

CLINICAL STUDY

GAS6 intron 8 c.834 + 7G > A gene polymorphism in diabetic nephropathy

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Abstract

Background - Aim: In animal experiments, growth arrest-specific 6 (Gas6) protein plays a key role in the development of mesangial cell and glomerular hypertrophy in the early phase of diabetic nephropathy, and diabetic nephropathy is prevented by warfarin-induced inhibition of GAS6 protein. It was shown that GAS6 intron 8 c.834 + 7G > A polymorphism is protective against type 2 diabetes mellitus, and AA genotype is associated with higher blood levels of GAS6 protein. Our aim is to investigate whether this polymorphism is a risk factor for diabetic nephropathy in type 2 diabetes mellitus. **Method:** Eighty-seven patients with diabetic nephropathy were compared with 66 non-diabetic controls in terms of GAS6 intron 8 c.834 + 7G > A polymorphism. Patients with history of stroke, ischemic heart disease were excluded. Each patient was examined by the ophthalmologist to determine diabetic retinopathy. **Results:** Frequency of GG, GA and AA genotypes are similar in diabetic nephropathy and control groups according to GAS6 intron 8 c.834 + 7G > A polymorphism ($p = 0.837$). Rate of diabetic retinopathy was 54.02%. In the subgroup analysis, GA genotype was significantly more frequent than GG genotype in patients with diabetic retinopathy when compared to without diabetic retinopathy ($p = 0.010$). **Conclusion:** In our study, GAS6 intron 8 c.834 + 7G > A polymorphism was not associated with diabetic nephropathy in type 2 diabetes mellitus. However, heterozygous state of this polymorphism may be a risk factor for diabetic retinopathy in patients with diabetic nephropathy.

Keywords

Albuminuria, diabetes mellitus, diabetic nephropathy, GAS6 gene, polymorphism

History

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Introduction

Diabetic nephropathy is the most common cause of chronic kidney disease, and it accounts 45% of end-stage renal disease cases.¹ Diabetic nephropathy develops in about 20 to 40% of type 2 diabetes patients, and it is not known why it is not seen in all of the patients with diabetes mellitus type 2.² Many mechanisms may contribute to the emergence of diabetic nephropathy and its clinical course. It involves mainly the interaction between metabolic and hemodynamic changes caused by hyperglycemia and genetic predisposition.² Growth arrest-specific 6 (GAS6) was first discovered as a protein produced by growth arrested fibroblasts.³ GAS6 is present at very low levels in the bloodstream (0.16–0.28 nm)

in humans.³ GAS6 protein is a member of the vitamin K-dependent coagulation proteins group, but in contrast to the other members, it plays a minor role in hemostasis GAS6 has a number of different functions concerning cellular survival, proliferation, migration and adhesion.⁴ GAS6 exerts most of its effects over AXL, SKY and Mer2, which are members of Axl subfamily of tyrosine kinase receptors.⁴ Animal studies showed that GAS6 protein plays a key role in the development of mesangial cell and glomerular hypertrophy in the early phase of diabetic nephropathy.^{5,6} When GAS6 is inhibited by warfarin, it was demonstrated that mesangial and glomerular hypertrophy is prevented, and hyperfiltration and albuminuria are reduced.^{5,6} Diabetic nephropathy was less severely observed in GAS6 knockout mice.^{5,6} A positive correlation was shown between albuminuria and serum levels of GAS6 in type 2 diabetic individuals.¹ Chromosomal localization of GAS6 gene is 13q34.⁷ A single nucleotide polymorphism (c.834 + 7G > A) of this gene in intron 8 was demonstrated to be protective against stroke, diabetes and acute coronary

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syndrome.^{4,8,9} We suggest that *GAS6* intron 8 c.834 + 7G > A gene polymorphism might be a risk factor for diabetic nephropathy in type 2 diabetic individuals. In this study, we compared frequency of *GAS6* intron 8 c.834 + 7G > A gene polymorphism between non-diabetic individuals and type 2 diabetic patients with diabetic nephropathy.

