Original Article

Effects of Angiotensin II Type 1 Receptor Gene Polymorphisms on Insulin Resistance in a Japanese General Population: The Tanno-Sobetsu Study

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Although gene polymorphisms in the renin-angiotensin system (RAS) are predisposing factors for cardiovascular diseases, the precise mechanisms and interactions among confounding factors have not been clarified. We investigated whether genetic variants of RAS are involved in insulin sensitivity in a Japanese general population. During a medical checkup in 2001, participants (*n*=550) were recruited from among the residents of the towns of Tanno and Sobetsu, and written informed consent was obtained to participate in the genetic analysis and the epidemiological study. The insertion/deletion (I/D) polymorphism of the angiotensin-converting enzyme gene (*ACE*), the Met235Thr polymorphism of the angiotensinogen gene (*AGT*), and the A1166C polymorphism of the angiotensin II type 1 receptor gene (*AGTR1*) were determined by gel electrophoresis or the TaqMan PCR method. We assessed insulin sensitivity using the homeostasis model assessment insulin resistance (HOMA-IR). The RAS gene polymorphisms were not associated with log-transformed values of HOMA-IR, whereas borderline association (*p*=0.02) was found between the A1166C polymorphism and dichotomous categorization of insulin resistance (defined as HOMA-IR ≥1.73). Our results suggested that the A1166C polymorphism of *AGTR1* might affect insulin resistance by altering the responsiveness to angiotensin II signaling, though this mechanism is as yet inconclusive. Further study is required to confirm these findings in a larger, multi-ethnic population. (*Hypertens Res* 2006; 29: 961–967)

***Key Words*: gene polymorphisms, renin-angiotensin system, insulin resistance, epidemiology**

Introduction

The metabolic syndrome, which includes visceral obesity, hypertension, glucose intolerance, and dyslipidemia, is known to be a risk factor for cardiovascular diseases (*1*). Using the criteria defined by the National Cholesterol Education Program–Adult Treatment Panel III (NCEP-ATP III), the prevalence of the metabolic syndrome is about 25% of the general populations of both Western countries (*2*) and Japan (*3*). Prospective epidemiological studies indicate that the metabolic syndrome increases the risk of cardiovascular disease 3- to 6-fold (*4*, *5*). Similarly, in our prospective epidemiological study the subjects with metabolic syndrome had a 2.2-times greater risk of developing cardiac disease than the subjects without metabolic syndrome (*6*). Therefore, intensive

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preventive measures must be implemented against this condition. Insulin resistance is closely linked to the metabolic syndrome (*7*), and the renin-angiotensin system (RAS) plays a central role in the regulation of insulin sensitivity (*8–10*), as well as in the regulation of blood pressure and sodium homeostasis (*11*). Many studies have examined the genetic involvement of homozygous deletion polymorphism (DD) in exon 16 of the angiotensin-converting enzyme gene (*ACE*) in insulin resistance, but their results have been controversial (*12–14*). The association between *ACE* insertion-deletion (I/D) polymorphism in intron 16 and cardiovascular phenotypes has also been studied, with the main result being that the D allele is a genetic risk for coronary artery disease or left ventricular hypertrophy (*15*, *16*).

Hypertension is one of the most common traits of the metabolic syndrome, and is also closely linked to insulin resistance. Numerous studies have reported an association between RAS gene polymorphisms and hypertension (*17–20*). One study has indicated that the RAS gene polymorphisms have a synergistic effect on the predisposition to myocardial infarction (*21*). In this study, we investigated the association between RAS gene polymorphisms and insulin resistance to detect a genetic risk for cardiovascular disease among the Japanese general population, and whether RAS polymorphisms synergistically affect insulin resistance.

