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Relation of RAS Gene Polymorphisms with Impaired Glucose Tolerance in Patient with End Stage Renal Failure

Derya Oz[1], Aysun Toraman Avcu[1], Seyhun Kursat[2], Hulya Colak Bahadir [2] and F. Sirri Cam[3]

[1]Department of Internal Medicine, Celal Bayar University Medical Faculty, Manisa, Turkey
[2]Department of Nephrology, Celal Bayar University Medical Faculty, Manisa, Turkey
[3]Department of Medical Genetics, Celal Bayar University Medical Faculty, Manisa, Turkey

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ABSTRACT Renin angiotensin system (RAS) is a system that is the role of renal hemodynamics, blood pressure and fluid electrolyte balance regulation with progression to end-stage renal disease (ESRD). Angiotensin II (AII),the main mediatör of this system, is thought to cause a functional impairment in insulin receptor and post receptor signaling pathways. Our aim; was to show the relation of angiotensin-converting enzyme (ACE), angiotensin II receptor type 1 (AT1 R) and angiotensinogen gene (AGT) polymorphisms, that cause genetic susceptibility to over activation of RAS, to glucose intolerance in patient with end stage renal failure. The study included one hundredth patients. Based on fasting plasma glucose values and oral glucose tolerance test second hours glucose values, patients were grouped as normal patients and patients with glucose intolerance [impaired fasting glycemia (IFG), impaired glucose tolerance (IGT) and diabetes mellitus (DM)]. Insulin resistance was calculated by HOMA-IR method and insülin sensitivity index was calculated by ISI-S method. ACE, AT1 R, AGT polymorphisms were detected by polymerase chain reaction (PCR) method. There was no statistically significant difference between distribution of ACE, AT1R and AGT genotypes and glucose intolerance groups, and insulin resistance. There was a statistically significant difference between MM and TT genotypes and average insulin resistance values ($p < 0,04$). We have obtained results suggesting a relation between ACE gene D allele and glucose intolerance in patients with end stage renal failure ($p=0.06$). Moreover, a relation has been shown between AGT gene T allele and insulin resistance ($p=0.04$).

INTRODUCTION

Chronic renal failure is a multifactorial disease in which genetic factors, in addition to major risk factors such as hypertension and diabetes, play a role (Narita and Gejyo 2004). In the development of end stage renal failure, metabolic abnormalities, in addition to glycemic state which is a strong and independent risk factor and known diabetes, contribute to progressive renal disease and cardiovascular events that are among the primary causes of mortality in haemodialysis patients (Lovati et al. 2001). Among these renal and cardiovascular risk factors, there is a well know relation between hypertension and insulin resistance and hyperinsulinemia (Lovati et al. 2001). Activation of phosphatidyl inositol-3-Kinase (PI3-K) enzyme that is involved in postreceptor signalling pathway by insulin causes an increase in NO (nitric acid) in endothelial cells and decrease in the myosin light chain activity which leads to vasoconstriction in vascular smooth muscle cells. This normal response disappears in the presence of insulin resistance (Lovati et al. 2001; Steinberg et al. 1996). Moreover, in insulin resistance, a decrease occurs in the quantity of local AT1 R and AII production in tissues through posttranscriptional mechanisms. Insulin and A II play a molecular role as negative regulators in PI3-K pathway.

It has been thought that A II causes a functional impairment by increasing serine phosphorylation in some enzymes involved in insulin receptor and post-receptor signalling pathways and decreasing tyrosine phosphorylation that is needed for a normal cascade of events, and thus leads to a decrease in intake of glucose into cell (Lovati et al. 2001; Steinberg et al. 1996).

It has been shown in vivo in animal studies that using agents blocking AT1 R causes an improvement in postprandial insulin-mediated glucose metabolism (Lovati et al. 2001). RAS is an enzyme cascade playing a role in renal hemodynamia, blood pressure and fluid electrolyte balance and determining target organ damage (Mizuno et al. 2002; El-Atat et al. 2004). Angiotensin I (AI) and AII, final products from

Address for correspondence:
Dr. F. Sirri Cam
Celal Bayar University,
Faculty of Medicine,
Department of Medical Genetics,
45010 Manisa, Turkey
Phone: +90 236 233 19 20
Fax: +90 236 233 14 66
E-mail: sirri.cam@cbu.edu.tr