Methods

Study design and study population

This study was designed as a prospective, cross-sectional, case-control study. Records of diabetic patients followed-up with a diagnosis of type 2 diabetic nephropathy in Bezmialem Vakıf University Medical Faculty Hospital, Internal Medicine Outpatient Clinic between December 2011 and December 2013 were screened. Patients meeting diabetic nephropathy diagnosis criteria according to the American National Kidney Foundation KDOQI Guidelines considering both clinical and routine laboratory findings were eligible for the study.¹⁰ Patients who met all of the inclusion and exclusion criteria and gave informed consent were enrolled in this study. The control group consisted of non-diabetic patients accepted to give informed consent, admitted to internal medicine outpatient clinic for any reason subsequently, meeting the inclusion/exclusion criteria.

Inclusion criteria

Patients with type 2 diabetes mellitus and diabetic nephropathy or non-diabetic individuals between 18 and 80 years of age.

Exclusion criteria

Following individuals were excluded from the study: possibility of any nephropathy other than diabetic nephropathy associated with type 2 diabetes (nephrotic syndrome, rapidly decreasing glomerular filtration rate (GFR), rapidly increasing proteinuria, refractory hypertension, active urinary sediment, GFR decrease of more than 30% in 2 or 3 months following initiation of angiotensin converting enzyme inhibitor or angiotensin receptor blocker treatment), history of ischemic heart disease and/or stroke.

Criteria for diabetic nephropathy

Diagnostic criteria accepted for defining diabetic nephropathy in type 2 diabetic individuals is detection of urinary albumin/creatinine ratio of ≥ 300 mg/g creatinine in a first-morning void and detection of ≥ 300 mg/g creatinine in a first-morning void at the subsequent 2 measurements with 3 months interval together with no active urinary sediment, and provided that there is no other comorbidity that may cause proteinuria.

Criteria of being a non-diabetic subject

Persons who have not previously diagnosed with diabetes, provided that they are not on a diet, demonstrated that they have no diabetes mellitus following the oral glucose tolerance test performed with 75 g of oral glucose in the morning after overnight fasting were accepted as non-diabetic individuals.

Sampling and storing

Venous blood samples were obtained in the early morning after overnight fasting. Routine blood analyses were performed on the same day. Blood samples in EDTA containing tubes taken for *GAS6* polymorphism was sent to the genetic laboratory immediately and DNA extraction was performed on the same day. A first morning void sample was taken for routine urinalysis and urine albumin/creatinine ratio, and it was analyzed on the same day.

Blood analysis

Complete blood cell (CBC) analysis was done with Sysmex XT 1800i apparatus (ROCHE-2011, Kobe, Japan). Biochemical assays were done on COBAS 8000 apparatus (ROCHE-2007, Tokyo, Japan) using COBAS-C system kits.

DNA extraction and genotype analysis

Blood samples were taken to the tubes containing EDTA and the genomic DNA was isolated using a DNA isolation kit (Invitrogen, Carlsbad, CA). All purified DNA samples were stored at +4°C. Growth arrest-specific 6 (*GAS6*) gene polymorphism was analyzed with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay.¹¹ For the genetic polymorphism rs8191974 (*GAS6* c.834 + 7G > A in intron 8), 25 µl of total PCR mixtures containing 10.0 pmol of each primers, 1.0 mM dNTP (deoxynucleotide triphosphates), 25 mM MgCl₂, 2.5U Taq DNA polymerase in the supplied reaction buffer (11615010, Invitrogen) and 100–200 ng of human genomic DNA were used. PCR was performed with the forward (5'-TTCCCTCAAGAAAGAGCCCG-3') and reverse primers (5'-TCTCATCCCAAACCTCCACA-3') with the initial denaturation at 94°C for 5 min, following 33 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 30 s and additionally the final step at 72°C for 5 min. PCR products digested by AlwNI (R0514 S, NEB) were run on 2% agarose gel with ethidium bromide staining, then genotyped. Three genotypes for *GAS6* gene polymorphism were defined by distinct banding patterns (Figure 1); 481 bp for GG genotype; 481, 345 and 136 bp for GA genotype; and 345, 136 bp for AA genotype.

