METABOLISM CLINICAL AND EXPERIMENTAL 60 (2011) 1150–1157

available at www.sciencedirect.com

ScienceDirect

www.metabolismjournal.com

The association of the angiotensinogen gene with insulin sensitivity in humans: a tagging single nucleotide polymorphism and haplotype approach

Patricia C. Underwood[a,b,*], *Bei Sun*[a], *Jonathan S. Williams*[a], *Luminita H. Pojoga*[a], *Benjamin Raby*[c], *Jessica Lasky-Su*[c], *Steven Hunt*[d], *Paul N. Hopkins*[d], *Xavier Jeunemaitre*[e], *Gail K. Adler*[a], *Gordon H. Williams*[a]

[a] *Division of Endocrinology, Diabetes, and Hypertension, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA*
[b] *William F. Connell School of Nursing, Boston College, Chestnut Hill, MA, USA*
[c] *Channing Laboratory, Brigham and Women's Hospital, Boston, MA, USA*
[d] *Cardiovascular Genetics Research Unit, Department of Medicine, University of Utah School of Medicine Salt Lake City, UT, USA*
[e] *AP-HP, Centre d'Investigation Clinique, Hopital Européen Georges Pompidou; INSERM U970, and University Paris Descartes, Paris, France*

ARTICLE INFO

Article history:
Received 15 September 2010
Accepted 15 December 2010

ABSTRACT

The purpose of this study was to clarify the association of the angiotensinogen gene (AGT) with insulin sensitivity using single nucleotide polymorphism (SNP) and haplotype analyses in a white cohort. A candidate gene association study was conducted in white persons with and without hypertension (N = 449). Seventeen SNPs of the AGT gene and their haplotypes were analyzed for an association with homeostasis model assessment of insulin resistance (HOMA-IR). Multivariate regression model accounting for age, sex, body mass index, hypertension status, study site, and sibling relatedness was used to test the hypothesis. Nine of the 17 SNPs were significantly associated with lower HOMA-IR levels. Homozygous minor allele carriers of the most significant SNP, rs2493134 (GG), a surrogate for the gain-of-function mutation rs699 (AGT p.M268T), had significantly lower HOMA-IR levels ($P = .0001$) than heterozygous or homozygous major allele carriers (AG, AA). Direct genotyping of rs699 in a subset of the population showed similar results, with minor allele carriers exhibiting significantly decreased HOMA-IR levels ($P = .003$). Haplotype analysis demonstrated that haplotypes rs2493137A|rs5050A|rs3789678G|rs2493134A and rs2004776G|rs11122576A|rs699T|rs6687360G were also significantly associated with HOMA-IR ($P = .0009$, $P = .02$), and these results were driven by rs2493134 and rs699. This study confirms an association between the AGT gene and insulin sensitivity in white humans. Haplotype analysis extends this finding and implicates SNPs rs2493134 and rs699 as the most influential. Thus, AGT gene variants, previously shown to be associated with AGT levels, are also associated with insulin sensitivity; suggesting a relationship between the AGT gene, AGT levels, and insulin sensitivity in humans.

Author contribution: PCU: wrote manuscript, study design and conduct, data collection and analysis, data interpretation. BS: data collection and analysis. JSW: study design and conduct, data collection and analysis, data interpretation. LP: data collection and analysis. BR: study design and conduct, data interpretation. JL-S: data interpretation. SH: data interpretation. PNH: study design and conduct, data collection and analysis. XJ: study design and conduct, data collection and analysis. GKA: data interpretation. GHW: study design and conduct, data collection and analysis, data interpretation.

* *Corresponding author*. Tel.: +617 525 7361; fax. +617 732 5764.
E-mail address: punderwood1@partners.org (P.C. Underwood).

0026-0495/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2010.12.009

1. Introduction

Angiotensinogen (AGT) is the initial component of the renin-angiotensin-aldosterone system (RAAS) and a precursor to both angiotensin I (AngI) and angiotensin II (AngII). Variants of the AGT gene are associated with plasma angiotensinogen levels, hypertension, and adrenal and renal blood flow [1-3], likely through the downstream effects of AGT on AngII. A possible role for AGT and AngII in the development of altered glucose metabolism is unclear. Pharmacologic blockade of the RAAS exhibits conflicting results, demonstrating a decrease in the incidence of new-onset type 2 diabetes mellitus in some [4,5] but not other studies [6]. Furthermore, gene association studies fail to clarify this issue, reporting both positive and negative associations for genes of the RAAS with insulin resistance (IR) and insulin sensitivity [7-10].

