Original Paper

Angiotensinogen Polymorphisms and Post-Transplantation Diabetes Mellitus in Korean Renal Transplant Subjects

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Key Words

ACE • AGT • Polymorphism • Post-transplantation diabetes mellitus • Renal transplantation

Abstract

Background: Post-transplant diabetes mellitus (PTDM) is a common and serious metabolic complication. Genetic polymorphisms of angiotensin-converting enzyme (ACE) and angiotensinogen (AGT) genes have been reported to be related to diabetes mellitus and insulin sensitivity; however, the role of these genes in the development of PTDM is not known. For this purpose, we investigated the association of ACE and AGT genetic polymorphisms with PTDM. **Methods:** A total of 302 subjects without previously diagnosed diabetes who had received kidney transplants were included. One ACE single nucleotide polymorphism (SNP) (rs4291) and two AGT SNPs (rs 699 and rs 4762) were genotyped from genomic DNA with direct sequencing. **Results:** PTDM developed in 49 (16.2%) of 302 subjects. Subjects in the PTDM were older than those in the non-PTDM. There was a significant difference between the two groups in tacrolimus use ($p=0.03$). Of the three SNPs, the rs4762 of the AGT gene was significantly associated with the development of PTDM in the dominant models ($p = 0.03$) after adjusting for age and tacrolimus usage. **Conclusions:** AGT gene rs4762 polymorphisms may serve as genetic markers for the development of PTDM. The exact molecular mechanisms still need to be clarified.

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Introduction

Post-transplant diabetes mellitus (PTDM) is a common and serious metabolic complication after kidney transplantation. Transplant recipients are at a particularly high risk of developing PTDM as a consequence of factors that are in addition to those that affect the general population, including the use of immunosuppressive agents in transplant management protocols. In addition, PTDM is thought to develop in response to a relative insulin deficiency resulting from increased insulin resistance or impaired insulin production, or a combination of both [1]. The risk factors for PTDM reportedly include old age, hepatitis C infection, and the use of corticosteroids and tacrolimus [2, 3]. Previous studies have found that several genetic factors may influence the development of PTDM. The *TCF7L2* and *SLC30A8* genetic variations are associated with the development of PTDM in renal allograft recipients [4, 5].

Recently, the renin-angiotensin system (RAS) has attracted interest with regard to the pathogenesis of insulin resistance and DM in the general population. Several studies have suggested that angiotensin (Ang) II may affect glucose metabolism through insulin signaling pathways, blood flow, oxidative stress, and adipogenesis [6, 7]. Angiotensinogen (AGT) is the initial component of the RAS and a precursor to both Ang I and Ang II. A possible role for AGT in the development of DM is unclear; however, *AGT* gene variants of rs699 (Met235Thr) that were previously shown to be associated with AGT levels were found to be associated with insulin sensitivity in a recent study [8]. It has been also reported that the *AGT* (rs699) polymorphism is related to visceral obesity and hyperinsulinemia in obese Japanese women [9]. Additionally, rs4762-rs699 haplotype of *AGT* was also reported to play the important role in the increase risk for pregnancy induced hypertension [10]. Angiotensin converting enzyme (ACE), a key enzyme in the RAS, catalyzes the conversion of Ang I to Ang II in the liver and inactivates bradykinins in many tissues. In the GenHAT study [11], an *ACE* promoter polymorphism (rs4291) was associated with lower fasting glucose levels. Many reports suggest that genetic variants of *ACE* or *AGT* influence the development of DM and insulin resistance or sensitivity. However, whether the genetic polymorphisms of *ACE* and *AGT* are involved in the pathogenesis of PTDM has not yet been clarified.

We hypothesized that the polymorphisms of *ACE* and *AGT* play an important role in determining PTDM susceptibility in renal transplant subjects. Polymorphisms/genes associated with diabetes and insulin sensitivity and other related disease in published studies were treated as candidates for PTDM. Therefore, in the present study, we genotyped one *ACE* SNP (rs4291 in promoter -262) and two *AGT* SNPs (rs 699 in Met235Thr and rs 4762 in Thr207Met), and investigated the association between polymorphisms in the *ACE* and *AGT* genes and the development of PTDM.