Ophthalmological examination

Each patient was examined for evaluation of diabetic retinopathy by the same ophthalmologist blinded to the

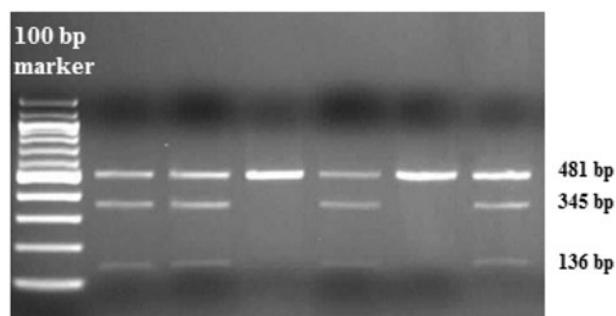


Figure 1. Genotyping for the *GAS6* gene polymorphism. Representative gel showing the genotype for *GAS6* 834 + 7G/A. The first lane is for 100 bp DNA ladder.

study. The Airlie House classification modified by the Diabetic Retinopathy Study Research Group developed for the Early Treatment Diabetic Retinopathy Study was used for grading retinopathy.^{12,13}

Statistics

Numerical variables are presented as means with standard deviations, and nominal variables in ratios. Nominal and numerical independent variables were compared between the diabetic nephropathy group and the control group. The chi-square test was used to compare nominal independent variables. One sample Kolmogorov-Smirnov test was done to see if the continuous independent variables were normally distributed. Normally distributed independent continuous variables were compared with Student's *t*-test; whereas non-normally distributed independent continuous variables were compared with the Mann-Whitney *U*-test. A two-tailed *p*-value of <0.05 was considered to be statistically significant.

Ethics

The study was approved by the Ethics Committee of the Bezmialem Vakif University and written informed consents were obtained from all the patients. The study was conducted in accordance with the Helsinki Declaration of 2009.

Results

There were 153 individuals in the study. Nephropathy and control groups consisted of 87 and 66 individuals, respectively. In the nephropathy group there were 56 female and 31 male subjects, and in the control group there were 41 female and 25 male subjects ($p=0.775$). In the study group, mean age was 57.9 ± 10.2 years (19–80 years) ($p>0.05$, Table 1). Mean BMI was 31.6 ± 5.8 kg/m² (16.9–44.9 kg/m²) in our study ($p>0.05$, Table 1).

Hypertension was significantly more frequent in the diabetic nephropathy group, also arterial blood pressure was significantly higher (Tables 1 and 2). According to *GAS6* intron 8 c.834+7G>A gene polymorphism analysis, there is no significant difference in terms of hypertension frequency among GG, GA and AA genotypes in both diabetic nephropathy group and control group ($p=0.365$ and $p=0.124$, respectively). Also in diabetic nephropathy group albuminuria was similar among GG, GA and AA genotypes ($p=0.313$).

Frequency of GG, GA and AA genotypes are similar in diabetic nephropathy and control groups in the analysis of *GAS6* intron 8 c.834+7G>A polymorphism (Table 3).

The frequency of GA or AA genotype versus GG genotype in diabetic nephropathy and control groups according to *GAS6* intron 8 c.834+7G>A polymorphism analysis were compared by the chi-square test, and found similar (Table 3). So the frequency allele A was similar in diabetic nephropathy and control groups.

