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Original Article

Association of ACE, AGT, eNOS gene single nucleotide polymorphism with Immunoglobulin A nephropathy in Xinjiang Uygur

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Abstract: The aim of the present study was to explore the correlation between single nucleotide polymorphisms ACE I/D (angiotensin-converting enzyme), AGT M235T (angioten-sinogen), eNOS G894T (endothelial nitricoxide synthase) and the susceptibility to Immunoglobulin A nephropathy (IgAN) in Xinjiang Uygur. The distributions of genotypes of SNPs ACE I/D, AGT M235T, eNOS G894T were detected by direct sequencing. The frequencies of genotype and allele distribution in ACE I/D and AGT M235T were no difference (P>0.05) between IgAN and control groups. eNOS G894T GG genotype and G allele of frequencies (62.20% and 75.60% , respectively) in IgAN were significantly higher than that of healthy controls (P=0.005, P<0.0001, respectively). The ACE DD genotype with Serum creatinine was significantly higher than that of ACE II genotype patients, the difference was statistically significant (P=0.018). The association of AGT M235T/eNOS G894T genotypes and serum creatinine was no statistical difference between the two groups. 24 h proteinuria with ACE DD genotype was significantly higher than that of ACE genotype II patients (P=0.023), with no differences between AGT M235T and eNOS G894T gene. ACE I/D, AGT M235T and eNOS G894T genotype and hematuria were not statistically different between the two groups. Conclusion: ACE I/D, AGT M235T gene are irrelevant to the susceptibility of patients with IgAN in Xinjiang Uygur. eNOS gene may be a risk factor in patients with IgAN. DD genotype in ACE gene is associated with the progression of IgAN in Xinjiang Uygur.

Keywords: Immunoglobulin A nephropathy, angiotensin-converting enzyme, angioten-sinogen, endothelial nitricoxide synthase, single nucleotide polymorphism

Introduction

IgA nephropathy is the most common primary glomerular disease, accounting for 30-40% of primary glomerular disease, the incidence rate increased year by year [1]. About 15%-25% of patients progressed to end-stage renal disease (ESRD) in the 10 years after the initial diagnosis [2]. The clinical manifestations, pathological types and prognosis of IgA nephropathy show diversity, pathogenesis is not very clear.

Some randomized controlled trials show that renin angiotensin system (RAS) blockers including angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARB) can reduce albuminuria and control the progression of IgA nephropathy [3, 4]. Genetic factors have been the focus of susceptibility and prognosis in glomerular disease [5-7].

The present study analyzed the correlation of polymorphism of ACE I/D, AGT M235T, eNOS G894T and the susceptibility of the Uyghur population to IgAN, so as to get a better understanding of the pathogenesis and genetic background of IgAN in the Uyghur region.

Subjects and methods

Subjects

Subjects of study: A total of 45 cases of hospital patients of Uyghur ethnicity (23 males and 22 females, aged 18-67 years, mean age 42.89±12.29 years), diagnosed with IgAN by renal biopsy in the Nephrology Department

Table 1. Primers for ACE I/D, AGT M235T and eNOS G894T

Locus	Forward primer (5'-3')	Reverse primer (5'-3')
ACE I/D	CTGGAGACCACTCCCATCCTTTCT	GATGTGGCCATCACATTCGTCAGAT
AGT M235T	CCGTTTGTGCAGGGCCTG	TGCTGTCCACACTGGACCCC
eNOS G894T	AAGGCAGGAGACAGTGGATGGA	CCCAGTCAATCCCTTTGGTGCTCA

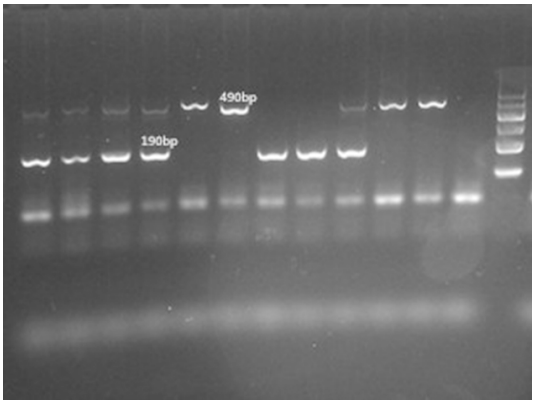

Figure 1. ACE I/D Polymorphism.