AGT, are produced through two enzymes of this enzymatic cascade, which are renine and ACE (Fox et al. 2005). AII, which is an important element of RAS, is a strong vasoconstrictor. AII shows its vasoconstrictor effect mainly on postglomerular arteriole and increases glomerular hydraulic pressure. Cell growth directly contributes to the onset and acceleration of chronic renal damage (Mizuno et al. 2002). Thus, inappropriate activation of RAS plays a central role in the pathogenesis of chronic renal failure (El-Atat et al. 2004). Genetic susceptibility to over activation of this system may cause loss of filtration rate in a shorter time and susceptibility to the development of renal failure (Mallamaci et al. 2000). For this reason, the importance of the RAS-related gene polymorphisms in individuals having a risk of progressive renal failure has been emphasized.

One of the gene polymorphisms concerned with over-activation of RAS is the ACE gen polymorphism which is an insertion (I)/deletion (D) polymorphism in intron 16 (Mallamaci et al. 2000). There exist studies showing that ACE gene I/D polymorphism can be associated with the development and progression of renal failures having different etiologies (Merta et al. 2003). Individuals who are homozygous for deletion polymorphism (DD) have more plasma ACE activity when compared to those who are homozygous for insertion polymorphism (II) (Mallamaci et al. 2000). Increased ACE activity is associated with over-production of AII (El-Atat et al 2004). The contribution of AT1 R gene polymorphism in the development of renal disease and progression of end stage renal failure could be associated with the genetic diversity in target organ sensitivity to AII which acts through AT1 R (Merta et al. 2003). AGT gene, which is another gene polymorphism associated with RAS activation, has two variants which are T174M and M235T. Both of these molecular variants have been observed more frequently in all hypertensive patients when compared to controls. Moreover, M235T variant has been found to be associated with higher plasma levels of AGT in hypertensive individuals (Mallamaci et al. 2000).

It has been known that renal failure and insulin resistance increase both the total and cardiovascular mortality and morbidity (Chahwala et al. 2009; Dogra et al. 2006; Guarnieri et al. 2009). The researchers' aim was to establish whether ACE, AGT and AT1 R gene polymorphisms had an association with impaired glucose tolerance, as establishing the relation between insulin resistance and genetic polymorphisms concerned with RAS would light the way for future studies on preventing mortality and morbidity and slowing down the progression of the disease through non-pharmacological and pharmacological treatments in cases having an increased risk of renal disease and those in whom the disease diagnosed at a late stage.

MATERIAL AND METHODS

100 pre-dialytic and dialytic patients with end-stage renal failure, 50 of which had a known diabetes or having no previously known diabetes but found to have diabetes after glucose tolerance test, were included in the study. The patients were being followed up by the Nephrology Service of Celal Bayar University, Medical Faculty. In patients with no known diabetes, 75-gram oral glucose tolerance test was performed. Based on the results of oral glucose tolerance test and fasting plasma glucose values, patients were grouped into four categories: normal glucose tolerance group (fasting plasma glucose < 100 mg/dl and a 2-hour glucose value of < 140 mg/dl after oral glucose tolerance test), IFG group (fasting plasma glucose between 100-125 mg/dl), IGT group (a 2-hour glucose value between 140-199 mg/dl after oral glucose tolerance test) and DM group (a 2- hour plasma glucose of 200 mg/gl and over after oral glucose tolerance test). Insulin resistance and insulin sensitivity index (ISI) were calculated in all the patients that had undergone oral glucose tolerance test. Insulin resistance was determined by HOMA-IR using fasting glucose and fasting insulin sensitivity index [fasting glucose (mmol/lt) x fasting insulin (microunit/mlt) /22.5)] while insulin sensitivity index was determined by ISI-S [0.226 – 0.0032 x BMI – 0.0000645 x insulin (120th minute) - 0.0375 x glukoz (90th minute)] method.

Those having an active malignancy or a history of malignancy, chronic renal failure associated with urinary system obstruction or a chronic or active infection were excluded from the study.The present study was approved by the ethics committee of Celal Bayar University Medical Faculty and each patient was given an informed consent form.