Diabetic retinopathy was present in 47 out of 87 patients with diabetic nephropathy (54.02%). Fourteen of the patients with diabetic retinopathy have mild non-proliferative retinopathy, 12 have moderate non-proliferative retinopathy, 12 have severe non-proliferative retinopathy and nine have proliferative retinopathy. The frequency of *GAS6* intron 8 c.834+7G>A polymorphism was compared in diabetic nephropathy patients with and without diabetic retinopathy using the chi-square test. AA and GG genotypes were found with similar frequency in the two groups ($p=0.745$). However, GA genotype was significantly more frequent than GG genotype in diabetic nephropathy patients with diabetic retinopathy (Table 4). GA genotype represents a significantly increased risk ratio for diabetic retinopathy in patients with diabetic nephropathy (OR: 1.68, 95% Confidence interval: 1.12 to 2.50, $p=0.010$). AA genotype was observed less frequently in diabetic nephropathy patients

Table 1. Demographics and routine laboratory tests in two groups.

Independent variables	Diabetic nephropathy group	Control group	Normal range	<i>p</i> Value	Type of distribution
Age (years)	59.06 ± 10.14	56.50 ± 10.1		0.125	ND
BMI (kg/m ²)	32.08 ± 6.01	30.88 ± 5.40		0.217	ND
White blood cell × 10 ⁹ /L	9.020 ± 2.518	6.693 ± 1.617	3.8–10.0	<0.001	ND
Hemoglobin (g/L)	125.2 ± 20.7	134.8 ± 14.7	130–175	0.002	ND
Hematocrit (%)	38.30 ± 5.63	40.78 ± 3.96	40–52	0.008	ND
Platelet × 10 ⁹ /L	275.333 ± 81.017	240.020 ± 56.577	150.0–400.0	0.008	ND
FT4 (pmol/L)	14.77 ± 1.40	14.61 ± 1.24	10–18.7	0.541	ND
Total protein (g/L)	72.1 ± 6.5	71.1 ± 3.8	60–80	0.312	ND
Urea (mmol/L)	17.55 ± 10.69	7.28 ± 2.64	2.9–8.2	<0.001	ND
Median-Min-Max					
Blood pressure systolic (mmHg)	130–110–200	120–100–150	<135	<0.001	NND
Blood pressure diastolic (mmHg)	80–55–110	70–50–90	<85	<0.001	NND
Creatinine (μmol/L)	88.4–35.4–521.6	70.7–44.2–122.0	<91.50	<0.001	NND
CRP (nmol/L)	6.7–0.0–197.2	1.9–0.0–148.2	0–4.57	<0.001	NND
TSH (mIU/L)	1.78–0.40–7.60	2.43–0.43–7.70	0.63–4.82	0.068	NND
Glucose (mmol/L)	8.77–4.83–22.26	5.44–4.38–6.11	3.9–5.55	<0.001	NND
HbA1C (%)	7.5–5.30–13.2	5.6–5.1–6.2	4.5–5.7	<0.001	NND
Albumin (g/L)	44–26–51	45–40–52	35–50	0.251	NND
AST (IU/L)	19–9–53	22–14–58	10–30	0.983	NND
ALT (IU/L)	18–2–71	19.5–10–135	10–40	0.282	NND
Urine albumin-creatinine ratio (mg/g creatinine)	706–327–5973	7.16–0.15–23	<30	<0.001	NND

Note: ND: Normal distribution; NND: Non-normal distribution.

Table 2. Frequency of hypertension and antihypertensive drugs, which patients used regularly between two groups.

Variables	Diabetic nephropathy group of 87 patients, no. (%)	Control group of 66 patients, no. (%)	<i>p</i> Value
Hypertension	74 (85.1)	14 (21.2)	<0.001
Dihydropyridine calcium antagonist	15 (17.2)	2 (3)	0.006
Non-dihydropyridine calcium antagonist	15 (17.2)	2 (3)	0.006
ACE inhibitor	25 (28.7)	8 (12.1)	0.013
Angiotensin receptor blocker	22 (2.5)	1 (1.5)	<0.001
Beta blocker	22 (2.5)	2 (3)	<0.001
Alpha blocker	12 (13.8)	0 (0)	0.002
Thiazide	39 (44.8)	9 (13.6)	<0.001
Furosemide	33 (37.9)	0 (0)	<0.001

Table 3. Frequency of *GAS6* intron 8 c.834 + 7G > A gene polymorphism in diabetic nephropathy and control groups.