Methods

Study Subjects

We recruited 550 subjects who had undergone medical checkups in the towns of Tanno and Sobetsu in Hokkaido, Japan in 2001 (the Tanno-Sobetsu Study). The detailed epidemiological findings have already been reported (*22–24*). Subjects completed a standard questionnaire regarding their medical history and smoking and drinking habits. We measured the systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), total cholesterol, triglyceride, high density lipoprotein (HDL) cholesterol, plasma glucose, immunoreactive insulin (IRI), and highly-sensitive C-reacting protein (hs-CRP) of all participants. The homeostasis model assessment insulin resistance (HOMA-IR) was used to determine insulin sensitivity, and was calculated as plasma glucose (mmol/l) × immunoreactive insulin (mU/l)/22.5. Blood samples were collected during fasting in the early morning.

Blood pressure was measured twice after 5 min of rest, with the subjects seated. Hypertension was defined as SBP ≥140 mmHg, DBP ≥90 mmHg, or the current use of antihypertensive agents. One hundred and fifty of the 550 subjects (27.3%) were taking antihypertensive agents, and these subjects were included in the study. The precise types of antihypertensive agents were unknown. Individuals in the acute phase of coronary heart disease ($n=4$) or of cerebrovascular disease ($n=15$), and individuals undergoing medical treatment for diabetes mellitus ($n=44$) were excluded from the study. Individuals receiving diet therapy or exercise therapy alone for diabetes were not counted among those receiving medical treatment for diabetes. All participants gave written informed consent to participate in the genetic analysis and in all other procedures associated with the study. The Ethics Committee of the Osaka University approved the study protocol. The final number of subjects participating in the genetic study was 550.

Table 1. Correlation Coefficient between log-Transformed HOMA-IR and Clinical Parameters

Term	*r*	*p*
Age	0.071	0.096
Gender	0.013	0.75
BMI	0.47	<0.0001
Prevalence of hypertension	0.21	<0.0001
Total cholesterol	0.050	0.24
Triglyceride	0.36	<0.0001
HDL-cholesterol	−0.30	<0.0001
hs-CRP	0.16	0.0002

HOMA-IR, homeostasis model assessment of insulin resistance; BMI, body mass index; HDL-cholesterol, high density lipoprotein cholesterol; hs-CRP, highly-sensitive C-reacting protein.

In the Tanno-Sobetsu Study, Oimatsu *et al*. attempted to determine the optimal cut-off point of the HOMA-IR as a practical index for evaluating insulin resistance (*25*). The tests using the glucose clamp (GC) method and 75-g glucose tolerance tests (OGTTs) were carried out in 57 men and women with normotension or essential hypertension. The *M* value is the rate of infusion of glucose in the GC method and serves as an index of insulin resistance; we used an *M* value of 167.3 mg/m^2/min (the mean value minus 1 SD of the mean value) as a cut-off point to divide the subjects into an insulin resistance group and insulin non-resistance group, and compared the data between the two groups. Also, from data on temporal plasma glucose levels, insulin values and HOMA-IR values obtained from simultaneously performed OGTTs, the cut-off values for distinguishing insulin resistance, classified according to the results of the GC tests, were examined using the receiver operator characteristic (ROC) curve. As a consequence, HOMA-IR 1.73 was adopted. In consideration of the fact that the 75-g OGTT is sometimes difficult to perform for large numbers of people, the examinations were carried out using HOMA-IR. The sensitivity and specificity of HOMA-IR 1.73 were 64.7% and 78.9%, respectively. Therefore, in this study, dichotomous classification of insulin resistance was made at HOMA-IR ≥1.73, in addition to the evaluation of HOMA-IR as a continuous variable.