Molecular Analysis

Genomic DNA was extracted from 200 µl of EDTA-anticoagulated peripheral blood leucocytes using the QUIAmp Blood Kit (QUIAGEN, Ontaria Canada, Cat. no: 51106). Amplification of DNA for genotyping the ACE I/D polymorphism was carried out by PCR in a final volume of 15 µl containing 200 µM dNTP mix, 1.5 mM $MgCl_2$, 1x Buffer, 1 unit of AmpliTaq polymerase (PE Applied Biosystems) and 10 pmol of each primer. The primers used to encompass the polymorphic region of the ACE were 5'-CTGGAGACCACTCCCATCC TTTCT-3' and 5'-ATGTGGCCATCACATT CGTCAGAT-3' (Rigat et al. 1992).

DNA is amplified for 35 cycles, each cycle comprising denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extention at 72 °C for 1 min with final extention time of 7 min. The initial denaturation stage was carried out at 95°C for 5 min. The PCR products are separated on 2.5% agarose gel and identified by ethidium-bromide staining. Each DD genotype was confirmed through a second PCR with primers specific for the insertion sequence (Shanmugam et al. 1993).

To analyse AGT M235T polymorphism we amplified genomic DNA with primers 5'-GATGCGCACAAGGTCCTG-3' and 5'-CAGGGTGCTGTCCACACTGGCTCGC-3', respectively. The PCR mixture (50 microliters) contained 2.5 mM $MgCl_2$, 100 µmol of each dNTP and 0.5 U of Taq polymerase. The amplification conditions were 94 °C for 5 min, followed by 25 cycles of one minute at 94 °C, one minute at 61 °C, and one minute at 72 °C. We determined the AGT genotype by 2.5% agarose gel electrophoresis after digestion of 5 microliters PCR product by 2.5U SfaNI (Caulfield et al. 1994).

The AT1R A1166C polymorphism was analysed with primers 5'-GCAGCACTTCA CTACCAAATGAT-3' and 5'-TGTTCTT CGAGCAGCCGT-3' as previously described (Alvarez et al. 1998). Amplification by PCR was carried out in a total volume of 25 ml using 50–200 ng of genomic DNA, 1.5 mM MgCl , 100 mM dNTP, 1.5 units of Taq polymerase and 10 pmol of each primer. 10 µl of each PCR were digested with BclI and electrophoresed on a 2.5 % agarose gel. The AT1R alleles were visualized as fragments of 176 bp (C) and 156 bp (A).

All the statistical analyses were conducted by SPSS 14.0 for Windows software. Continuous variables were expressed as ± SD. The intra-group distribution of genotypes was evaluated by Chi-square, Kruskal Willis and Mann Whitney tests. $p<0.05$ was considered as statistically significant.

RESULTS

A total of 100 pre-dialytic and dialytic patients with end-stage renal failure were included in our study. Oral glucose tolerance test was performed in patients included in the study and having no known diabetes. Frequency of patients having normal glucose tolerance or glucose intolerance according to fasting plasma glucose and oral glucose tolerance test are given in Table 1.

When the relation between distribution of ACE, AT1 R and AGT genotypes and glucose tolerance status was evaluated in patients grouped as having normal glucose tolerance and having glucose intolerance, no statistically significant difference was found between normal glucose tolerance and glucose intolerance groups although the percentage of those having ACE II genotype was 45% in normal glucose tolerance group and 21.3% in intolerance group (Table 2). Similarly, in terms of genotypes distribution, there was no statistically significant difference among the groups when the relation between distribution of ACE, AT1 R and AGT genotypes and glucose intolerance groups (IFG, IGT and DM) were evaluated.

In all the patients that had undergone oral glucose tolerance test (n=68) and in those having normal glucose tolerance (n=20), insulin resistance was calculated by HOMA-IR method,

Table 1: Average values of the demographic features and the frequency laboratory parameters of the patients

	Average ± SD		*N (%)*		*N (%)*
Age	62.92 ± 12.71	Normal glucose tolerance	20	IFG	5
Body Mass Index	24.95 ± 4.38	Glucose Ýntolerance	80	IGT	25
Creatine Clearance	10.77 ± 2.40	Total	100	DM	50

IFG: Impaired Fasting Glycemia, IGT: Impaired Glucose Tolerance, DM: Diabetes Mellitus

Table 2: The relation between distribution of ACE genotypes and glucose tolerance status.

	ACE gen polymorphýsm			
	II Genotype N (%)	*ID Genotype N (%)*	*DD Genotype N (%)*	
Normal	9 (45.0)	7 (35.0)	4 (20.0)	*P* 0.09
Having intolerance	17 (21.3)	39 (48.8)	24 (30.0)	

and any value above 2,7 was considered as insulin resistance. When the relation between ACE, AT1 R and AGT genotypes and insulin resistance was evaluated in the said two patient groups whose insulin resistance had been calculated, no statistically significant difference was found among the genotypes in terms of insulin resistance.