of the People's Hospital of Xinjiang Uyghur Autonomous Region (Urumqi, China) were collected. Renal biopsy pathological diagnostic criteria established by Zou in 2011 [8] were used as diagnostic criteria for IgAN. Patients with secondary IgA deposition diseases, such as systemic lupus erythematosus (SLE), allergic purpura, chronic liver diseases, ankylosing spondylitic renal damage and psoriatic renal damage were excluded. The healthy controls were 45 healthy individuals (27 males and 18 females, aged 23-84 years, mean age 43. 71±13.46 years) who went to the aforementioned hospital for medical examination from June 2011 to May 2015. All the selected patients were unrelated, with permanent Uyghur residency, of three different generations and all lived in Xinjiang. All subjects provided informed consent and participated voluntarily. This study was approved by the Medical Ethics Committee of The People's Hospital of Xinjiang Uygur Autonomous Region.

Method

Collection of blood samples: A 5 ml sample of venous blood was collected from each patient on an empty stomach in the morning. EDTA was used for anticoagulation. The samples were numbered and registered. Whole blood samples were placed at -80°C for cryopreservation.

DNA extraction: DNA specimens were extracted with Epzu pillar type whole blood genomic DNA extraction kit from Shanghai Biological company according to the kit instructions and Ultraviolet spectrophotometer measured A260 nm/A280 nm absorbance, determine the purity of more than 1. 80, preserved at -20°C.

PCR amplification: Retrieving the DNA sequences of PLA2R SNP rs35771982 loci, HLA DQA1 SNP rs2187668 site in the U. S. national center for biotechnology information (NCBI). Chose Primer5.0 software design of forward primer and reverse primers in this experiment. As shown in **Table 1**. Primers synthesized by Shanghai sangon biotechnology company. PCR amplification kits were provided by the Shanghai sangon biotechnology company. The total volume of the amplifcation stage of the PCR was 35 μl (containing 3 μl DNA, 20 μl ddH_2O, 5 μl buffer, 2 μl dNTP, and 1.2 and 2 μl Taq polymerase in the upstream and downstream directions, respectively). The amplifcation reaction conditions of PCR were: denaturation at 95°C for 5 min; main cycling at 95°C for 45 sec, 56. 50°C for 1 min and 72°C for 45 sec, 30 cycles in total; followed by 72°C for 10 min and preservation at 4°C. The PCR reaction was performed on the GeneAmp® PCR System 9700 Thermal cycler from Applied Biosystems®, Invitrogen Life Technologies (Foster City, CA, USA).

Genotyping: Adding 5 ul PCR amplification products in 2% agarose gel previously added GelGreen nucleic acid dye electrophoresis (Voltage180 V, time 20 min). Uv transmission gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to observe banding distribution condition, taking digital photos classification preservation. All PCR products for each SNP locus were genotyped by direct sequencing (conducted by Beijing Dingguo Biotechnology Co., Ltd.). Analysised sequencing results with Chromas software, obtained PLA2R SNP rs35771982 locus, HLA-DQA1 SNP rs2187668 locus genotype distribution.

Statistical analysis: The genotype frequencies of SNP were tested separately for Hardy-Weinberg equilibrium in patients and controls.