It is possible that studies analyzing components of RAAS with glucose metabolism conflict because of the heterogeneity of the populations studied and the inconsistent measurements of IR, blood pressure (BP), and type 2 diabetes mellitus. To address this possibility, we took advantage of a cohort where influential confounders (medication, activity, diet) were controlled. We examined whether single nucleotide polymorphisms (SNPs) of the AGT gene are associated with IR as determined by homeostasis model assessment (HOMA–IR) in a white cohort. To our knowledge, this is the first study that evaluates the relationship between SNPs representing coverage of the entire AGT gene and IR. To extend these observations, we also conducted haplotype analyses.

2. Research design and methods

2.1. Population

The 449 white participants studied were part of the Hypertensive Pathotype (HyperPATH) cohort, a data set of participants with and without mild hypertension. Four international centers contributed to this data set: Brigham and Women's Hospital (Boston, MA) (n = 155), University of Utah Medical Center (Salt Lake City, UT) (n = 179), Hospital Broussais (Paris, France) (n = 84), and Vanderbilt University (Nashville, TN) (n = 31) [11-13]. Individuals included in this analysis were participants with measured fasting glucose values and fasting insulin values and were genotyped for variants of the AGT gene. Individuals with and without hypertension were included to examine the relationship between the AGT gene and IR within a range of metabolic risk. Furthermore, we examined whether hypertension status affected the genotype-phenotype relationship.

2.2. Phenotype protocol

Hypertension was defined as a diastolic blood pressure (DBP) of at least 100 mm Hg on no medications, DBP of at least 90 mm Hg on 1 antihypertensive medication, or the use of 2 or more antihypertensive medications at the time of screening [11-13]. Normotensives, in addition to having BP less than 140/90, reported no first-degree relatives diagnosed with hypertension before the age of 60 years [11]. Nondiabetic participants (fasting glucose <126 mg/dL, 2-hour oral glucose tolerance test glucose <200 mg/dL, no oral hypoglycemic agents) were excluded in this analysis because of small sample size. All participants were placed on an isocaloric high-salt diet (200 mmol/d sodium, 100 mmol/d potassium, 1000 mmol/d calcium) for 5 days before study to control for known effects of salt on measurements of RAAS and insulin sensitivity [14]. On the final day of the diet, participants were admitted to the General Clinical Research Center and remained fasting and supine overnight. Participants were washed off of antihypertension medications as previously described to minimize the effects of these medications on study outcome measurements [11-13].

All inclusion and exclusion criteria for the HyperPath protocol are described elsewhere [11-13]. In brief, participants with known or suspected secondary hypertension, coronary artery disease, stroke, overt renal insufficiency (serum creatinine >1.5 mg/dL), psychiatric illness, current oral contraceptive use, current tobacco/illicit drug use, or moderate alcohol use were excluded. Participants with abnormal electrolyte or thyroid/liver function test results or electrocardiographic evidence of heart block, ischemia, or prior coronary events at the screening examination were excluded. All participants were between the ages of 18 and 65 years [11-13].

Baseline systolic, diastolic, and mean arterial BPs were taken as the mean of 3 consecutive readings (by Dinamap; Critikon, Tampa, Fl.) separated by 5 minutes each. Plasma glucose, serum insulin, and lipids levels were measured after an 8-hour fast and collected between 8:00 AM and 9:00 AM. Serum insulin, glucose, and lipids were measured as previously described [11]. The HOMA-IR was calculated as (fasting glucose mmol × fasting insulin in microunits per milliliter)/22.5 [15].

The HyperPath project was approved by the institutional review boards of each participating site. All participants were recruited through institutional review board–approved advertisements in the general population, and informed consent was obtained before participant enrollment.

2.3. Outcome measurement

The primary phenotype, IR as measured by HOMA-IR, was initially assessed in the population.