Similarly, when the relation between ACE, AT1 R and AGT genotypes and average insulin resistance values were evaluated, no statistically significant difference was found among genotypes in terms of average insulin resistance values. In all the patients that had undergone oral glucose tolerance test (n=68) and in those having normal glucose tolerance (n=20), insulin sensitivity index was calculated by ISI-S method. When the relation between ACE, AT1 R and AGT genotypes and average insulin sensitivity index values were evaluated, no statistically significant difference was found among genotypes in terms of average insulin sensitivity index values.

The relation between I and D allele genoypes for ACE gen polymorphism and glucose tolerance status was evaluated. When distribution of II and DD genotypes, II and ID genotypes and ID and DD genotypes in normal and glucose intolerance groups were evaluated, it was observed that the results were almost statistically significant for D allel in II ve DD genotype ($p = 0.08$) and II and ID genotype ($p=0.06$) groups (Table 3) (statistical significance $p<0.05$). When the relation between genotypes for AT1R and AGT gen polymorphism and glucose tolerance status were evaluated, there was no statistically significant difference in normal glucose tolerance and glucose intolerance groups in terms of genotype distribution.

When the relation between I and D allel genotypes for ACE gene polymorphism and A and C allel genotypes for AT1R gene polymorphism and average insulin resistance values (Table 4) were evaluated, there was no statistically significant difference. When the relation between M and T allel genotypes for AGT gene polymorphism and average insulin resistance values was evaluated, there was a statistically significant difference between MM and TT genotypes and average insulin resistance values ($p < 0.04$) (Table 5).

Table 3: The relation between I allele and D allele genotypes and glucose tolerance in ACE gen polymorphism

	ACE gen polymorphism		
	II Genotype N (%)	*DD Genotype N (%)*	
With normal	9 (34.6)	4 (14.3)	*P* 0.08
intolerance	17 (65.4)	24 (85.7)	
	II Genotype N (%)	*ID Genotype N (%)*	
With normal	9 (34.6)	7 (15.2)	*P* 0.06
intolerance	17 (65.4)	39 (84.8)	
	ID Genotype N (%)	*DD Genotype N (%)*	
With normal	7 (15.2)	4 (14.3)	*P* 0.91
intolerance	39 (84.8)	24 (85.7)	

Table 4: Average insulin resistance values in all the patients carrying ACE, AT1 R and AGT genotypes and undergoing OGTT

ACE Gen	*N*	*HOMA-IR Average ± SD*	*p*
II Genotype	19	2.92±2.19	0.49
ID Genotype	29	2.63±2.79	
DD Genotype	20	3.17±3.27	
AT1 R Gene	*N*	*HOMA-IR Average ± SD*	*p*
AA Genotype	40	2.88±2.64	0.63
AC Genotype	24	3.04±3.16	
CC Genotype	4	1.73±0.88	
AGT Gene	*N*	*HOMA-IR Average ± SD*	*p*
MM Genotype	9	1.89±1.83	0.24
MT Genotype	23	3.01±3.17	
TT Genotype	36	3.03±2.69	

Table 5: The relation between AGT genotypes and average insulin resistance values

	HOMA Average ± SD	
AGT Gen		
MM Genotype	1.89 ± 1.83	*P* 0.11
MT Genotype	3.01 ± 3.17	
AGT Gen		
MM Genotype	1.89 ± 1.83	*P* 0.04
TT Genotype	3.03 ± 2.69	
AGT Gen		
MT Genotype	3.01 ± 3.17	*P* 0.37
TT Genotype	3.03 ± 2.69	

When the relation between I and D allel genotypes for ACE gen polymorphism, A and C allel for AT1 R gene polymorphism and M and T allel for AGT gene polymorphism and average insulin resistance values (Table 6) were evaluated, there was no statistically significant difference.

