Association of *COL4A3* (rs55703767), *MMP-9* (rs17576) and *TIMP-1* (rs6609533) gene polymorphisms with susceptibility to type 2 diabetes

SAMIRA SARAVANI[1], DAVOOD YARI[2], RAMIN SARAVANI[2,3] and CHANGIZ AZADI AHMADABADI[4]

[1]Department of Biology, Zabol University, Zabol 98615-538; [2]Department of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Zahedan 98167-43463; [3]Cellular and Molecular Research Center, [4]Department of Cardiovascular Surgery, Zahedan University of Medical Sciences, Zahedan 98167-43463, Iran

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Abstract. Type 2 diabetes (T2D) is defined by high levels of glucose in the blood. The collagen IV level is associated with conditions of hyperglycemia and insulin resistance. Collagen type IV α3 chain (COL4A3) is a structural protein of the extracellular matrix (ECM). Matrix metallopeptidase 9 (MMP-9) is an enzyme that degrades the extracellular matrix and its activity is moderated by TIMP metallopeptidase inhibitor 1 (TIMP-1). The aim of the current study was to examine the association between genetic polymorphisms of *COL4A3* (rs55703767), *MMP-9* (rs17576) and *TIMP-1* (rs6609533) in patients with T2D. This case-control study was performed on 120 Iranian patients with T2D and 120 healthy individuals. Genotypes were analyzed using the amplification refractory mutation system-polymerase chain reaction technique. The findings demonstrated significant differences between genotypic and allelic distributions of *COL4A3* (G/T) and *MMP-9* (A/G) polymorphisms as follows: *COL4A3* (G/T); TT vs. GG, odds ratio (OR)=0.235, 95% confidence interval (CI)=0.063-0.0802 (P=0.013) and T vs. G, OR=0.592, 95% CI=0.371-0.943 (P=0.026); *MMP-9* (A/G); AG vs. GG, OR=2.429, 95% CI=1.232-4.820 (P=0.008) and A vs. G, OR=2.176, 95% CI=1.155-4.130 (P=0.013). No significant association was identified between *TIMP-1* (A/G) polymorphism and T2D in females and males. Thus, the genotypic and allelic distributions of *COL4A3* (G/T) and *MMP-9* (A/G) polymorphisms were associated with T2D. In addition, no significant association was identified in the genotypic distribution of the *TIMP-1* (A/G) gene in females and in males. Further studies in other ethnic groups are required to confirm these findings.

Correspondence to: Dr Ramin Saravani, Cellular and Molecular Research Center, Zahedan University of Medical Sciences, Khalij Fars Boulevard, Zahedan 98167-43463, Iran
E-mail: saravaniramin@yahoo.com

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Introduction

Type 2 diabetes (T2D) is a metabolic disease, which is distinguished by high levels of blood sugar and occurs when the pancreas is unable to produce enough insulin or the body is unable to use the insulin that is produced (insulin resistance) (1,2). The signs and symptoms of T2D include excessive urination, polydipsia, polyphagia, weight loss, blurry vision and fatigue (3,4). The prevalence of diabetes has been estimated to increase from 171 million in 2000 to 366 million by 2030 and is associated with macro- and microvascular disorders (3,5,6). T2D has numerous risk factors, including ageing, genetics, family history, previous gestational diabetes, ethnicity, nourishment and poverty (3,7). As one of the most well-known polygenic diseases, certain candidate genes have been detected for the risks and complex traits of T2D (8). The extracellular matrix is important to the structure and function of various cell types; furthermore, it contributes to processes, such as cell adhesion, cellular proliferation, differentiation, migration and apoptosis (9). Type IV collagen is a structural glycoprotein and the primary component of basement membranes (BMs). Each collagen molecule comprises three chains (10,11). In diabetes mellitus, marked alterations in the synthesis and structure of the extracellular matrix have been detected. A previous study has shown that collagen IV levels are associated with hyperglycemia and insulin resistance (12). Collagen type IV α3 chain (*COL4A3*) is expressed in the alveolar BM (13). It is located at position 2q35-q37 and contains 51 exons (14). Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases that degrade matrix and non-matrix proteins (15). MMPs regulate numerous normal and pathological activities (16). The *MMP-9* gene is located on chromosome 20q12.2-13.1 and contains 13 exons and 12 introns (17). The activities of MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs) (18). TIMP-1 is a secreted glycoprotein, which binds to active MMPs and inhibits their proteolytic activity (19,20). In addition, *TIMP-1* was mapped to X11p11.23-11.4 (21). The purpose of the current study was to investigate the possible association between T2D and *COL4A3* (rs55703767, G/T), *MMP-9* (rs17576, A/G) and *TIMP-1* (rs6609533, A/G) gene polymorphisms in an Iranian population.