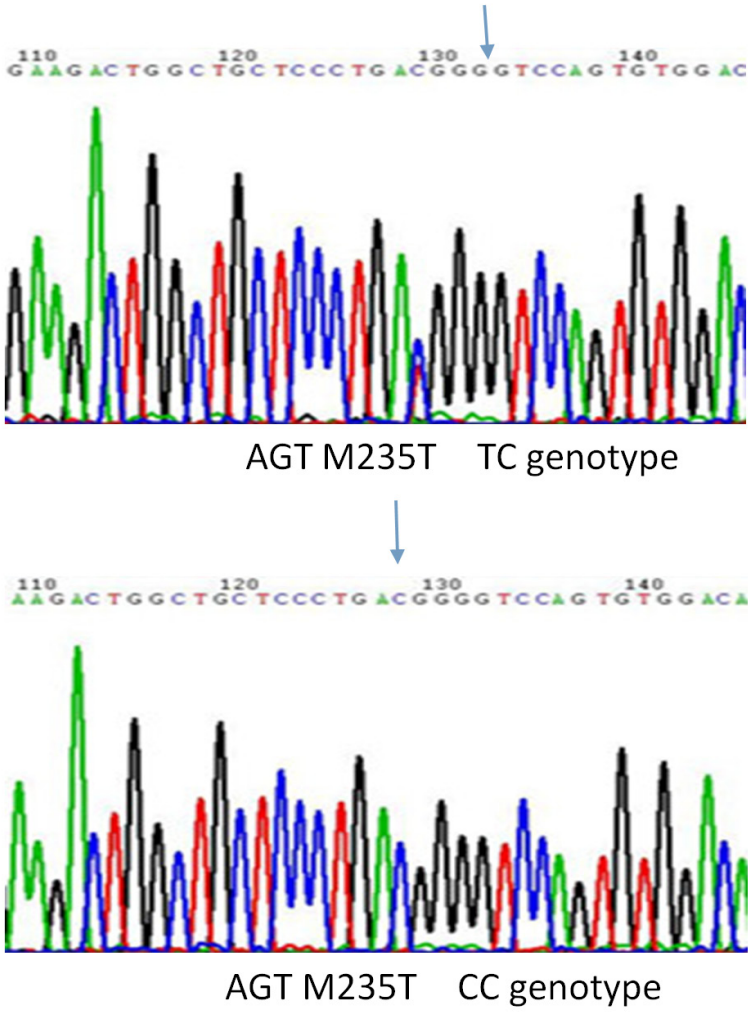

Figure 2. AGT M235T polymorphism.

Other Statistical analyses were performed with the SPSS17.0 software package (SPSS, Inc. , Chicago, IL, USA). The allele and genotype frequencies were calculated using the chi-squared test. A t-test was applied for comparison of measurement data between groups. Logistic regression analysis was applied to analyze the correlation between polymorphism and IgAN. All statistical analyses were 2-sided, at a test level of α=0.05; P<0.05 indicated that the difference was statistically signifcant.

Results

ACE I/D polymorphism results of agarose gel electrophoresis

As shown in **Figure 1**. ACE I/D polymorphism shows three genotypes, insert homozygous: II, homozygous deletion: DD, heterozygous: ID. II genotypes have a 490 bp bands, DD genotype 190 bp 1 bands, ID genotype 490 bp and 190 bp 2 bands.

Gene sequencing results

As shown in **Figure 2**. AGT M235T gene have a T/C mutation. In this study only shows CC and CT. As shown in **Figure 3**. eNOS G894T gene have a T/C mutation. In this study shows GG, GT and TT.

Hardy-Weinberg equilibrium test

The distributions of the genotypes in the IgAN and control groups were in accordance with Hardy-Weinberg equilibrium (P>0.05).

General patient characteristics

There was no statistically signifcant difference in terms of gender, age and BMI between the IgAN group and the control group (P>0.05; **Table 2**).

Comparison of distributions of ACE/AGT/eNOS gene polymorphisms among IgAN and control groups.

A comparison of the genetypes and frequencies between the IgAN and control groups was performed. As shown in **Table 3**.

There was a significant difference in eNOS genotype GG and allele G between the IgAN and control groups (P=0.005, P<0.0001), but no differences for the ACE and AGT genes (P>0.05).

Correlationa analysis of ACE/AGT/eNOS gene polymorphisms and clinical characteristics in IgAN group.

As shown in **Table 4**. The ACE DD genotype (178.00 (101.99, 204.24)) with Serum creatinine was significantly higher than that of ACE II genotype (78.27 (64.23, 112.78)) patients, the difference was statistically significant (P=0.018). The association of AGT M235T/eNOS G894T genotypes and serum creatinine

