Renin-angiotensin system polymorphisms and renal graft function in renal transplant recipients

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ABSTRACT

Objectives: To analyze the role of 3 polymorphisms of the renin-angiotensin system (RAS) in renal transplant recipients (RTRs) and correlate them with graft function.

Methods: The present study was performed in the Drug Applied Research Center, Tabriz Medical University, Tabriz, Iran from September 2003 to December 2005 on 108 RTRs (66 males and 42 females, with a mean age of 37.34±4.97 years) with stable allograft function (creatinine ≤2.2 mg/dl). Following the DNA extraction from the blood leukocytes, the genotypes of the angiotensin converting enzyme (ACE I/D), angiotensinogen (ANG M235T), and angiotensin II type 1 receptor (ATR1 A1166C) were determined by polymerase chain reaction. The magnitude of clearance of creatinine (ClCr) in the setting of each of the above RAS polymorphisms was determined. The ClCr was measured by modification of diet in renal disease formula. Values were expressed as mean±SD; $p \leq 0.05$ was considered to indicate statistical significance.

Results: There was no association of each genotype of the RAS alone with ClCr, serum urea, cyclosporine through level and the degree of urinary protein excretion rate. However, patients with the DD genotype of angiotensin converting enzyme + CC genotype of angiotensin II type I receptor polymorphisms had lower ClCr ($p=0.05$) and a higher urinary protein excretion rate ($p=0.03$). Other combination genotypes of RAS had no effect on allograft function. Interestingly, the percent of hypertensive patients in the C allele (70%) was more than the A allele (30%) of ATR1 polymorphism ($p=0.04$).

Conclusion: Although none of the single gene polymorphisms of the RAS affected renal allograft function, combinations of these genotypes were associated with the outcome of allograft function.

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The renin-angiotensin system (RAS) is an important mediator of renal and cardiovascular physiology.[1] In the RAS cascade, angiotensinogen (ANG) is synthesized primarily by the liver and released into the circulation, where it is cleaved by renin to generate angiotensin I (ANGI). The latter is subsequently converted to angiotensin II (ANGII) by the angiotensin-converting enzyme (ACE). It is the major effector of the RAS cascade, and primarily acts on the angiotensin receptors type 1 and type 2 (ATR1 and ATR2).[2] The classical opinion of ANGII function, the effector peptide of the RAS, which is considered a vasoactive hormone that regulates the vascular tone, homeostasis, salt balance, and blood pressure, has started to be changed recently. Research in nephrology and cardiology sciences demonstrated that ANGII regulates cell growth, remodeling, extracellular matrix production, and many intracellular signaling mechanisms with which it shares classic cytokines. It also has been shown that ANGII is involved in key events of inflammation and thrombosis.[3,4] The RAS has been considered one of the probable pathophysiologic mechanisms governing disease progression.[5] It has an important role in the pathogenesis of cardiovascular disease.[6] Recent studies have demonstrated some association between RAS, activity and the development and progression of different entities such as diabetes mellitus (DM) and chronic allograft nephropathy (CAN).[7] This system regulates blood pressure and may affect chronic kidney disease through

controlling of renal function, both in physiological and pathological conditions by induction of tissue growth and fibrosis.[8,9] These observations are particularly important while the RAS has been established as an independent risk factor for progression of renal damage and development of end-stage renal disease.[10] Substantial evidence exists for the involvement of the RAS in diabetic nephropathy,[11] gA nephropathy,[10] adult autosomal dominant polycystic kidney disease (ADPKD),[12] focal segmental glomerulosclerosis (FSGS),[5] hypertension,[9] coronary artery disease,[6] congestive heart failure,[13] dilated cardiomyopathy,[2] chronic obstructive pulmonary disease,[14] systemic lupus erythematosus,[15] and sarcoidosis.[16] Recently, several investigators have reported that over-activation of the RAS is linked to poorer long-term renal transplant function and decreased graft survival rate.[17,18] Thus, polymorphism of the responsible genes, which regulate the activation of the RAS, may be an important determinants for renal transplant outcomes.[17] Among these forms of RAS polymorphisms, the ANG as a first step in the system with M and T alleles, ACE as the key enzyme for converting of ANGI to ANGII, which has I and D alleles, and ATR1 as an effector point for RAS with A and C alleles, are of particular interest. Research has shown that RAS activity is significantly higher in some genotypes, including ANG-AA, ANG-TT, ACE-DD, and ATR1-CC.[19] Moreover, clinical studies have shown that the ANG-AA and ACE-DD genotypes are associated with poorer long-term renal allograft function.[20] We hypothesized that the RAS plays a significant role in renal function, and several polymorphisms of the RAS system have been associated with renal disease. The aim of this study was to analyze the role of 3 polymorphisms in renal transplant recipients (RTRs) and correlate them with graft function.