Genotyping

Genomic DNA was extracted from 200 μl of buffy coat using

Table 2. Comparison between Insulin Resistance Group (HOMA-IR ≥1.73) and Insulin Non-Resistance Group (HOMA-IR <1.73) on Clinical Characteristics

	Insulin resistance (–) (n=434)	Insulin resistance (+) (n=116)	p
Age (years)	63.2±0.5	65.2±0.9	0.04
Gender (male (%))	35.7	37.9	0.66
BMI (kg/m^2)	23.0±0.1	25.9±0.2	<0.0001
Prevalence of hypertension (%)	43.3	62.1	0.0003
Total cholesterol (mmol/l)	5.2±0.04	5.2±0.07	0.57
Triglyceride (mmol/l)	1.1±0.03	1.7±0.07	<0.0001
HDL-cholesterol (mmol/l)	1.4±0.02	1.2±0.04	<0.0001
hs-CRP (mg/l)	0.67±0.05	1.1±0.09	0.0003
IRI (mU/l)	4.2±0.1	10.8±0.3	<0.0001
HOMA-IR	0.97±0.0	2.85±0.1	<0.0001

Values are expressed as %, or means±SEM. HOMA-IR, homeostasis model assessment of insulin resistance; BMI, body mass index; HDL-cholesterol, high density lipoprotein cholesterol; hs-CRP, highly-sensitive C-reacting protein; IRI, immunoreactive insulin.

a QIAamp DNA Blood Kit (QIAGEN Inc., Hilden, Germany). The insertion-deletion polymorphisms in intron 16 of the angiotensin-converting enzyme gene (*ACE* I/D) were determined by gel electrophoresis. The Met→Thr transversion at codon 235 of the angiotensinogen gene (*AGT* Met235Thr) and the A→C transversion at nucleotide position 1166 of the angiotensin II type 1 receptor gene (*AGTR1* A1166C) were determined by the TaqMan polymerase chain reaction (PCR) method.

To amplify the intron 16 region where the I/D fragment of *ACE* is located, the following primer pairs were used: forward, 5′-CTGGAGACCACTCCCATCCTTTCT-3′; and reverse, 5′-GATGTGGCCATCACATTCGTCAGAT-3′. Genomic DNA was amplified for 45 cycles, each comprising denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The products were separated by electrophoresis on 1.5% agarose gels and identified by ethidium bromide staining.

The *AGT* Met235Thr polymorphism was detected using the following primers and probes: forward, 5′-GCTGTGACA GGATGGAAGACT-3′ and reverse, 5′-AGTGGACGTAGG TGTTGAAAGC-3′; cytosine base (C) specific probe, 5′-FAM-CTGGCTCCCGTCAGG-MGB-3′ and thymine base (T) specific probe, 5′-VIC-CTGGCTCCCATCAGG-MGB-3′. The *AGTR1* A1166C polymorphism was detected using the following primers and probes: forward, 5′-CATTCCTCT GCAGCACTTCACT-3′ and reverse, 5′-CGGTTCAGTCCA CATAATGCAT-3′; adenine base (A) specific probe, 5′-FAM-CAAATGAGCATTAGCTAC-MGB-3′; cytosine base (C) specific probe, 5′-VIC-CAAATGAGCCTTAGCTACT-MGB-3′. PCR was conducted using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, USA). The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 60 s. The fluorescence level of PCR products measured using an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) differentiated the three genotypes of the two polymorphisms.

Statistical Analysis

Associations between the polymorphisms and clinical variables were analyzed using one-way analysis of variance (ANOVA). Differences in genotype or allele distribution were examined by χ^2 analysis. In order to evaluate the association between gene polymorphisms and insulin resistance represented as HOMA-IR, we used the stepwise method as follows. First, a multiple-comparison procedure using ANOVA was done between genotypes and log-transformed HOMA-IR. Second, χ^2 analysis was done between genotypes and insulin resistance was defined using the dichotomous qualitative trait of HOMA-IR ≥1.73 or HOMA-IR <1.73. Third, stepwise analysis was done between genotypes and quintiles of log-transformed HOMA-IR to confirm whether or not the result of the second step was a chance effect. Multiple logistic regression analysis was used to assess the contribution of confounding factors. All numerical values are expressed as the mean±SEM. Values of $p<0.05$ were considered to indicate statistical significance. To adjust for multiple-testing of the three gene polymorphisms by Bonferroni's correction, we arbitrarily adopted $p<0.017$ as the level of statistical significance. All statistical analyses were conducted using JMP software version 5.0.1 for Windows (SAS Institute Inc., Cary, USA).