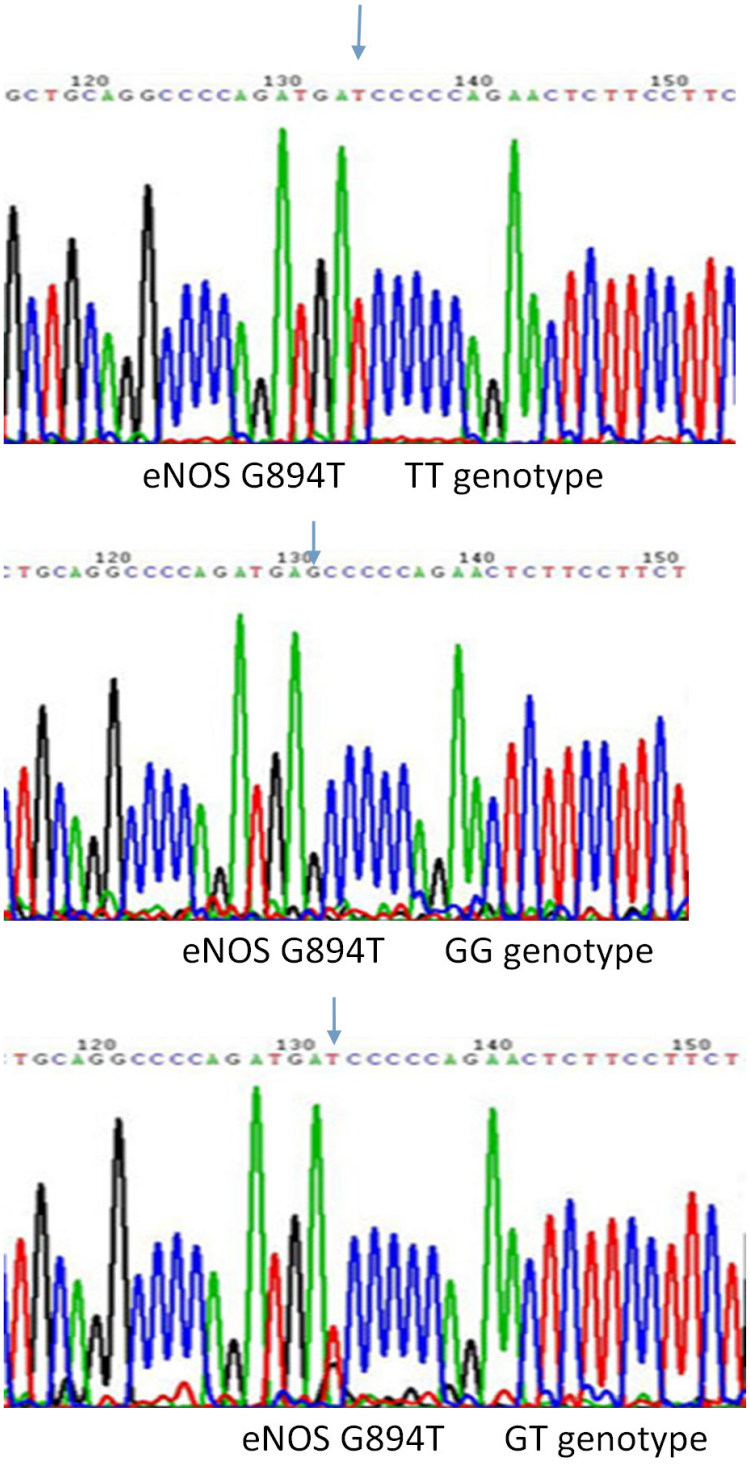

Figure 3. eNOS G894T polymorphism.

was no statistical difference between the two groups.

As shown in **Table 5**. 24 h proteinuria with ACE DD genotype (2.66 (1.44, 3.87)) was significantly higher than that of ACE genotype II (1.31 (0.14, 2.65)) patients (P=0.023), with no differences between AGT M235T and eNOS G894T gene.

As shown in **Table 6**. ACE I / D, AGT M235T and eNOS G894T genotype and Hematuria were not statistically different between the two groups.

Discussion

RAS system plays an important role in the development of kidney disease, cardiovascular disease and diabetes [9-11]. The AGT, ACE is a core component of the RAS. The study found single nucleotide polymorphisms of core component of the RAS is associated with prognosis and progression of kidney disease [12].

ACE gene is located on chromosome 17q23, long 21 kb, consist of the 26 exons and 25 introns. The insertion or deletion of a 287 bp DNA fragment in intron 16 of ACE gene while performes I/D polymorphism [13]. ACE, a metal zinc peptidase which catalyzes angiotensin I conversion into angiotensin II is the most important part of the RAS system [14]. High levels of Angiotensin II makes renal hemodynamic changed, expression of various cytokines and growth factors increased, mesangial cell proliferated, mesangial matrix increased, glomerular capsule pressure increased. Finally resulted in renal tubular interstitial fibrosis and glomerular sclerosis which affects kidney function [15]. There is a lot study of the ACE gene I/D polymorphism in IgA nephropathy. A meta analysis found that DD genotype and D allele is susceptible factor of

Table 2. Age, gender and BMI distribution in the IgAN and control groups

Item	IgAN group	Control group	P
Age	42.89±12.29	43.71±13.46	0.763
Gender (M/F)	23/22	27/18	0.396
BMI (kg/m^2)	26.58±4.14	25.95±3.32	0.425

IgAN, Immunoglobulin A nephropathy.