Methods. The present analytical cross-sectional study was performed in the Drug Applied Research Center (DARC), Tabriz Medical University, Tabriz, Iran from September 2003 to December 2005. After obtaining written informed consent and an approval from the ethical committee from DARC, Tabriz Medical University, Tabriz, Iran, a total of 108 RTRs (66 males and 42 females with the mean age of 37.34± 4.97 years, range 12-72) were enrolled in the study. They had stable serum creatinine (Cr) ≤2.2 mg/dl during the last 3 months despite underlying histological findings of the renal allograft including normal allograft, chronic rejection and recurrence of *denovo* glomerulonephritis, chronic cyclosporine toxicity, and chronic pyelonephritis. To rule out graft loss due to early surgical or early immunological problems, patients with an engrafted kidney earlier than one year were not included. Positive panel reactive antibody, post-transplantation dialysis (during one week after transplantation), taking of RAS blockade, re-transplantation, urologic problems and Cr more than 2.2 mg/dl were among the other exclusion criteria. Pre-renal (such as dehydration and renal artery stenosis) and post-renal (such as urolithiasis and ureter stricture) complications of allograft were excluded by physical examination and ultra-sonography. Blood pressure was measured twice each time at 2 separate visits. There were at least 15-minute intervals between the 2 blood pressures measured. Hypertension was defined when the patients were treated by anti-hypertensive drugs or systolic blood pressure was more than 140 mm Hg, or diastolic blood pressure was more than 90 mm Hg. The magnitude of ClCr in the setting of each of the above RAS polymorphisms was determined by the modification of diet in renal disease (MDRD) formula for patients above 18 years old. For children and adolescents up to 18 years old, the Schwartz formula for calculation of ClCr was used.[21,22] All the RTRs received kidneys from living donors. The mean post-transplantation period was 39.96±7.25, and the duration of dialysis before transplantation were 19.55±7.44 months, respectively. The underlying causes of end stage renal diseases were chronic glomerulonephritis (22), chronic pyelonephritis (14), hypertension (12), ADPKD (11), focal segmental sclerosis (10), DM (9), obstructive uropathy (8), collagen vascular disease (5), Alport syndrome (1), cystinosis (1), and unknown causes (15). They were under a triple immunosuppressive protocol with cyclosporine+prednisolone+azathioprine or mycophenolate mofetil.

Laboratory analysis. DNA extraction. For extraction of genomic DNA, buffy coats of nucleated cells obtained from anticoagulated blood (ethylenediamine-tetraacetic acid [EDTA]) were resuspended in 15 ml polypropylene centrifugation tubes with 3 ml of nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM Na2EDTA, pH 8.2). The cell lysates were digested overnight at 37°C with 0.2 ml of 10% sodium dodecyl sulfate and 0.5 ml of a protease K solution (1 mg protease K in 1% SDS and 2 mM Na2 EDTA). After digestion was complete, 1 ml of saturated NaCl (approximately 6M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 2500 rpm for 15 minutes. The precipitated protein pellet was left at the bottom of the tube, and the supernatant containing the DNA was transferred to another 15 ml polypropylene tube. Exactly 2 volumes of room temperature absolute ethanol were added, and the tubes inverted several times until the DNA precipitated. The precipitated DNA strands were removed with a plastic spatula or pipette and transferred to a 1.5 ml microcentrifuge tube containing 100-200 µl tris-EDTA buffer (10 mM

Tris-HCl, 0.2 mM Na2EDTA, pH 7.5). The DNA was allowed to dissolve for 2 hours at 37°C before quantitating.[23]