<i>GAS6</i> intron 8 c.834G > A polymorphism	Diabetic nephropathy group of 87 patients <i>n</i> (%)	Control group of 66 subject <i>n</i> (%)	<i>p</i> Value
GG genotype	41 (47.1%)	30 (45.5%)	0.837
GA genotype	34 (39.1%)	30 (45.4%)	
AA genotype	12 (13.8%)	6 (9.1%)	
GA or AA genotype	46 (52.9%)	36 (54.5%)	0.579
GG genotype	41 (47.1%)	30 (45.5%)	

Table 4. Frequency of *GAS6* intron 8 c.734 + 7G > A gene polymorphism in diabetic nephropathy patients with diabetic retinopathy and without retinopathy.

Genotypes	Patients with diabetic nephropathy, and with diabetic retinopathy 47 patient <i>N</i> (%)	Patients with diabetic nephropathy, but without diabetic retinopathy 40 patients <i>N</i> (%)	<i>p</i> Value
AA	4 (8.5)	8 (20)	0.011
GA	25 (53.2)	9 (22.5)	
GG	18 (38.3)	23 (57.5)	

with diabetic retinopathy compared to the diabetic nephropathy patients having no diabetic retinopathy, but without statistical significance (Table 4, *p* = 0.215).

Discussion

Revealing the pathogenesis of diabetic nephropathy has paramount importance since diabetic nephropathy is the most common cause of end-stage renal failure. Expansion of mesangium, hypertrophy of glomerulus, increased glomerular filtration rate and albuminuria are observed in early phase of diabetic nephropathy.^{5,6} If not intervened, consequences of these changes are glomerulosclerosis and reduced glomerular filtration.^{5,6} Since diabetic nephropathy is reversible in the early phase it is particularly important to reveal molecular

mechanisms of glomerular mesangial expansion and hypertrophy.^{5,6} The relationship of *GAS6* with diabetic nephropathy was shown in animal studies. In streptozotocin-induced diabetic rat's glomerular hypertrophy, increased GFR and albuminuria were observed 12 weeks after STZ injection.^{5,6} The expression of *GAS6* and *AXL* receptors were found elevated in the glomeruli of these rats.^{5,6} Since warfarin inhibits the synthesis of *GAS6* protein, low-dose warfarin administration was demonstrated to inhibit albuminuria, GFR increase and glomerular hypertrophy in streptozotocin-induced diabetic rats.^{5,6} When diabetes was induced in *GAS6* knockout and wild type mice, it was seen that mesangial expansion and glomerular hypertrophy were mild in *GAS6* $-/-$ mice.^{5,6} It was demonstrated that *GAS6* added to cell culture medium in concentrations lower than the required concentration to exhibit mitogenic activity causes mesangial cell hypertrophy.^{5,6} These studies suggest that *GAS6*-*Axl* pathway may have an important role in the early phase of diabetic nephropathy.^{5,6}

Besides the animal experiments and cell culture studies, our previous study has shown that in type 2 diabetic patients with micro- and macroalbuminuria, *GAS6* concentration was higher than the control group, and positive correlation exists between albuminuria and plasma *GAS6* levels.¹

In a recent study comparing *GAS6* intron 8 c.834 + 7G > A polymorphism in subjects with type 2 diabetes, impaired glucose tolerance and controls, it was shown that AA genotype is protective against type 2 diabetes, and for individuals with AA genotype, plasma *GAS6* protein levels are higher compared to individuals with GG genotype.⁸ This genotype was also found to be protective from type 2 diabetes as well as acute coronary syndrome and stroke.^{4,8,9} It was shown that fasting glucose, TNF- α , interleukin-6 and vascular cell adhesion molecule-1 levels decrease when *GAS6* protein plasma concentration increases. That means *GAS6* is related to diabetes, inflammation and endothelial function.¹⁴ For this reason, AA genotype associated with higher *GAS6* protein levels may protect against diabetes mellitus type 2, acute coronary syndrome and stroke. As in animal experiments it was shown that *GAS6* protein is responsible for mesangial expansion and glomerular hypertrophy during the early phase of diabetic nephropathy.^{5,6} In this case, higher *GAS6* protein levels may not be protective against diabetic nephropathy but contrarily may facilitate development of it. We suggested that AA or GA genotypes of *GAS6* intron 8 c.834 + 7G > A polymorphism associated with higher plasma *GAS6* protein levels might be associated with higher incidence of diabetic nephropathy. However, in this study, AA or GA genotype is not more frequent in diabetic nephropathy patients compared to the control group. Hence, we determined that allele A is not associated with diabetic nephropathy.