Table 3. Distribution of genotype and allele frequencies of ACE/AGT/eNOS gene in the IgAN and control groups

	IgAN group, n (%)	Control group, n (%)	P
ACE I/D			
Genotype			
II	18 (40.00)	12 (26.70)	
ID	18 (40.00)	18 (40.00)	0.417
DD	9 (20.00)	15 (33.30)	0.100
Allele			
I	54 (60.00)	42 (46.70)	
D	36 (40.00)	48 (53.30)	0.073
AGT M235T			
Genotype			
TT	0	0	-
TC	19 (42.20)	16 (35.60)	
CC	26 (57.80)	29 (64.40)	0.517
Allele			
T	19 (21.10)	16 (35.60)	
C	71 (78.90)	74 (64.40)	0.572
eNOS G894T			
Genotype			
GG	28 (62.20)	12 (26.70)	
GT	12 (26.70)	21 (46.70)	0.004Δ
TT	5 (11.10)	12 (26.70)	0.005ΔΔ
Allele			
G	68 (75.60)	45 (50.00)	
T	22 (24.40)	45 (50.00)	<0.0001

ΔP: GG vs GT; ΔΔP: GG vs TT; IgAN, immunoglobulin A nephropathy.

patients in Asia with IgA nephropathy and is irrelevant to patients in Europe and America, DD genotype and D allele has nothing to do with progression of patients with IgA nephropathy in Asia and Europe [16]. 2015 Junya et al. [5] n a retrospective multi-center study found that ACE DD genotype was associated with renal progression in patients with IgA nephropathy, compared with the ACE ID/II genotype. Using RAS blockers can inhibit the progression of renal function. That is to say, ACE I/D gene can serve as a useful noninvasive biomarkers that can predict whether the patient is effective with RAS blockers or not. The patients with the ACE DD genotype was more suitable for clinical treatment with RAS blockers.

AGT gene in human located on chromosome 1 q42-43, long 13 Kb, consist of the 5 exons and 4 introns. Its 2 exon region of T/C mutation makes encoding product of 235 amino acids from the methionine mutated into threonine, namely M235T. A meta analysis found that AGT M235T polymorphism associated with susceptibility to coronary artery disease in Han population [17]. The study of Masanori [18] found that compared with minimal change glomerular disease, patients in IgA nephropathy have a higher expression of AGT protein in glomerular mesangial cells and endothelial cells, and the level of glomerular AGT protein is associated with angiotensin, transforming growth factor, glomerular cell number and glomerular sclerosis. Therefore, RAS system is related to kidney disease and the severity of glomerular injury [19].

Compared with the former two genes, the research of eNOS gene is less. eNOS gene locates on chromosome 7 q35~36 with 25 exons and 26 introns, long 21 Kb. nitric oxide synthase Encoded by eNOS gene is a key enzyme to maintain the basal level of NO in the blood vessel wall. The mutation of the 7th exon in G894T may lead to the encoded glutamic acid at position 298 is replaced by aspartic acid, which affects the activity of encoding of eNOS, thereby affect nitric oxide (NO) synthesis. NO is an endothelium-derived relaxing factor, which play an important role in vasodilation, vascular smooth muscle relaxation, inhibition of endothelial cell proliferation and regulating renal hemodynamics and other aspects. One study found that eNOS G894T GG genotype in patients with IgAN related to the progression of the renal function [20].

2013 zhu [6] studied the association of the ACE/AGT/eNOS polymorphisms and IgAN, found that ACE, AGT and eNOS gene were associated with development of renal failure in patients with IgA nephropathy, whereas ACE and eNOS gene were associated with albuminuria and development of renal failure in patients with IMN. In this study, we analyzed the distri-

Table 4. Correlationa analysis of ACE/AGT/eNOS gene polymorphisms and Serum creatinine in IgAN group

Locus	Serum creatinine (mmol/L)	Z	P
ACE I/D			
II	78.27 (64.23, 112.78)	-2.73	0.018*
ID	89.04 (79.41, 103.75)	-2.62	0.009**
DD	178.00 (101.99, 204.24)	-2.75	0.006***
AGT M235T			
TT	0	0	0
TC	90.06 (77.80, 154.04)	-0.16	0.872
CC	89.61 (71.49, 136.64)		
eNOS G894T			
GG	93.74(69.27, 175.35)	-0.90	0.925ΔΔΔ
GT	89.03 (78.49, 96.03)	-0.47	0.637Δ
TT	109.05 (85.65, 150.91)	-0.60	0.547ΔΔ

DD vs II: *P; DD vs ID: **P; DD vs II+ID: ***P; GG vs GT: ΔP; GG vs TT: ΔΔP; GG vs GT+TT: ΔΔΔP; IgAN, immunoglobulin A nephropathy.