Determination of RAS genotypes. To determine the ACE genotype of the patients, the polymerase chain reaction (PCR) technique was used as described previously.[5] In brief, to amplify a DNA fragment in intron 16 of the ACE gene, the following primers (Invitrogen Inc, California, USA) flanking the insert sequence were used: 5_-CTGGAGACCACTCCCATCCTTTCT-3_ and 5_-GATGTGGCCATCACATTCGTCAGAT-3_. The PCR reaction was performed in a 20 µl reaction containing genomic DNA (250 ng), 10 mM of Tris-HCl, pH 9.0, 50 mM of KCl, 1 mM of MgCl2, 0.1% Triton-X-100, 200 <mu>M of dNTPs, 1 pM of each primer, and 0.5 U of Taq DNA polymerase (Cinnagen Inc, Tehran, Iran). Thirty amplification cycles were carried out according to the following temperature profile: 94°C for one minute, 58°C for one minute, and 72°C for one minute. This PCR reaction yields PCR products of 190 bp for the D allele, and 490 bp for the I allele. These were detected on an ethidium bromide stained 2% agarose gel. To study the polymorphism of AGT M235T, the method described by Ohno et al[11] was utilized. In brief, restriction endonuclease digestion was performed following PCR of genomic DNA. For PCR, genomic DNA (250 ng) was used in a final volume of 50µl containing 2.5 mmol/L MgCl2, 50 mmol/L KCl, 10 mmol/L Tris hydrochloride (pH 9.0), 1 pM of each primer (Invitrogen Inc, California, USA), 250µmol/L each of the 4 dNTPs, and 0.4 U Taq polymerase (Cinnagen Inc, Tehran, Iran). Primers were: 5_-GAGTCGCACAAGGTCCTG-3_ and 5_-CAGGGTGCTGTCCACACTGGCTCGC-3_. Amplification cycles were carried out according to the following temperature profiles: an initial denaturation at 94°C, followed by 25 cycles of one minute at 94°C, one minute at 61°C, and one minute at 72°C. The longer oligonucleotide is mismatched with genomic DNA, which creates a SfaN1 (Fermentas Inc, Burlington, Canada) restriction site during amplification. After enzymatic amplification of genomic DNA, each product was digested with SfaN1. If codon 235 is AGT (M235), digestion with SfaN1 yields a 266 bp product relative to undigested 303 bp products (T235). The genotypes were analyzed by electrophoresis in 8% polyacrylamide gel and visualized by ethidium bromide staining. The ATR1 A1166_C polymorphism was analyzed according to the technique described by Ohno et al.[11] Approximately 250 ng of DNA was amplified with the following primers (Invitrogen Inc, California, USA): 5_-AAGTCTTGTAGCCAAAGTCACCT-3_ and 5_-GGCTTTGCTTTGTCTTGTTG-3_, which yield PCR products of 850 bp. The PCR reaction mix was as follows: 10 mM of Tris-HCl, pH 9, 50 mM of KCl, 1 mM of MgCl2, 0.1% Triton X-100, 200 <mu>M of dNTPs, 1 pM of each primers and 0.5 U of Taq DNA polymerase (Cinnagen Inc, Tehran, Iran). Thermocycling consisted of 2 minutes of denaturation at 94°C, followed by 40 cycles of one minute (denaturation) at 94°C, one minute (annealing) at 60°C, 2 minutes (extension) at 72°C, and final extension of 10 minutes at 72°C. The PCR product was subsequently digested by the restriction enzyme DdeI (Roche Inc, Mannheim, Germany) at 37°C, which cuts the product into 2 pieces, 600 and 250 bp fragments. An additional DdeI recognition site is created in the C-type variant, at nucleotide 1166, which is found in the 250-bp fragment. Thus, the homozygote CC produces 3 bands (600, 140, and 110 bp long), the homozygote AA produces 2 bands (600 and 250 bp long), and the heterozygote produces all 4 bands. The amplification products were resolved by electrophoresis on a 3% agarose gel and visualized under UV light following staining with ethidium bromide.