2.4. Genotyping

DNA was extracted as previously described [2,3]. Genotyping was conducted using the Illumina Bead Station GoldenGate platform (Illumina Inc, San Diego, CA). Sixteen tagging SNPs were identified from HapMap (Phase II, November 2008) using the chromosomal coordinates chr1:228,904,892-228,916,564 and including 5-kilobase flanking regions. Sixteen SNPs captured 100% of the common HapMap white variation in this region defined as minor allele frequencies greater than 0.1 at $R^2 > 0.9$ [16]. Single nucleotide polymorphism rs2493134 was used as a surrogate for the well-known AGT SNP rs699 (p.M268T).

In addition, a subset of the study population (n = 385) was genotyped for rs699. This genotyping was conducted in an earlier HyperPATH genotyping sequence using Applied Biosystems (ABI) 3100 genetic analyzer. Genotypes were called using the Genescan and Genotyper software packages (ABI) (Life Technologies Corporation, Carlsbad, CA) [1]. Analyses of rs699 and rs2493134 were compared to evaluate the appropriateness of rs2493134 as a

marker for rs699 in the larger population. The linkage disequilibrium (LD) plot of AGT SNPs for this study demonstrates strong LD between rs2493134 and rs699 (R^2 = .9, Fig. 1), indicating that rs2493134 and rs699 are inherited together and that rs2493134 is an appropriate marker for rs699 in the larger cohort.

All genotyped SNPs had a completion rate of greater than 95%. All SNPs conformed to Hardy-Weinberg expectations in the study population. Furthermore, SNP allele frequencies did not differ by site ($P > .05$ for all SNPs tested via χ^2 analysis). A second genotyping for 10% of the SNPs demonstrated concordance with the original genotype call.

The AGT variants are numbered using the Human Genome Variation Society recommendations [17]. In Table 2, variants are numbered at the complementary DNA (cDNA) level. Position +1 corresponds to the A of the ATG translation initiation codon located at nucleotide 114 in the NM_000029.3 AGT reference sequence. Hence, rs699 with cDNA label c.803T>C and protein label p.M268T is identical to the previously described variant 4072T>C (cDNA) and M235T (protein label). The earlier reported naming of 4072T>C and M235T started numbering from the beginning of the mature peptide, creating the difference between labels [18].

2.5. *Statistical analyses*

Statistical analyses were performed using SAS 9.1 (SAS Institute; Cary NC). Hardy-Weinberg expectations testing was performed for each SNP using a χ^2 test. Pairwise linkage (D' and R^2) was estimated using Haploview. A mixed effect linear regression (PROC MIXED) with all phenotypes and individual SNPs was performed accounting for relatedness between participants and adjusted for age, sex, body mass index (BMI), hypertension status, and study site. The natural log of HOMA-IR was used as the primary phenotype of interest. Both HOMA-IR and fasting insulin levels were log transformed to meet the normality assumptions of the regression model. The HOMA-IR and fasting insulin estimates represented in the figures were untransformed to ensure that results were easily interpreted within a clinical perspective. Error is represented as 95% confidence intervals. Haplotypes were constructed using the Haploview program, and an association of each haplotype with HOMA-IR was assessed using PLINK [19]. PLINK estimates haplotype frequencies via the expectation-maximization algorithm, computing global and haplotype-specific score statistics for tests of association between a trait and haplotype weighted by their posterior possibility. Because PLINK is unable to account for relatedness, the haplotype analysis was conducted in unrelated individuals only. All statistical tests were 2-sided. Nominal significance is indicated for $P < .05$. A Bonferroni correction for multiple comparisons is conservative because of the LD between SNPS of the AGT gene [1]. However, significance at the Bonferroni-corrected level of .004 (.05/14 = .004) is indicated.

3. Results

3.1. *Population characteristics*

Population characteristics are summarized in Table 1. Seventy-one percent of the population had hypertension.