Although diabetic nephropathy seems in direct proportion with poor glycemic control or duration of diabetic state, this is not applicable for all patients with diabetic nephropathy.² Nevertheless, diabetic nephropathy is only present in about 30% of diabetic patients.² It is obvious that a genetic predisposition should be present for the emergence of diabetic nephropathy.² In a study conducted on Pima Indians, among those families having diabetes for two successive generations,

if proteinuria is not present in both parents, the probability of developing overt proteinuria in siblings is 14%; however, if proteinuria is present in one or both of the parents, then probability is 23 and 46%, respectively.² In Pima Indians, 7q21.3, 10p15.3, 14q23.1 and 18q22.3 chromosome gene loci were identified leading to a susceptibility to diabetic nephropathy.² It has been suggested that genetic variations in ACE gene are associated with diabetic nephropathy.² In type 2 diabetic patients, it was demonstrated that DD polymorphism of ACE gene is associated with the development of diabetic nephropathy, severe proteinuria, progressive renal failure and mortality during dialysis.² However, in other studies investigating the association between diabetic nephropathy and ACE gene polymorphism, conflicting results were obtained.²

The subgroup analysis we performed revealed that GA genotype in the *GAS6* intron 8 c.834 + 7G > A polymorphism is significantly more frequent in diabetic nephropathy patients with diabetic retinopathy compared to patients without retinopathy. As a result, GA genotype seems to be a risk factor for retinopathy in patients with diabetic nephropathy. AA genotype was tended to be present less frequently in diabetic nephropathy and retinopathy patients. If we have larger diabetic nephropathy patient population, it might be revealed that AA genotype is protective against retinopathy in diabetic nephropathy contrary to GA genotype. Diabetic retinopathy is the leading cause of blindness in the USA for adults aged 20–74.¹⁵ The risk of the development of diabetic retinopathy is related to poor glycemic and blood pressure control and the duration of diabetes similar to nephropathy.¹⁵ Similarly diabetic retinopathy does not develop in all patients with these risk factors.¹⁵ It is obvious that genetic predisposition is necessary for the development of diabetic retinopathy. Heritability was reported as 27% for diabetic retinopathy, and 52% for proliferative diabetic retinopathy and advanced diabetic proliferative retinopathy.¹⁵ Despite these findings, to our knowledge, linkage analyses, candidate gene association studies and genome wide association studies could not demonstrate a widely reproducible diabetic retinopathy locus. Whether *GAS6* gene polymorphism is a risk factor for the development of diabetic retinopathy is yet to be studied. In our study, we demonstrated that among diabetic nephropathy patients *GAS6* intron 8 c.834 + 7G > A polymorphism is associated with diabetic retinopathy. Whether *GAS6* intron 8 c.834 + 7G > A gene polymorphism is a risk factor for diabetic retinopathy independent from diabetic nephropathy should be investigated in broader patient populations.

In conclusion, there is no correlation between *GAS6* intron 8 c.834 + 7G > A gene polymorphism and diabetic nephropathy in type 2 diabetic individuals. Among diabetic nephropathy patients *GAS6* intron 8 c.834 + 7G > A

polymorphism is associated with diabetic retinopathy. It is suggested that genetic basis of type 2 diabetes involves interaction between many genes.¹⁶ Like this, genetic basis of diabetic nephropathy may not be explained with a single gene.

Declaration of interest

All authors declare there is no conflict of interest.

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