Biochemical data. Urea concentration was measured by the standard enzymatic method and Cr level was assayed by the Jaffe method. The through level of serum cyclosporine (C_0) was measured by radioimmunoassay (Kontron, Laborgerätebörse Inc, Switzerland). Urinary protein and Cr excretion was measured on 24-hour collections using the pyrogallol red reagent in Alcyon 300 automated analyzer (Abbott Inc, Los Angeles, USA).

Statistical analysis. The Statistical Package for Social Sciences for Windows 11.0 (SPSS Inc, Chicago, IL, USA) was used. Categorical data were measured by frequency analysis. Clinical characteristics were compared by parametric or non-parametric tests for independent samples as appropriate. Data are presented as percentage, mean±SD, or median (range). In this study, p=0.05 was considered to indicate statistical significance.

Results. The distribution of genotypes for the ACE gene in patients were DD 35 (32%), ID 54 (50%), and II 19 (18%). It was 64 (60%) for AA, 37 (34%) for AC, and 7 (6%) for CC genotypes for the ATR1 polymorphism, and 21 (19%) for MM, 56 (52%) for MT, and 31 (29%) for TT genotypes for the ANG polymorphisms. The frequency of D and I alleles of ACE I/D polymorphism were 57.40 and 42.59%. The distribution of M and T alleles of ANG M235T was 45.37 and 54.62%, and also for ATR1 A1166C polymorphism, the frequency of A and C alleles were 76.38 and 23.61%. There was no relationship between gender and RAS polymorphisms (p>0.05). Any discrete RAS genotype did not correlate with the degree of urinary protein excretion, cyclosporine through level,

Table 1 - Biochemical and physical findings base on ACE genotypes in RTRs (n=108).

Parameters	DD	DI	II	*P* value
Cr clearance (mls/min/1.73 m^2)	65.2 ± 12.5	64.5 ± 9.4	70.7 ± 9.6	0.79
Cr level (mg/dl)	1.40 ± 0.29	1.28 ± 0.25	1.18 ± 0.19	0.09
Urea level (mg/dl)	42.5 ± 6.8	39.4 ± 6.2	39.8 ± 7.4	0.79
MAP (mmHg)	100.1 ± 6.4	96.6 ± 4.9	95.8 ± 4.5	0.58
CsA level (ng/ml)	271.4 ± 41.1	242.4 ± 33.6	306.4 ± 52.1	0.26
U Pr/Cr	0.19 ± 0.06	0.16 ± 0.06	0.23 ± 0.06	0.75

ACE - angiotensin converting enzyme, RTRs - renal transplant recipients, Cr - creatinine, MAP - mean arterial pressure, CsA - cyclosporine A, U Pr/Cr - urine protein/creatinine.

Table 2 - Biochemical and physical findings base on ANG genotypes in RTRs (n=108).

Parameters	TT	MT	MM	*P* value
Cr clearance (mls/min/1.73 m^2)	63.3 ± 13.3	66.8 ± 8.4	67.3 ± 14.7	0.88
Cr level (mg/dl)	1.39 ± 0.13	1.27 ± 0.07	1.26 ± 0.17	0.30
Urea level (mg/dl)	43.2 ± 9.4	39.0 ± 4.9	40.7 ± 8.0	0.68
MAP (mmHg)	97.9 ± 7.2	98.0 ± 3.9	96.1 ± 10.0	0.92
CsA level (ng/ml)	257.2 ± 44.1	262.7 ± 43.5	280.0 ± 58.8	0.88
U Pr/Cr	0.23 ± 0.08	0.18 ± 0.05	0.13 ± 0.05	0.58

ANG - angiotensinigen, RTRs - renal transplant recipients, Cr - creatinine, MAP - mean arterial pressure, CsA - cyclosporine A, U Pr/Cr - urine protein/creatinine.

Table 3 - Biochemical and physical findings base on ATR1 genotypes in RTRs (n=108).