Materials and Methods

Study Design and Population

A total of 302 renal transplant recipients were recruited from 2000 to 2009 at three transplant centers in the Republic of Korea (Kyung Hee University Medical Center, Kyung Hee University Hospital at Gangdong, and Inje University Busan Paik Hospital). In this study, we retrospectively examined the genetic polymorphism in stored blood and reviewed the medical record in the renal transplant recipients. All studies were performed according to the Declaration of Helsinki guidelines and written informed consent was obtained from each subject. This study was approved by the ethics review committees of all three transplant centers.

As in our previous study [12], the subjects were classified into two groups according to the development of PTDM. PTDM was diagnosed according to the American Diabetes Association guidelines [13] after the third post-transplantation month. These criteria were fasting blood glucose levels ≥ 126 mg/dl (7.0 mmol/l); or symptoms of diabetes mellitus plus plasma glucose concentrations ≥ 200 mg/dl (11.1 mmol/l) at any time of day; or 2-h post-load glucose ≥ 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test; or insulin

Table 1. Primer Sequences and Expected Product Sizes

Chromo- some	Gene	SNP locus		Sequence (5'-3')	Products size (bp)
17	<i>ACE</i>	rs4291	sense	ACCATGGCCTGGTGAAGAAG	444
		Promoter -262	antisense	GGTTATAAAACCGACACACC	
1	<i>AGT</i>	rs 699	sense	TGTACAGGGCCTGCTAGTGG	369
		Met235Thr	antisense	ACTGGCTGATCTCAGCTACACA	
1	<i>AGT</i>	rs 4762	sense	CTCTCTCTATCTGGGAGCCTTG	323
		Thr207Met	antisense	CAATCTTCTCAGCAGCAACATC	

and/or oral hypoglycemic agents that were required for more than three months. Subjects were excluded if they had a history of diabetes before transplantation or if they had a severe metabolic or infectious disease. We included the patients with normal fasting glucose levels and HbA1c levels before transplantation. Patients were excluded if they had impaired fasting glucose levels (fasting plasma glucose 100-125 mg/dL). In this study, we analyzed the following demographic data: age, sex, body mass index (BMI) at the time of transplantation, human leukocyte antigen mismatching, hepatitis C infection, acute rejection episodes, follow up levels of creatinine and the types of immunosuppressive agents used.

SNP Selection and Genotyping

We selected SNPs in the *ACE* and *AGT* gene using the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP>) and that of the International Hapmap Project (<http://www.hapmap.org/index.html>). We excluded SNPs with unknown heterozygosity and a minor allele frequency below 5% in the general population. We included SNPs that were previously reported to be associated with diabetes or insulin sensitivity [8]. One *ACE* SNP (rs4291) and two *AGT* SNPs (rs 699 and rs 4762) were finally selected and used for genotyping the subjects.

Blood samples were collected from each subject and then stored in a -20°C refrigerator. Genomic DNA was extracted from the peripheral blood using a DNA extraction kit (Qiagen, Tokyo, Japan). SNP genotyping was conducted by direct sequencing. Genomic DNA was amplified with specific primers for the one *ACE* and two *AGT* gene SNPs (Table 1). The amplified products were sequenced with an ABI PRISM 3730XL automatic sequencer (PE Applied Biosystems, Foster City, CA, USA), and the sequence data were analyzed with SeqManII software (DNASTAR Inc., Madison, WI, USA).

Statistical Analysis

We analyzed the one *ACE* and two *AGT* SNPs in each of the 302 renal transplant subjects. SNPstats (<http://bioinfo.iconcologia.net/index.php?module=Snstats>) was used to analyze genetic data. Allelic frequencies were compared between the two groups with the χ^2 test. In the association study between SNPs and PTDM, we calculated the odds ratios (OR), 95% CI, and *p*-values with SNPstats, HapAnalyzer version 1.0, and SNPAnalyzer (ISTECH, Goyang, Korea), respectively. For multiple logistic regression analyses to determine the associations between single SNPs and PTDM, we used multiple inheritance models, including codominant 1 (major allele homozygotes vs. heterozygotes), codominant 2 (major allele homozygotes vs. minor allele homozygotes), dominant (major allele homozygotes vs. minor allele homozygotes plus heterozygotes), and recessive (major allele homozygotes plus heterozygotes vs. minor allele homozygotes) models. A linkage disequilibrium (LD) block of polymorphisms was tested with Haploview version 4.1 (<http://www.broadinstitute.org/haploview/haploview>). Clinical characteristics were compared with Student's unpaired *t*-test and the χ^2 test. Values of *p* < 0.05 were considered statistically significant.