Results

The mean age among the 550 subjects was 63.6±0.4 years, and the mean body mass index (BMI) was 23.6±0.1 kg/m^2. The mean SBP and DBP were 133.5±0.8 mmHg and 77.8±0.4 mmHg, respectively. The mean plasma levels of total cholesterol, triglyceride, HDL-cholesterol, hs-CRP, glu-

Table 3. Multiple-Comparison between log-Transformed HOMA-IR and RAS Gene Polymorphisms

Gene polymorphism	Genotype	*n*	*p*
ACE I/D	II: 0.12±0.04	214	0.92
	ID: 0.12±0.04	267	
	DD: 0.15±0.07	69	
AGT Met235Thr	Met/Met: −0.01±0.16	14	0.55
	Met/Thr: 0.15±0.04	165	
	Thr/Thr: 0.12±0.03	335	
AGTR1 A1166C	AA: 0.13±0.03	464	0.46
	AC: 0.09±0.06	82	
	CC: −0.19±0.29	4	

Values are expressed as means±SEM. HOMA-IR, homeostasis model assessment of insulin resistance; *ACE* I/D, insertion-deletion polymorphisms in intron 16 of the angiotensin-converting enzyme gene; *AGT* Met235Thr, Met→Thr transversion at codon 235 of angiotensinogen gene; *AGTR1* A1166C A→C transversion at nucleotide position 1166 of angiotensin II type 1 receptor gene.

cose, and IRI were 5.2±0.03 mmol/l, 1.3±0.03 mmol/l, 1.4±0.02 mmol/l, 0.75±0.04 mg/l, 5.4±0.03 mmol/l, and 5.5±0.2 mU/l, respectively. The mean calculated HOMA-IR was 1.3±0.05; this HOMA-IR value did not satisfy normal distribution, but did satisfy log-normal distribution.

Table 1 shows the correlation coefficient between the log-transformed HOMA-IR and the clinical parameters. BMI, prevalence of hypertension, triglyceride, HDL-cholesterol, and hs-CRP were significantly correlated with log-transformed HOMA-IR.

Table 2 shows the clinical characteristics of the insulin resistant ($n=116$) and insulin non-resistant ($n=434$) groups when we defined insulin resistance as HOMA-IR ≥1.73. Age, BMI, prevalence of hypertension, triglyceride, HDL-cholesterol, and hs-CRP were significantly higher in the resistant than in the non-resistant group.

The genotype frequencies of the gene polymorphisms examined did not significantly differ from the values predicted by Hardy-Weinberg equilibrium. The frequencies of the II, ID, and DD genotypes of *ACE* were 38.9%, 48.5%, and 12.5%, respectively. The frequencies of the Met/Met, Met/Thr, and Thr/Thr genotypes of *AGT* were 2.7%, 32.1%, and 65.2%, respectively. The frequencies of the AA, AC, and CC genotypes of *AGTR1* were 84.4%, 14.9%, and 0.7%, respectively. Although a multiple-comparison procedure was done among the three genotypes of the three genes and log-transformed HOMA-IR, no significant correlation was found (Table 3). When insulin resistance was defined as HOMA-IR ≥1.73, the A allele of the *AGTR1* A1166C polymorphism was significantly associated with insulin resistance, but not the *ACE* I/D or *AGT* Met235Thr polymorphisms (Table 4). We examined three modes of inheritance: dominant, co-dominant (additive) and recessive, and found that only the recessive model of *AGTR1* polymorphism (*i.e.*, AA *vs*. AC+CC) was associated with insulin resistance. The estimated odds ratio (OR) for insulin resistance in the subjects with AA was 2.25 (95% confidence interval [CI] 1.17–4.77, $p=0.02$) compared to those with AC or CC. Next, we examined the association between genotype or allelic frequencies and quintiles of log-transformed HOMA-IR, but no significant difference was found. Furthermore, no significant difference was found between genotype or allelic frequencies of the lowest and the highest quintile of log-transformed HOMA-IR.