Parameters	CC	AC	AA	*P* value
Cr clearance (mls/min/1.73 m^2)	74.4 ± 25.3	68.4 ± 13.3	63.2 ± 4.7	0.57
Cr level (mg/dl)	1.48 ± 0.14	1.28 ± 0.11	1.29 ± 0.09	0.33
Urea level (mg/dl)	36.4 ± 7.2	42.8 ± 9.0	40.0 ± 4.8	0.68
MAP (mmHg)	105.0 ± 10.9	102.3 ± 5.0	94.2 ± 4.3	0.04*
CsA level (ng/ml)	182.6 ± 43.6	276.5 ± 42.7	269.0 ± 38.6	0.21
U Pr/Cr	0.18 ± 0.07	0.24 ± 0.10	0.15 ± 0.05	0.44

ATR1 - angiotensin receptor type 1, RTRs - renal transplant recipients, Cr - creatinine, MAP - mean arterial pressure, CsA - cyclosporine A, U Pr/Cr - urine protein/creatinine, *significant.

and ClCr (**Tables 1-3**). However, homozygous D alleles (DD) for ACE when accompanied with homozygous C alleles (CC) for ATR1 were associated with lower ClCr (p=0.05) and higher urinary protein excretion rate (p=0.03). Any other bigenomic sets for RAS, including ACE+ATR1, ACE+ANG, and ANG+ATR1 did not impact on proteinuria and ClCr (**Tables 4 & 5**). Interestingly, the percent of hypertensive patients with the C allele (70%) was more than the A allele of ATR1 polymorphism (30%) (p=0.04), however, ANG and ACE genes polymorphism had no such association. The distribution of RAS polymorphisms was not different among the primary kidney disease result compared with kidney transplantation (p>0.05).

Discussion. Chronic allograft dysfunction (CAD) is the major cause of transplant loss in RTRs. The factors leading to CAD are not fully understood, however, they consist of a combination of the underlying kidney disease and superimposed environmental and genetic factors. Genes that affect blood pressure regulation, mesangial or vascular proliferation, or aspects of inflammatory response may play an important roles in this complex syndrome; genes determining the activity of a recipient's RAS may be alloantigen-independent factors that influence kidney allograft function.[24] Genetic polymorphisms of the various components of the RAS have been associated with the clinical course of several disease states in adults.[5] Genetic variants of the RAS have been implicated in the progression of native kidney diseases. A decreased in long-term renal allograft function has also been associated with increased activity of RAS, which may be genetically determined.[20] Gene coding for the main components of the RAS have been characterized and localized as angiotensinogen (ANG, chromosome 1q42), renin (REN, chromosome 1), angiotensin I-converting enzyme (ACE, chromosome 17), and angiotensin II receptors (ATR1, chromosome 3 and ATR2, chromosome X).[25] The present study revealed that the distribution of the ACE gene genotypes was DD 32%, ID 50%, and II 18%. Filler et al[26] showed the allele frequencies of ACE in renal transplant recipients were DD 31%, ID 41%, and II 28%.[26] Another research reported the distributions of genotypes for ACE were DD 33%, ID 48%, and II 19%.[7] The ACE D allele has been shown to be a risk factor for the development and progression of the diabetic nephropathy, and the progression of chronic renal failure in immunoglobulin-A nephropathy and in ADPKD.[24] The ACE D-allele seems to be associated with a poorer

Table 4 - Biochemical and physical findings base on ACE and ATR1 genotypes in RTRs.

Parameters	DD+CC	II+AA	*P* value
Cr clearance (mls/min/1.73 m^2)	48.0 ± 11.7	72.2 ± 12.3	0.05*
Cr level (mg/dl)	1.56 ± 0.05	1.15 ± 0.09	0.002*
Urea level (mg/dl)	39.3 ± 9.6	42.0 ± 9.8	0.76
MAP (mmHg)	109.4 ± 8.7	94.5 ± 5.8	0.07
CsA level (ng/ml)	207.3 ± 74.3	307.5 ± 75.0	0.45
U Pr/Cr	0.26 ± 0.07	0.07 ± 0.02	0.03*

ACE - angiotensin converting enzyme, ATR1 - angiotensin receptor type 1, RTRs - renal transplant recipients, Cr - creatinine, MAP - mean arterial pressure, CsA - cyclosporine A, U Pr/Cr - urine protein/creatinine, *significant.

Table 5 - Biochemical and physical findings base on ACE and ANG genotypes in RTRs.