Results

Clinical Characteristics

We enrolled 302 renal transplant recipients. The mean follow-up duration for all 302 subjects was 87.91 months (87.91±78.23 months), and 49 subjects (16.2%) developed PTDM. The clinical parameters of the transplant recipients are shown in Table 2. The mean ages at transplantation of the non-PTDM and PTDM subjects were 38.10±11.21 years and

Table 2. Comparison of Clinical Factors Between PTDM and non-PTDM Groups

Variable	PTDM (n = 49)	Non-PTDM (n = 253)	p
Age at transplantation (years)	45.18±9.39	38.10±11.21	<0.01
Sex			
Male	26	155	0.28
Female	23	98	
BMI (kg/m ²)	22.26±3.31	22.48±3.45	0.71
HLA total mismatches, n	3.00±1.55	3.27±1.51	0.28
Hepatitis C infection, n	0	6	0.25
Acute rejection with steroid boluses, n	13	49	0.25
Tacrolimus usage, n	23	78	0.03
Serum Creatinine (mg/dl)			
6 mos. after transplantation	1.31±0.58	1.31±0.46	0.95
12 mos. after transplantation	1.30±0.37	1.36±0.85	0.64

PTDM, posttransplantation diabetes group; non-PTDM, non-posttransplantation diabetes group;
Bold characters represent statistically significant values

Table 3. Allele Frequencies of Three SNPs of the *ACE* and *AGT* Genes in PTDM and Non-PTDM Subjects

SNP Gene	Allele	PTDM; n (%)	Non-PTDM; n (%)	OR (95% CI)	p
rs4291	A	61 (65%)	277 (56%)	0.69(0.44-1.10)	0.12
<i>ACE</i>	T	33(35%)	215 (44%)		
rs 699	C	70 (71%)	399 (80%)	1.61(0.98-2.63)	0.05
<i>AGT</i>	T	28 (29%)	99 (20%)		
rs 4762	C	78 (81%)	456 (90%)	2.10 (1.16-3.79)	0.01
<i>AGT</i>	T	18 (19%)	50 (10%)		

PTDM, posttransplantation diabetes group; non-PTDM, non-posttransplantation diabetes group; OR, odds ratio; CI, confidence interval; Bold characters represent statistically significant values

45.18±9.39 years, respectively. Subjects in the PTDM group were significantly older than those in the non-PTDM group ($p < 0.01$). The sex ratio between the two groups was similar ($p=0.28$). BMI, HLA mismatches, the frequency of hepatitis C-positivity, and episodes of acute rejection did not differ between the two groups; however, in the PTDM group, tacrolimus use as an immunosuppressive agent was significantly higher ($p=0.03$). However, two groups were statistically indistinguishable in the levels of serum creatinine after transplantation at 3 months and 6 months.

Genetic Associations between One ACE and Two AGT SNPs and PTDM

Genetic associations between the one *ACE* and two *AGT*s SNPs and PTDM in subjects that had undergone renal transplants were investigated. Genotyping success rates were 97.02%, 98.68%, and 99.67% for rs4291, rs 699, and rs 4762, respectively. The genotype distributions of the three polymorphic SNPs were all in Hardy-Weinberg equilibrium ($p>0.05$). The allele frequencies are shown in Table 3. The allele frequency of the one SNP of *AGT* (rs4762) was statistically associated with a risk of PTDM (OR = 2.10; 95% CI: 1.16–3.79; $p = 0.01$), where the presence of the T allele increased the risk of PTDM in kidney transplantation patients.