Table 5 shows the results of multiple logistic regression

Table 4. Allelic Frequency of RAS Gene Polymorphism

	Insulin resistance (−)	Insulin resistance (+)	*p*
ACE I/D			
Polymorphism (*n* (%))			0.92
II	167 (38.5)	47 (40.5)	
ID	212 (48.8)	55 (47.4)	
DD	55 (12.7)	14 (12.1)	
Total	434	116	
Allele (*n* (%))			0.71
I	546 (62.9)	149 (64.2)	
D	322 (37.1)	83 (35.8)	
Total	868	232	
AGT Met235Thr			
Polymorphism (*n* (%))			0.59
Met/Met	12 (3.0)	2 (1.8)	
Met/Thr	133 (32.8)	32 (29.4)	
Thr/Thr	260 (64.2)	75 (68.8)	
Total	405	109	
Allele (*n* (%))			0.32
Met	157 (19.4)	36 (16.5)	
Thr	653 (80.6)	182 (83.5)	
Total	810	218	
AGTR1 A1166C			
Polymorphism (*n* (%))			0.054
AA	358 (82.5)	106 (91.4)	
AC	72 (16.6)	10 (8.6)	
CC	4 (0.9)	0 (0.0)	
Total	434	116	
Allele (*n* (%))			0.015
A	788 (90.8)	222 (95.7)	
C	80 (9.2)	10 (4.3)	
Total	868	232	

Values are expressed as *n* (%). RAS, renin-angiotensin system; *ACE* I/D, insertion-deletion polymorphisms in intron 16 of the angiotensin-converting enzyme gene; *AGT* Met235Thr, Met→Thr transversion at codon 235 of angiotensinogen gene; *AGTR1* A1166C A→C transversion at nucleotide position 1166 of angiotensin II type 1 receptor gene.

Table 5. Multiple Logistic Regression Analysis for Insulin Resistance

Term	β	SEM	p
Gender	−0.69	0.28	0.01
BMI	0.31	0.05	<0.0001
Prevalence of hypertension	0.60	0.25	0.02
Triglyceride	0.008	0.002	0.0002
HDL-cholesterol	−0.047	0.012	<0.0001
hs-CRP	2.29	1.0	0.03
AGTR1 AA *vs.* AC+CC	0.81	0.39	0.04

r^2=0.27 (n=550). BMI, body mass index; HDL-cholesterol, high density lipoprotein cholesterol; hs-CRP, highly-sensitive C-reacting protein.

analysis for the risk of insulin resistance. The confounding factors were selected by using the stepwise method. After adjusting for confounding factors such as gender, BMI, prevalence of hypertension, triglyceride, HDL-cholesterol, and hs-CRP, the *AGTR1* AA genotype was independently associated with insulin resistance (OR 2.25; 95% CI 1.04–4.84).

Since the *ACE* I/D and *AGTR1* A1166C polymorphisms have been reported to have a synergistic effect on determining the risk of myocardial infarction, we examined whether pairs of polymorphisms would have a similar synergistic effect on insulin resistance. However, none of the RAS polymorphisms exerted a synergistic effect on insulin resistance (data not shown).