Parameters	DD+TT	II+MM	*P* value
Cr clearance (mls/min/1.73 m^2)	70.4 ± 23.5	59.3 ± 11.4	0.74
Cr level (mg/dl)	1.62 ± 0.25	1.36 ± 0.27	0.28
Urea level (mg/dl)	46.0 ± 8.3	40.6 ± 4.3	0.66
MAP (mmHg)	101.6 ± 8.4	98.8 ± 6.0	0.81
CsA level (ng/ml)	308.0 ± 35.4	314.0 ± 43.4	0.91
U Pr/Cr	0.30 ± 0.08	0.11 ± 0.03	0.81

ACE - angiotensin converting enzyme, ANG - angiotensinigen, RTRs - renal transplant recipients, Cr - creatinine, MAP - mean arterial pressure, CsA, - cyclosporine A, U Pr/Cr - urine protein/creatinine.

kidney graft long-term outcome. It may be implicated in glucose metabolism disorders after transplantation.[7] Reversibly, in a research by Slowinski et al,[24] ACE polymorphisms were not associated with any of the tested markers of early and long-term graft function. Our findings indicated that there is no association between ACE gene polymorphisms and kidney graft outcome. An unresolved issue concerning the effect of the D allele on renal transplant is the underlying mechanism: does it itself increase the incidence of CAN or just modulate the progression to renal failure in patients who already display chronic lesions? Some authors have demonstrated an association of ACE polymorphism with CAN following a recessive model, DD versus ID-II. Rodriguez-Moreno et al[7] found significant results with a dominant model DD-ID versus II.

Our study reported that the distribution of the ATR1 genotypes was AA 60%, AC 34%, and CC 6%. The observed allele frequencies in Filler et al[26] study were AA 51%, AC 38%, and CC 11%. This polymorphism has been shown to be associated with essential hypertension.[27] Our study demonstrated that the percent of hypertensive patients in the C allele was more than the A allele of ATR1 polymorphism. Furthermore, in the study by Filler et al,[26] no association was found with disease progression after renal transplant. Similarly, current research demonstrated, that there was no significant relationship between ATR1 gene polymorphism and kidney graft function. In humans, variance in the ATR1 gene may affect blood pressure.[27] The ATR1 knocked-out mice show a decrease of the infiltration of inflammatory mononuclear cells in ischemic tissue and decreased expressions of monocyte chemoattractant protein-1 and vascular endothelial growth factor.[28] Regarding this phenotype, polymorphisms regulating the activity of ATR1 may influence the development and progression of CAD, as well as acute rejection and delayed graft function.[24] This research showed that the distribution of the ANG genotypes was MM 19%, MT 52%, and TT 29%. A previous study showed that the distribution of ANG genotypes were TT 15%, MT 45%, and MM 40%.[7] Another report in renal transplant recipients demonstrated that the observed allele frequencies were MM 42%, MT 37%, and TT 21% for the M235T polymorphism of ANG.[26] Previous studies have shown that plasma ANG concentrations correlate with blood pressure, and are higher in hypertensive patients than in normotensive subjects. The Met 235_Thr gene polymorphism has been shown to be associated with hypertension, and the 235T variant is associated with increased plasma ANG concentrations.[29] A rise or fall in ANG can lead to a parallel change in the formation of angiotensin II, promoting the development of hypertension, which might influence the progression of renal disease.[29] Our data could fully establish previous reports, that the Met235_Thr variant of the ANG gene was not a risk factor for the development of hypertension after renal transplant.[30] However, in Rodriguez-Moreno et al[7] study, there was no association between CAN and ANG polymorphism, similar to our findings; so that no association was found between ANG gene polymorphism and renal allograft function. Slowinski et al[24] reported that none of the investigated gene polymorphisms of the RAS in recipients of kidney allograft had an impact on kidney allograft dysfunction, alone or in combination. Our report revealed that none of the gene polymorphisms of the RAS had an impact on renal allograft dysfunction alone, however, in the addition of genotypes, the patients homozygous for the ACE D allele (DD) and ATR1 C allele (CC) in comparison with patients homozygous for the ACE I allele (II) and for the ATR1 A allele (AA), had higher renal allograft dysfunction.

In conclusion, although none of the gene polymorphisms of the RAS had any impact on renal allograft function, evaluation of the combinations of these genotypes can predict the outcome of allograft function. What we have shown in our present study is only a preliminary results, thus further investigation with larger number of patients are required to answer these questions.

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