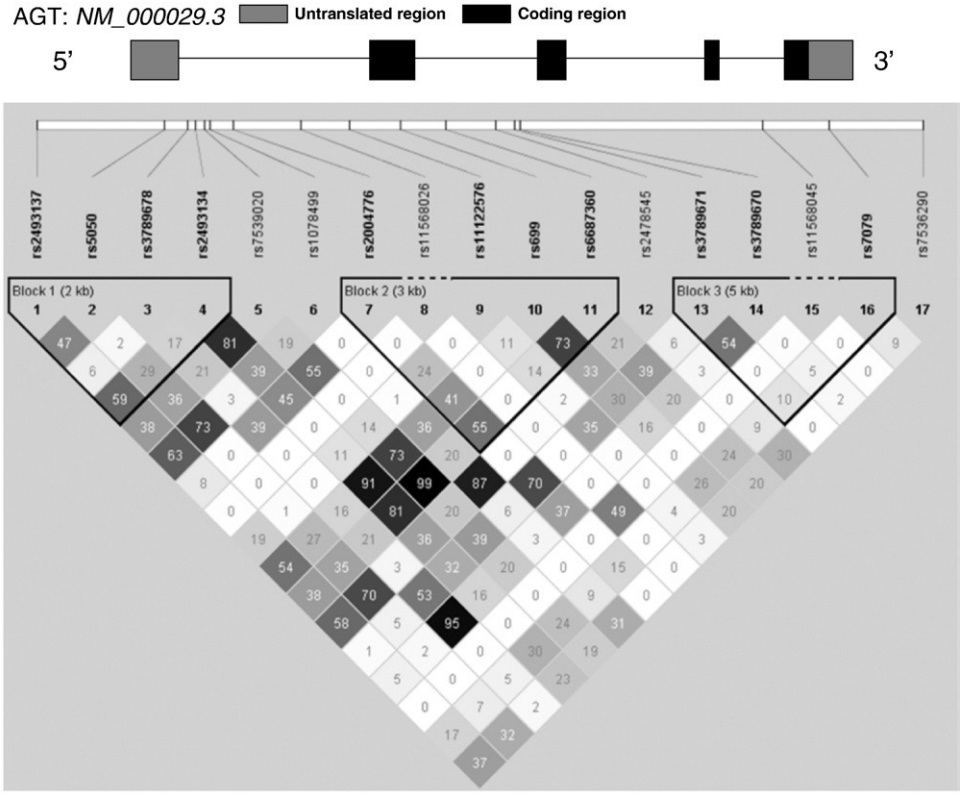

Fig. 1 – Linkage disequilibrium plot of 17 SNPs. Numbers represent R^2 values. Population includes participants with and without hypertension. The SNP location along the gene was determined using the NM_000029.3 AGT reference sequence.

Table 1 – Clinical characteristics of the HyperPath cohort by hypertension status

Baseline characteristics	NTN	HTN	P value
	n = 132	n = 317	
Age (y)	39.07 ± 11.07	48.60 ± 8.07	<.0001 *
Female (n [%])]	67 (50.76)	130 (41.01)	.06
BMI (kg/m^2)	25.08 ± 3.83	28.04 ± 3.81	<.0001 *
Fasting glucose (mg/dL)	85.25 ± 10.74	90.78 ± 11.19	<.0001 *
Fasting insulin (mg/dL)	10.4 ± 4.89	9.79 ± 5.72	<.0001 *
Impaired fasting glucose (≥100 mg/dL) (n [%])	11(8.3%)	67(21.14%)	<.0001 *
SBP (mm Hg)	109.48 ± 11.05	145.58 ± 20.24	<.0001 *
DBP (mm Hg)	65.74 ± 8.08	86.52 ± 11.19	<.0001 *
Mean arterial BP (mm Hg)	80.32 ± 8.36	106.20 ± 13.27	<.0001 *
HDL (mg/dL)	47.11 ± 18.27	40.47 ± 12.68	.0002 *
LDL (mg/dL)	96.18 ± 29.46	123.53 ± 36.38	<.0001 *
Total cholesterol (mg/dL)	165.5 ± 32.79	198.46 ± 36.22	<.0001 *
Triglycerides (mg/dL)	115.52 ± 73.81	164.90 ± 111.27	<.0001 *

Data are means ± SD. NTN indicates normotensive; HTN, hypertensive; SBP, systolic blood pressure; HDL, high-density lipoprotein, LDL, low-density lipoprotein.
* P value < .05.