Table 6: Average insulin sensitivity index values of ACE, AT1 R and AGT genes in patients carrying the said genotypes and undergoing OGTT

ACE Gen	*ISI-S Average ± SD*	*p*
II Genotype (N=19)	-6.51±1.36	0.32
ID Genotype	-6.94±2.43	
II Genotype	-6.51±1.36	0.19
DD Genotype (N=20)	-6.10±1.70	
ID Genotype (N=29)	-6.94±2.43	0.12
DD Genotype	-6.10±1.78	
AT1 R Gene	*ISI-S Average ± SD*	*p*
AA Genotype (N=40)	-6.51±2.10	0.38
AC Genotype	-6.58±1.60	
AC Genotype (N=24)	-6.58±1.60	0.43
CC Genotype	-7.07±3.43	
AA Genotype	-6.51±2.10	0.49
CC Genotype (N=4)	-7.07±3.43	
AGT Gene	*ISI-S Average ± SD*	*p*
MM Genotype (N=9)	-6.66±1.37	0.23
MT Genotype	-6.20±2.24	
MM Genotype	-6.66±1.37	0.48
TT Genotype (N=36)	-6.78±1.98	
MT Genotype (N=23)	-6.20±2.24	0.17
TT Genotype	-6.78±1.98	

No statistically significant difference was found when the distribution of ACE, AT1 R and AGT genotypes were evaluated in the patients having known diabetes, in those found to have diabetes as a result of oral glucose tolerance test (n=50) and in those having glucose tolerance (n=20). Similarly, for ACE, AGT, AT1 R gen polymorphism, it was found that there was no statistically significant difference in terms of genotype distribution between those having normal glucose tolerance and diabetic patients.

DISCUSSION

RAS is a very important system in regulating vascular and renal functions. Recently, some gene polymorphisms have been identified to play a role in the regulation of the said system. Three gene polymorphisms, which are AGT M235T, ACE I/D and AT1 R gene A1166C polymorphism have been widely studied in the literature.

ACE gene I/D polymorphism can be the cause of the difference in plasma ACE activity, which may lead to 50% or more among individuals. DD homozygotes have the highest plasma ACE activity while II homozygotes have the lowest plasma ACE activity. AGT gen polymorphism and AT1 R gen polymorphism have been associated with increased plasma AGT level and increased response, respectively (Young et al. 1998).

The meta analysis of all the published studies on the association of ACE gen polymorphism and cardiovascular diseases have supported the association between ACE gen D allele and myocard infarct. Moreover, increased hypertension and ischemic heart disease risk has been shown in individuals who are homozygous for C allele of AT1 R gen polymorphism and those who are homozygous for T allele of AGT gene polymorphism (Young et al. 1998).

The last product of RAS activity which is effector peptide A II has been shown to have vascular and myocardial effects and to precipitate glomerular sclerosis in experimental models. Both in type 1 diabetes and type 2 diabetes patients, antiproteinuric and renoprotective effects of ACE inhibitors have suggested that RAS has an important pathophysiological role in diabetic renal patients (Young et al. 1998).

It has been shown that a treatment with ACE inhibitor and AT1 R antagonist may decrease the risk of cardiovascular disease which is an important mortality and morbidity cause and also effect pathogenesis of metabolic syndrome components such as atherosclerosis and insulin resistance in end-stage renal failure patients. Showing that treatment with ACE inhibitors cause an improvement in insulin resistance and atherosclerosis have led to the consideration that RAS activation may increase the development of dyslipidemia and diabetes. In a study of Dogra et al., insulin resistance, in addition to inflammation, systolic hypertension and increased blood pressure, has been associated with vascular dysfunction in CRF patients (2006). All of these metabolic syndrome criteria are the factors contributing to the mortality, morbidity and progression of renal failure. In this regard, in a study where the association between RAS activity and metabolic syndrome was studied, it was found that ACE gen I/D polymorphism and

glucose intolerance had an association and I allele frequency was observed to be higher in the group having glucose intolerance (Thomas et al. 2001).

Although there exist studies in the literature on RAS gene polymorphism in end-stage renal failure patients (Lovati et al. 2001; Buraczyñska et al. 2006), the role of RAS genes in type 2 diabetes patients having CRF (Prasad et al. 2006), RAS gene polymorphisms due to other etiological reasons such as IgA nephropathy (Lau et al. 2004), interstitial nephritis (Buraczyñska et al. 2002), chronic glomerulonephritis (Buraczyñska et al. 2001), focal segmental glomerulosclerosis (Luther et al. 2003) and polycystic renal disease (Lee and Kim 2003) that may lead to end-stage renal failure and glucose intolerance of RAS genes, no studies could be found on the relation between glucose intolerance and ACE, AT1 R and AGT gene polymorphisms in end-stage renal failure patients.