Materials and methods

Subjects. This case-control study was performed on 120 patients with T2D and 120 healthy individuals. The mean ages were 56.34±10.8 years. Approval was obtained from the ethics committee of Zahedan University of Medical Sciences (Zahedan, Iran) and written informed consent was obtained from all participants. T2D was diagnosed according to medical records and fasting blood glucose (FBG) levels using the American Diabetic association (ADA) criteria (22); our studies have been described previously (23,24) and confirmed by at least two endocrinologists from the Diabetic Clinic at the Ali-Asghar Hospital (Zahedan, Iran). Healthy subjects were in good health and free of any comorbidity. Blood samples (5 ml) were collected from all patients and controls after a 12-h fast (between 7:00 and 9:00 am), and stored in ethylenediaminetetraacetic acid-containing tubes for DNA extraction and measurement of clinical characteristics. The FBG, cholesterol, triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), creatinine, and urea are presented in Table I.

Table I. Demographic characteristics of T2D patients.

Characteristic	T2D (n=120)	Controls (n=120)	P-value
Age (years)	56.57±10.602	56.11±11.075	0.744
Gender (f/m)	91/29	88/32	0.767
Duration of illness (years)	9.91±6.90	-	-
Fasted blood sugar (mg/dl)	172.51±77.32	75.53±12.04	0.001
TG (mg/dl)	152.47±73.50	120.25±10.12	0.001
TC (mg/dl)	175.79±38.29	190.09±15.04	0.001
HDL-C (mg/dl)	59.02±6.74	60.36±4.53	0.116
LDL-C (mg/dl)	89.82±13.00	90.22±6.03	0.878
Urea (mg/dl)	31.33±10.95	30.23±6.12	0.185
Creatinine (mg/dl)	1.02±0.264	1.01±0.233	0.352

T2D, type 2 diabetes; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; f, female; m, male.

COL4A3, MMP-9 and TIMP-1 polymorphism genotyping. Genomic DNA was extracted from peripheral blood according to the salting-out method (25). The quality of the extracted DNA was confirmed using electrophoresis (at 80 V for ~30-40 min) on 2% agarose gel and the quantity of DNA by spectrophotometry (ratio, 260/280 nm; absorption, 1.7-1.9), the isolated DNA was stored at -20˚C until further use. The *COL4A3* (rs55703767), *MMP-9* (rs17576) and *TIMP-1* (rs6609533) polymorphisms were genotyped using the amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method.

Genotyping the rs55703767 polymorphism of the COL4A3 (G/T) gene. Evaluation of the polymorphism, *COL4A3* (rs55703767) was performed using ARMS-PCR (the primers) (Pishgam Biotech Co., Tehran, Iran) are presented in Table II. For this purpose, two tubes were used to define the variant as follows: Tube 1 contained wild-allele-specific reverse primer G and a common forward primer; tube 2 contained mutant-allele-specific reverse primer T and a common forward primer. PCR was performed in a final volume of 20 μl using 2 μl genomic DNA, 7 DNase-free water (SinaClon BioScience Co., Tehran, Iran), 0.5 μl each primer and 10 μl Master Mix (Ampliqon A/S, Odense, Denmark). The amplification was performed with a primary denaturation step at 95˚C for 5 min, followed by 33 cycles at 95˚C for 30 sec, 54˚C for 35 sec, and 72˚C for 30 sec with a final extension at 72˚C for 10 min. The PCR products were assayed by electrophoresis (at 50 V for ~30-40 min) on a 2% agarose gel and the product size was 216 bp.