In the univariate analyses, age and use of tacrolimus were significantly associated with the development of PTDM. Therefore, we performed multiple logistic regression analysis to analyze genotype data for associations with PTDM, with adjustment for age and the use of tacrolimus. Table 4 shows that the effect of genotype on PTDM remained significant after these multiple logistic regression adjustments. The rs4762 of the *AGT* gene was significantly associated with the development of PTDM. We observed significant higher distribution of C/T plus T/T genotypes in PTDM subjects than in control subjects under a dominant model

Table 4. Logistic Regression Analysis of the *ACE* and *AGT* Polymorphisms in PTDM and Non-PTDM Subjects Adjusted for Age and Tacrolimus Usage

Gene	SNP	Genotype	PTDM n (%)	Non-PTDM n (%)	Models	OR (95% CI)	p
<i>ACE</i>	rs4291	A/A	20(42.5%)	75(30.5%)	Codominant1	0.57(0.28-1.15)	0.11
		T/A	21(44.7%)	127(51.6%)	Codominant2	0.51(0.18-1.42)	0.20
		T/T	6(12.8%)	44(17.9%)	Dominant	0.56(0.29-1.09)	0.08
					Recessive	0.61(0.24-1.55)	0.28
<i>AGT</i>	rs 699	C/C	24(49%)	157(63%)	Codominant1	1.68(0.87-3.26)	0.12
		T/C	22(44.9%)	85(34.1%)	Codominant2	3.58(0.78-16.38)	0.10
		T/T	3(6.1%)	7(2.8%)	Dominant	1.80(0.95-3.42)	0.07
					Recessive	2.89(0.65-12.81)	0.19
<i>AGT</i>	rs 4762	C/C	37(69.8%)	202(79.5%)	Codominant1	1.99(0.95-4.15)	0.06
		C/T	14(26.4%)	47(18.5%)	Codominant2	5.10(0.61-42.33)	0.13
		T/T	2(3.8%)	5(2.0%)	Dominant	2.15(1.06-4.36)	0.03
					Recessive	4.34(0.53-35.63)	0.18

PTDM, posttransplantation diabetes group; non-PTDM, non-posttransplantation diabetes group; OR, odds ratio; CI, confidence interval; Models, Multiple inheritance models, namely codominant 1 (major allele homozygotes vs. heterozygotes), codominant 2 (major allele homozygotes vs. minor allele homozygotes), dominant (major allele homozygotes vs. minor allele homozygotes plus heterozygotes), and recessive (major allele homozygotes plus heterozygotes vs. minor allele homozygotes) models were used for association analysis of single SNPs

C/C vs C/T + T/T) with an odds ratio of 2.15 (95% CI, 1.06-4.36; $p = 0.03$). The significance was permitted after controlling for covariates age and tacrolimus usage.

We then tested whether the *AGT* haplotype was associated with PTDM. To demonstrate pair-wise linkage disequilibrium (LD), we analyzed two SNPs and found that they were in linkage equilibrium. The D' value between *AGT* SNP rs699 and rs4762 was 0.652. The r^2 value between *AGT* SNPs rs699 and rs4762 was 0.578.

In order to predict the functional significance of missense SNP (rs4762) in *AGT* gene, we performed bioinformatic software tool using PolyPhen (<http://genetics.bwh.harvard.edu/pph2/index.shtml>). Result based on PolyPhen, the function and protein structure of AGT was significantly affected by the Thr207Met missense SNP (rs4762).

Discussion

PTDM is a serious metabolic complication after kidney transplantation. Multiple risk factors for the development of PTDM have been identified: type of immunosuppression, ethnicity, age, and body mass index [14]. However, PTDM can also develop without these risk factors, and even if they are present, the rate of PTDM occurrence may be influenced by genetic factors. Many studies have been performed to analyze SNPs as genetic markers. The results of the present study indicate that an *AGT* polymorphism (rs 4762) is associated with PTDM in Korean renal transplant patients.

The *AGT* gene (specifically rs4762) polymorphism, was previously reported to be associated with essential hypertension and pregnancy hypertension [10, 15]. The association between this gene and glucose metabolism is controversial. Perhaps the mechanism underlying our finding of an association between the *AGT* gene and PTDM may be explained by insulin resistance. Molecular and genetic studies demonstrate a relationship between variants of the *AGT* gene, *AGT* gene expression, and plasma AGT levels [16, 17]. In our study, the Thr207Met missense SNP affects the function and protein structure of AGT. It is possible that increased plasma AGT levels and the effect of *AGT* gene variants increase Ang II levels inappropriately and thereby impair glucose transport mainly via the inhibition of insulin signaling [19]. However, we could not evaluate the AGT levels and/or insulin resistance in this study. The relationship between *AGT* gene polymorphisms and insulin resistance has been

reported in other studies. Takakura et al. [9] demonstrated that *AGT* gene polymorphisms are linked to visceral obesity and insulin resistance in obese Japanese women.

