6]. Also, our result showed that C allele was significantly higher in diabetic nephropathy patients with >10 years duration of DM), while T allele was significantly higher in diabetic nephropathy patients with <10 years duration of DM compared to each other. The risk of occurrence of nephropathy in < 10 years duration of DM was higher in TT genotype with an odds ratio of 5.00 (95% CI 1.866-18.861, *p*=0.01) than in CT genotype with an odds ratio of 1.19 (95% CI 1.0031.413, *p* <0.001). Also, the risk of occurrence of nephropathy in < 10 years duration of DM was higher in risk allele carrier genotypes (CT+TT) with an odds ratio of 1.429 (95% CI 1.13- 1.806, *p*=0.03). The risk of occurrence of nephropathy in < 10 years duration of DM was higher in T allele with an odds ratio of 5.5 (95% CI 1.594-18.979, *p*=0.01).

In the present study, we evaluated the diagnostic performance of AGEs for prediction of nephropathy in diabetic subjects. Our results showed that AGEs at AUC of 0.97 was 97.2% sensitive with. Our results agreed with those of Sun et al., 2019, who reported that the area under the ROC curve of AGEs was 0.82 and had high predictive values for diabetic nephropathy.[11] The sensitivity and specificity of AGEs were high, indicating their significant clinical value.

In conclusion, the T allele of SNP rs7903146 C/T of TCF7L2 gene was predominant in diabetic patients with nephropathy especially those with macroalbuminuria. Thus, the T allele of SNP rs7903146 C/T of TCF7L2 gene and risk allele carrier genotypes (CT+TT) were associated with increased susceptibility to development of nephropathy in T2DM patients. Also, risk allele carrier genotypes (CT+TT) had significantly higher frequency in diabetic nephropathy patients with <10 years duration of DM (100%) compared to diabetic nephropathy patients with ≥ 10 years duration of DM (77.8%). Screening of diabetic patients for early detection of AGEs would be a useful and relatively inexpensive laboratory test for early clinical diagnosis of nephropathy in T2DM patients as AGEs (AUC 0.97) had good diagnostic performance for prediction of nephropathy in diabetic patients. AGEs were significantly elevated in diabetic patients with nephropathy, so they could be good biomarkers in monitoring progression of DN disease.

Author Contributions

EFE, GA, EMA; performed the laboratory work. MYB, GA, EMA; made the statistical analysis. WAK, GA; examined the patients. EFE, GA, EMA; collected samples. All authors participated in Writing and reviewing the paper.

Declaration of Conflicting Interests

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Ethical approval

The study protocol was reviewed and approved by the Institutional review board (IRB) of the Faculty of Medicine, Assiut University (approval dated October 2019).

Informed consent

All study participants provide an informed consent before included in the study.

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