Genotyping the rs17576 polymorphism of the MMP-9 (A/G) gene. To detect the *MMP-9* (rs17576) variant, two tubes were used as follows: Tube 1 contained wild-allele-specific forward primer G and a common reverse primer, and tube 2 contained mutant-allele-specific forward primer A and a common reverse primer. PCR was performed in a final volume of 20 μl using 1 μl each primer, 10 μl Master Mix, 2 μl genomic DNA and 6 μ DNase-free water and the PCR cycling condition were as follows: Primary denaturation at 95˚C for 5 min, followed by 30 cycles at 95˚C for 30 sec, 53˚C for 25 sec, and 72˚C for 30 sec with a final extension at 72˚C for 5 min. The PCR products were assayed by electrophoresis (at 50 V for ~30-40 min) on a 2% agarose gel and the product size was 275 bp.

Genotyping the rs6609533 polymorphism of the TIMP-1 (A/G) gene. DNA amplification was performed using two tubes. Tube 1 contained wild-allele-specific forward primer A and a common reverse primer, and tube 2 contained mutant-allele-specific forward primer G and a common reverse primer. PCR was performed in a final volume of 20 μl using 0.5 μl each primer, 10 μl Master Mix, 2 μl genomic DNA and 7 μl DNase-free water. The PCR cycling conditions were as follows: Primary denaturation at 95˚C for 5 min, followed by 32 cycles at 95˚C for 30 sec, 61˚C for 25 sec, and 72˚C for 35 sec with a final extension at 72˚C for 5 min. The PCR products were assayed by electrophoresis (at 50 V for ~ 30-40 min) on a 2% agarose gel and the product size was 306 bp.

Statistical analysis. The statistical analysis of the data was calculated using the SPSS version 19.0 software (IBM SPSS, Armonk, NY, USA). The association between genotypes and T2D was assayed by calculating the OR and 95% CI using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference. In addition, Hardy Weinberg equilibrium (HWE) was calculated through comparison between observed and expected frequencies of genotypes associated with the investigated polymorphisms.

Results

Study population. The case and control groups were matched regarding age (patient group: 56.57±10.602 years; control

Table II. Primers used for the identification of SNPs.

SNP	Orientation	Sequence (5'-3')	Amplicon size (bp)
COL4A3 (rs55703767) G/T	R (G allele)	AGGATTACCTTAATGCCACC	
	R (T allele)	AGGATTACCTTAATGCCACA	216
	F (universal)	CTGCATTTGGGAATCATAGT	
MMP-9 (rs17576) A/G	F (A allele)	CCCAGGACTCTACACCAA	
	F (G allele)	CCCAGGACTCTACACCAG	275
	R (universal)	GTGGAAAGACAAACTGATGG	
TIMP-1 (rs6609533) A/G	F (A allele)	CTGTGTCCAATACCGTGTGATAA	
	F (G allele)	CTGTGTCCAATACCGTGTGATAG	306
	R (universal)	GGCTTCAAGATAGTCACTGG	

SNP, single nucleotide polymorphism; *COL4A3*, collagen type IV α3 chain; *MMP-9*, matrix metallopeptidase 9; *TIMP-1*, TIMP metallopeptidase inhibitor 1; F, forward; R, reverse.

Table III. Genotype and allele frequencies of selected polymorphisms in *COL4A3*, *MMP-9* and *TIMP-1* genes.