It was reported that Ang II induced mononuclear leukocyte interaction are mediated by release of several CC chemokines such as chemokine ligand 5 (CCL5) [18]. As in our previous study [12], genetic polymorphisms of the *CCL5* gene were associated with PTDM. But, we could not predict any relationship between *CCL5* and *ACE*, *AGT* gene in the PTDM. Further study is necessary for evaluation of relation of the RAS SNPs and cytokine such as CCL5.

Earlier studies have reported the influence of *ACE* promoter polymorphisms on fasting glucose levels [11]. Previous data on the association between *ACE* polymorphisms and insulin resistance are contradictory; these studies have included limited numbers of participants and/or only used surrogate markers of insulin resistance [20, 21]. A recent study demonstrated that *AGT* Met235Thr (rs 699) and *ACE* I/D polymorphisms were linked to the increased risk of PTDM [22]; this result was not replicated in the present study. Study designs and populations may explain the contradictory results and are important to emphasize in future investigations. The previous study was performed using subjects of a different ethnicity, suggesting that the association with *AGT* polymorphisms may be ethnically dependent. We used a purely Korean population to eliminate false positive results due to population stratification. Furthermore, our regression approach permitted adjustment for the effects of environmental risk factors of PTDM, such as age and tacrolimus use.

Older age and tacrolimus use were significant risk factors in this study. Previous studies have consistently found that PTDM developed much more frequently in older transplant recipients than in younger recipients [23]. Tacrolimus as a main immunosuppressive drug was also shown to be a risk factor for PTDM in renal transplant patients [23]; however, the effect of *AGT* polymorphisms on PTDM remained significant after adjustment for age and tacrolimus use. Hjelmestaeth et al showed that the risk of developing PTDM increases by 5% when the corticosteroid dose is raised by 0.01 mg/kg/day [24]. In addition, high corticosteroid doses such as steroid boluses, given for acute rejection episodes, might partly account for the development of PTDM. However, the acute rejection episodes with boluse steroid therapy were not significantly different in this study. Further studies are necessary to evaluate the association of steroid doses and development of PTDM. Previous study has reported the influence of hepatitis C virus (HCV) infection on the development of PTDM [25]. According to our results, however, the HCV infection was not different between PTDM and non-PTDM groups. Possible explanations for this discrepancy may be due to small number of HCV in our study compared to previous study.

There were several limitations to this study. First, our analysis was conducted in a relatively small sample. To confirm or refute the associations between the *AGT* gene and PTDM, validation studies with an adequate sample size and studies with additional SNPs not analyzed in the present study may be required. Second, we did not assay the levels of *AGT* in vivo or examine the functional effects of the *AGT* polymorphisms. Further studies are necessary to elucidate whether *AGT* SNPs can affect the expression of *AGT*. Finally, we didn't performed regularly oral glucose tolerance tests before transplantation or study registration. So, preexisting impaired glucose tolerance could have led to an overestimation of PTDM incidence. We minimized overestimation by excluding PTDM when hemoglobin A1c was over 6.0%, fasting plasma glucose concentration was over 126 mg/dl before transplantation. Nevertheless, this study is meaningful because this is the first study to reveal a significant association between PTDM and *AGT* SNP (specifically rs4762), and we suggest that these results show potential for clinical application and better explanation of PTDM episodes.

Conclusion

We examined the possible association between *ACE* and *AGT* gene polymorphisms and PTDM in Korean subjects who had undergone renal transplants. We found a significant

association between SNPs of the *AGT* gene (rs4762) and PTDM in kidney transplantation recipients. These results suggest that AGT rs4762 polymorphisms may act as genetic markers for the development of PTDM. Additional genetic studies are needed to provide an understanding of the precise mechanisms underlying the pathogenesis of PTDM in kidney transplantation patients.

Conflict of Interests

No potential conflict of interest relevant this article was reported.

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