Table 5. Correlationa analysis of ACE/AGT/eNOS gene polymorphisms and 24h proteinuria in IgAN group

Locus	24 h proteinuria (g)	Z	P
ACE I/D			
II	1.31 (0.14, 2.65)	-2.28	0.023*
ID	1.97 (1.38, 4.67)	0.00	1.00**
DD	2.66 (1.44, 3.87)	-1.67	0.095**
AGT M235T			
TT	0	0	0
TC	1.48 (0.89, 3.34)	-0.51	0.613
CC	2.15 (1.19, 3.33)		
eNOS G894T			
GG	2.45 (1.00-4.31)	-1.05	0.292ΔΔΔ
GT	1.67 (1.31-3.08)	-0.30	0.768Δ
TT	0.77 (0.13-1.75)	-1.76	0.079ΔΔ

DD vs II: *P; DD vs ID: **P; DD vs II+ID: ***P; GG vs GT: ΔP; GG vs TT: ΔΔP; GG vs GT+TT: ΔΔΔP; IgAN, immunoglobulin A nephropathy.

bution of polymorphisms of ACE/AGT/eNOS gene in Xinjiang Uygur. There was a significant difference in eNOS genotype GG and allele G between the IgAN and control groups (P=0.005, P<0.0001), but no differences for the ACE and AGT genes (P>0.05). The ACE DD genotype was positively associated with serum creatinine and 24 h proteinuria, the difference was statistically significant (P=0.018, P=0.023), with no differences between AGT M235T and eNOS G894T gene. This result is inconsistent with zhu, the reason may be related to our small sample size and ethnic, geographical and other relevant factors. We need to expand the sample size and repeated studies to further explore.

In summary, ACE/AGT/eNOS gene are not susceptible gene of patients with IMN in Xinjiang Uygur, but may be the functional gene. They are closely associated with the clinical manifestations, nephrologists need attention. The DD genotype in ACE gene has a higher 24 h proteinuria and the Serum creatinine than II genotype in Xinjiang Uygur patients with IMN. The CC genotype in AGT gene has a higher Serum creatinine than TC genotype and the GG genotype in eNOS gene has a higher Serum creatinine than GT genotypein Xinjiang Uygur patients with IMN. This is the first study of association between ACE/AGT/eNOS gene and patients with IgAN in Xinjiang Uygur. AGT M235T locus detected only CC and CT genotype, no TT genotype in this study. Analysis of the reasons may be related to sample size, ethnic, gender, place of residence, living habits, environmental factors, diet, clinic time, diagnosis and intervention effect and so on. We will continue to expand the sample size to verify association of ACE I/D, AGT M235T, eNOS G894T gene polymorphism with IgAN patients, the proposed joint research mode multiple sites for risk prediction in patients with IgAN and target gene therapy Provide evidence. Multi-site study so as to provide new evidence for risk prediction as well as prevention and treatment.

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Disclosure of conflict of interest

None.

Table 6. Correlationa analysis of ACE/AGT/eNOS gene polymorphisms and hematuria in IgAN group

Locus	Hematuria (/ml)	Z	P
ACE I/D			
II	38.50 (15.50, 147.75)	-0.70	0.487*
ID	38.00 (18.75, 98.50)	-0.75	0.456**
DD	87.00 (24.00, 110.50)	-0.80	0.427***
AGT M235T			
TT	0	0	0
TC	45.00 (31.00, 339.00)	-1.20	0.232
CC	33.50 (12.75, 90.00)		
eNOS G894T			
GG	36.00 (12.25, 87.00)	-1.12	0.261ΔΔΔ
GT	61.50 (23.75, 306.50)	-1.08	0.281Δ
TT	71.00 (27.00, 91.00)	-0.58	0.563ΔΔ

DD vs II: *P; DD vs ID: **P; DD vs II+ID: ***P; GG vs GT: ΔP; GG vs TT: ΔΔP; GG vs GT+TT: ΔΔΔP; IgAN, immunoglobulin A nephropathy.

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