SNP	Patients, n (%)	Controls, n (%)	OR (95% CI)	P-value
COL4A3 (rs55703767) G/T				
GG	83 (69.2)	73 (60.8)	Ref.	-
GT	33 (27.5)	32 (26.7)	1.1 (0.593-2.05)	0.769
TT	4 (3.3)	15 (12.5)	0.235 (0.063-0.0802)	0.013
Allele frequency				
G	199 (82.9)	178 (74.1)	Ref.	-
T	41 (17)	62 (25.8)	0.592 (0.371-0.943)	0.026
MMP-9 (rs17576) A/G				
GG	84 (70)	102 (85)	Ref.	-
AG	36 (30)	18 (15)	2.429 (1.232-4.820)	0.008
AA	0	0	-	1.000
Allele frequency				
G	204 (85)	222 (92.5)	Ref.	-
A	36 (15)	18 (7.5)	2.176 (1.155-4.130)	0.013
TIMP-1 (rs6609533) A/G in females				
AA	27 (29.7)	27 (30.7)	Ref.	-
AG	44 (48.3)	25 (28.4)	1.760 (0.852-3.634)	0.126
GG	20 (22)	36 (40.9)	0.556 (0.259-1.192)	0.131
Allele frequency				
A	98 (53.8)	79 (44.9)	Ref.	-
G	84 (46.2)	97 (55.1)	0.698 (0.461-1.058)	0.092
TIMP-1 (rs6609533) A/G in males				
AA	17 (58.6)	15 (46.9)	Ref.	-
GG	12 (41.4)	17 (53.1)	0.62 (0.23-1.72)	0.360

P<0.05 was considered to indicate statistically significant differences. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; *COL4A3*, collagen type IV α3 chain; *MMP-9*, matrix metallopeptidase 9; *TIMP-1*, TIMP metallopeptidase inhibitor 1.

group: 56.11±11.075; P=0.744) and gender [patient group (female/male)=91/29 and control group (female/male)=88/32; P=0.767] (Table I).

Frequency of the COL4A3 (rs55703767) genetic polymorphism. Table III presents the allelic and genotypic distributions of the *COL4A3* (rs55703767), *MMP-9* (rs17576) and *TIMP-1*

Table IV. Single nucleotide polymorphism association with T2D patients and control subjects.

SNP	T2D, n (%)	Control, n (%)	P-value	OR (95% CI)
COL4A3 (rs55703767) G/T				
Dominant				
GG	83 (69.2)	73 (60.8)		
GT + TT	37 (30.8)	47 (39.2)	0.18	0.69 (0.41-1.18)
Recessive				
GG + GT	116 (96.7)	105 (87.5)		
TT	4 (3.3)	15 (12.5)	0.0068	0.24 (0.07-0.75)
Overdominant				
GG + TT	87 (72.5)	88 (73.3)		
GT	33 (27.5)	32 (26.7)	0.88	1.04 (0.59-1.85)
TIMP-1 (RS6609533) A/G				
Dominant				
AA	39 (32.5)	44 (36.7)		
AG + GG	81 (67.5)	76 (63.3)	0.498	1.202 (0.706-2.048)
Recessive				
AA + AG	83 (69.2)	69 (57.5)		
GG	37 (30.8)	51 (42.5)	0.062	0.603 (0.355-1.025)
Overdominant				
AA + GG	76 (63.3)	95 (79.2)		
AG	44 (36.7)	25 (20.8)	0.062	0.603 (0.355-1.025)

P<0.05 was considered to indicate a statistically significant difference. T2D, type 2 diabetes; OR, odds ratio; CI, confidence interval; OR, odds ratio; CI, confidence interval; *COL4A3*, collagen type IV α3 chain; *MMP-9*, matrix metallopeptidase 9; *TIMP-1*, TIMP metallopeptidase inhibitor 1.

(rs6609533) polymorphisms in the patients and control subjects. The frequency distribution of the *COL4A3* (G/T) genotypes in T2D patients were as follows: GG, 69.2%; GT, 27.5%; and TT, 3.3% and the distribution in the controls were: GG, 60.8%; GT, 26.7%; and TT, 12.5%. Statistically significant differences concerning the genotypic distribution of the COL4A3 polymorphism (rs55703767) were identified. The frequency of the TT genotype was significantly different between the patients and the control subjects (OR=0.235, 95% CI=0.063-0.0802; P=0.013). In addition, a statistically significant difference in the T allele frequency was observed between patients with T2D and the control subjects (OR=0.592, 95% CI=0.371-0.943; P=0.026). These results demonstrated that the *COL4A3* (rs55703767) polymorphism was associated with T2D.