Discussion

Angiotensin II plays a pivotal role in the pathogenesis of hypertension, vascular remodeling and insulin resistance. Our previous investigations revealed that RAS polymorphisms are genetically predisposing factors for cardiovascular diseases (*26–28*). Although many studies have examined the association between RAS polymorphisms and hypertension, the relationship between RAS polymorphisms and insulin resistance has not yet been clarified. Numerous studies have examined candidate genes for insulin resistance, such as genes of the angiotensin-converting enzyme (ACE) (*12*, *14*), β-3 adrenergic receptor (*29*), uncoupling protein (UCP) 2 (*30*), lamin A/C (LMNA) (*31*), adiponectin (*32*), and peroxisome proliferator–activated receptor (PPAR)-γ 2 (*33*), but the findings of these studies were controversial.

A recent topic in clinical trials is that inhibitors of RAS, such as ACE inhibitors and angiotensin II type 1 (AT1) receptor blockers (ARBs), may reduce the incidence of new-onset diabetes in patients with or without hypertension and at high risk of developing diabetes (*34*). Since the insulin resistance is associated with upregulation of the AT1 receptor and an increase in oxygen free radicals in endothelial tissue caused by activation of NADPH oxidase (*35*), therapy using ACE inhibitors or ARBs may normalize oxidase stress and improve endothelial function. Angiotensin II activates various intracellular protein kinases, such as receptor or non-receptor tyrosine kinases and serine/threonine kinases (*36*), and these AT1-activated kinases are involved in vascular remodeling, vascular contractility, endothelial dysfunction and insulin resistance. Furthermore, the AT1 receptor undergoes rapid phosphorylation, desensitization, and internalization upon angiotensin II stimulation. Recent studies with site-directed mutagenesis of the AT1 receptor also demonstrated a structural requirement of the receptor for downstream signal transduction, suggesting that AT1 mutants provide an excellent means for examining the mechanisms of signal transduction and their significance in mediating angiotensin II function (*37*).

We identified a borderline significant association between insulin resistance and the *AGTR1*/AA genotype and considered the mechanisms of this relationship. van Geel *et al*. reported that the *AGTR1* polymorphism is associated with an increased response to angiotensin II and not with increased *AGTR1* expression (*38*). A hemodynamic study by Miller *et al*. found that normotensive individuals with the *AGTR1*/AA genotype have a higher glomerular filtration rate (GFR) than those with the *AGTR1*/AC or CC genotype at baseline, whereas the AA genotype is more responsive to an infusion of angiotensin II in the sodium-replete state, and the response to losartan is blunt (*39*). We therefore postulate that individuals with the *AGTR1*/AA genotype are more responsive to angiotensin II, which together with increased RAS activity, blunts insulin sensitivity. Recent studies have revealed that a RAS blockade increases insulin sensitivity and improves impaired insulin signaling due to angiotensin II (*40*), thereby activating the glucose transporter *via* translocation from the intracellular membrane compartment to the plasma membrane fraction (*9*, *41*).

There were several limitations to this study. First, the significance of association with insulin resistance was only observed for the recessive model and for allele frequency. Even though the p value (0.015) of the genetic predisposition to insulin resistance in the subjects with an A allele was less than 0.017, which was estimated as the level of significance from Bonferroni's correction, we have to keep in mind that the significance was borderline. In addition, the *AGTR1* polymorphism was significantly associated with dichotomous categorization of HOMA-IR as a qualitative trait, not as a continuous trait, suggesting that the polymorphism only affects insulin resistance in the advanced state. Furthermore, we did not find a significant association between quintiles of the log-transformed HOMA-IR and genotype or allelic frequencies. This suggested that the result of a significant association between gene polymorphism and insulin resistance as a qualitative trait might be a chance effect. To clarify this matter, the genetic involvement of *AGTR1* should be examined in larger epidemiological studies.

In conclusion, like other investigations into the risk of hypertension, the present study indicates the possibility that

AGTR1 gene polymorphism affects the risk of insulin resistance. The AA genotype of *AGTR1* in the general Japanese population might be an independent risk for insulin resistance. Further investigation is required to confirm these findings in a larger, multiethnic population, and to confirm the risk of RAS gene polymorphism for cardiovascular diseases in a longitudinal prospective study.

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