In our study, pre-dialytic and dialytic patients with end-stage renal failure were divided into two groups as normal glucose tolerance group and glucose intolerance group (IFG, IGT and DM) based on fasting plasma glucose and 2nd hour oral glucose tolerance test plasma glucose values. When the distribution of ACE, AT1 R and AGT genotypes were studied in terms of their association with glucose tolerance, no statistically significant difference was found between normal glucose tolerance and glucose intolerance groups. Similarly, when the association of genotype distribution with the subgroups of glucose intolerance (IFG, IGT and DM) was studied, no statistically significant difference was found. When the same evaluation was performed in diabetic patients and those having normal glucose tolerance, no statistically significant difference was found in terms of genotype distribution.

The reason of not finding a statistically significant difference between the groups in terms of genotype distribution even though those having ACE II genotype was % 45 in normal glucose tolerance group and 21.3% in intolerance group was thought to be the small number of patients in two compared groups and it was considered that larger number of patients could have yielded statistically significant values.

In all the patients having no known diabetes and that had undergone oral glucose tolerance test, insulin resistance was calculated by HOMA-IR method while the insulin sensitivity index was calculated using the ISI-S method suggested by Stumvoll et al. (Kanauchi et al. 2004; Stumvoll et al. 2000). There was no statistically significant difference when the association of ACE, AT1 R and AGT genotypes with insulin resistance, the average values of insulin resistance and the average values of insulin sensitivity index were studied individually in normal glucose tolerance group and all the patients that had undergone OGTT. When the association of I and D allele genotypes and glucose tolerance state was studied for ACE I/D polymorphism in the patients, the results obtained in the groups having II and DD genotype ($p=0.081$) and II and ID genotype ($p=0.057$) was almost statistically significant. When it was taken into consideration that ACE gen II genotype was 45% in normal glucose tolerance group and 21,3% in the intolerance group, it was thought that ACE gen D allele was associated with glucose intolerance in end-stage renal failure patients and larger number of patients could have yielded statistically significant results.

As mentioned before, in a study of Thomas et al. (2001) where they studied the association between RAS activity and metabolic syndrome in a population composed of 853 patients meeting the metabolic syndrome criteria, I allele was found to be associated with glucose intolerance.

When A allele and C allele genotypes in AT1 R gen polymorphism and M allele and T allele genotypes in AGT gen polymorphism were studied in terms of their association with glucose tolerance, the difference obtained between normal and glucose intolerance groups was not statistically significant. Similarly, when I allele and D allele genotypes in ACE gen polymorphisms, M allele and T allele in AGT gen polymorphism, A and C allele in AT1 R gene polymorphisms were studied in terms of their association with normal glucose tolerance and having diabetes, the difference between normal and diabetes groups was not statistically significant.

In our study, insulin resistance of I and D allele genotypes for ACE gen polymorphism, A and C allele genotypes for AT1 R gen polymorphism and M and T allele genotypes for AGT gene polymorphism were studied. The only statistically significant difference was obtained between average values of insulin resistance in individuals who were homozygous for T allele and those who were homozygous for M allele

($p<0.043$). When the number of patients included in the study was taken into consideration, it was thought that increasing the number of patients could also increase the statistical significance for the association between T allele and insulin resistance. Evaluation of the association between genotypes and average values of insulin sensitivity indices revealed no statistically significant results in three of the genetic polymorphisms.

The role of RAS components in progression into end-stage renal failure, in the pathophysiology of the hypertension that may occur as a result of renal failure, in ischemic heart diseases, insulin resistance and diabetic renal diseases have been determined in previous studies.

It has been thought that the results obtained in our study are unique in the sense that determination of genetic polymorphisms coding RAAS components in those having a risk of renal failure or in those having renal failure may emphasize the importance of a change in the life-style and early-initiation of pharmacological treatments against factors playing a role in the development of the disease and its progress into end-stage renal failure especially in patients meeting metabolic syndrome criteria. With regard to pharmacological treatment methods, drugs that could be taken into consideration was thought to be renin inhibitors or A II receptor blockers due to increased serum ACE level in individuals having ACE gene polymorphisms and especially in those who are homozygous for DD, directly renin inhibitors due to expected increased AGT levels in individuals with AGE gene polymorphism and especially in those who are homozygous for TT, and ACE inhibitor or renin inhibitors due to expected increased AII receptor response in individuals having AT1 R gene polymorphism and especially in those who are homozygous for CC genotype.

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