Frequency of the MMP-9 (rs17576) genetic polymorphism. In the current study, the allelic and genotypic distributions of the MMP-9 (rs17576) polymorphism were significantly different between the T2D patients and control subjects. Significant differences in the distribution of the AG genotype frequency were observed in the patients with T2D and the healthy individuals (OR=2.429, 95% CI=1.232-4.820; P=0.008). The current findings indicated that the AG genotype increased the risk of susceptibility to T2D. The AA genotype was not observed in the current study. Furthermore, a statistically significant difference was identified in the A allele frequency (OR=2.176, 95% CI=1.155-4.130; P=0.013). The A allele was a risk factor for susceptibility to T2D.

Frequency of the TIMP-1 (rs6609533) genetic polymorphism. The *TIMP-1* gene is located on the X chromosome; therefore, males and females were analyzed separately. No significant difference in the distribution of the AG genotype frequency was identified between the T2D patients and control subject groups in females. In addition, no significant difference in distribution of the genotype frequency was identified for *TIMP-1* (A/G) in males. Furthermore, no statistically significant differences were identified in the allele frequencies in females.

The Hardy-Weinberg equilibrium (HWE) of the *COL4A3*, *MMP-9* and *TIMP-1* polymorphism were evaluated using the χ^2 test for each of the single nucleotide polymorphisms (SNPs) as follows: *COL4A3* (case; P=0.748, χ^2=0.1, control; P<0.05, χ^2=11.1), *MMP-9* (case; P=0.0532, χ^2=3.74, control; P=0.374, χ^2=0.79), and *TIMP-1* (case; P<0.05, χ^2=8.52, control; P<0.05, χ^2=40.63).

Dominant, recessive and overdominant model analyses were performed (Table IV) for the *COL4A3* (rs55703767) G/T and the *TIMP-1* (RS6609533) A/G, and the result indicated that, of the *COL4A3* (rs55703767) G/T, the TT genotype was associated with T2D in the recessive model (P=0.0068) while,

in *TIMP-1* (RS6609533) A/G, the AG genotype was associated with T2D in the overdominant model (P=0.062). No significant association with T2D was identified in the other models.

Discussion

T2D is a growing health challenge worldwide and is the most widespread form of diabetes (26). T2D is characterized by hyperglycemia and results from a combination of resistance to insulin action and insufficient insulin secretion (27). Previous studies demonstrated that genetic polymorphisms may reveal individual differences in T2D risk (28). Type IV collagen is a major component of the ECM and various different physiological situations, including ageing, diabetes, scarring, and fibrosis are associated with it (29); in T2D, the biosynthesis of type IV collagen is increased (30). The levels of extracellular matrix components are a reflection of the balance between the rate of synthesis and the degradation of matrix proteins. Degradation is attained through MMPs, which are regulated by TIMPs (9). Alterations in connective tissue metabolism may associate with the development of diabetic complications, such as neuropathy, nephropathy and retinopathy (12). Rana *et al* (31) proposed that thin BM nephropathy (TBMN) results from mutations in *COL4A3*. Numerous different *COL4A3* mutations cause TBMN and the identification of polymorphisms in this gene is particularly important (31). Hou *et al* (32) identified that a novel mutation (3725G>A, G1242D) of *COL4A3* exerted an underlying pathogenic role in the heterozygous form in TBMN (29). Ahluwalia *et al* (33) demonstrated that *MMP-9* polymorphisms were associated with the risk of diabetic nephropathy (DN). Nazir *et al* (34) found that genetic variants of *MMP-9* had a significant positive association with DN and that this gene may contribute to the pathophysiology of DN (34). The results of Pan *et al* (35) indicated that the *TIMP-1* SNPs, rs4898 and rs6609533 were associated with an increased risk of early aseptic loosening susceptibility (35). Kumar *et al* (36) observed that the SNP rs6609533 of the *TIMP-1* gene interacted with pim-3 proto-oncogene, serine/threonine kinase, resulting in a possible risk for the development of chronic obstructive pulmonary disease (36).

In conclusion, the T allele of *COL4A3* (G/T) (with a protective role) and the A allele of *MMP-9* (A/G) (as a risk factor) were associated with T2D. However, no significant association was identified between the G allele of *TIMP-1* (A/G) and the risk/protective characteristics of T2D in females in the evaluated population. However, further studies with larger sample sizes and various ethnic groups are required to confirm these findings.

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