Seventeen percent of the total population had impaired fasting glucose according to the American Diabetes Association criteria (fasting glucose ≥100 mg/dL) [20]. Individuals with hypertension were more likely to have impaired fasting glucose when compared with normotensives. As expected, individuals with hypertension had significantly higher fasting glucose values, greater BMI, higher triglyceride and total cholesterol levels, and elevated BP than individuals without hypertension.

3.2. *Gene characterization and SNP association with HOMA-IR*

Seventeen SNPs were genotyped (Table 2). Three SNPs were removed before the start of analyses because of monomorphism in the population (rs11568045, rs11568026) and a minor allele frequency (MAF) less than 0.1 (rs11122576), resulting in 14 SNPs. Five of the SNPs were in LD ($R^2 > 0.80$) with other SNPs (Fig. 1). Nine SNPs were significantly (P = .0001-.03) associated with lower HOMA-IR and, therefore, insulin sensitivity (Table 2).

3.3. *Association of rs2493134 with HOMA-IR, fasting insulin, and fasting glucose levels*

We used rs2493134 for further analyses because it is the most significant SNP, was genotyped in the entire population, and is in complete LD with p.M268T (M235T) and the promoter variant –44G>A [–6G>A] [21]. Fig. 2 demonstrates an association of rs2493134 with HOMA-IR (AA = 2.07 [1.79-2.39], AG = 1.82 [1.58-2.09], GG = 1.59 [1.35-1.87]; P = .0001) and fasting insulin levels (AA = 9.40 [7.66-11.53] mU/mL, AG = 8.29 [6.79-10.12] mU/mL, GG = 7.29 [5.88-9.04] mU/mL; P = .0001) accounting for age, sex, BMI, study site, and hypertension status in the entire population. No significant association was seen between rs2943134 and fasting glucose levels (P = .3) or 2-hour glucose levels after an oral glucose tolerance test (P = .5). This initial analysis demonstrated that hypertension status, BMI, and sex were significantly contributing to the variance of HOMA-IR (.005, <.0001, and <.0001, respectively).

Because of the effects of hypertension status on the association, we dichotomized the population by hypertension status and examined the association between rs2493134 and insulin phenotypes (HOMA-IR and fasting insulin) (Fig. 2). A significant association between rs2493134 and HOMA-IR (AA = 2.2 [1.84-2.64], AG = 1.84 [1.55-2.18], GG = 1.65 [1.36-1.99]; P = .0004) and fasting insulin (AA = 9.97 [7.69-13.46]

Table 2 – Sixteen SNPs analyzed for association with log HOMA-IR values

No.	Polymorphism	Location: cDNA NM_000029.3	Amino acid change	MAF	Log HOMA-IR P values
1	rs2493137	Upstream T>C	–	0.31	.01 *
2	rs5050	c.–58A>C	–	0.17	.03 *
3	rs3789678	c.–4+350G>A	–	0.14	.7
4	rs2493134	c.–4+473A>G	–	0.44	.0001 †
5	rs7539020	c.–4+642G>A	–	0.40	.005 *
6	rs1078499	c.–4+736T>C	–	0.22	.007 *
7	rs2004776	c.–4+11306G>A	–	0.28	.02 *
8	rs11568026	c.–3–924T>C	–	0.00	Monomorphic
9	rs11122576	c.–3–80A>G	–	0.08	MAF <0.10
10	rs699	c.803T>C	p.Met268Thr (M235T)	0.42	.0003 †
11	rs6687360	c.856+749G>A	–	0.40	.0003 †
12	rs2478545	c.856+1620C>T	–	0.21	.01 *
13	rs3789671	c.857–1854C>A	–	0.22	.1
14	rs3789670	c. 857–1768G>A	–	0.13	.07
15	rs11568045	c.1270_408A>C	–	0.00	Monomorphic
16	rs7079	c.556C>A	–	0.31	.4
17	rs7536290	Downstream A>G	–	0.15	.2

Minor allele frequencies did not differ significantly between study sites. The SNP location was determined using the NM_000029.3 AGT reference sequence. Data are adjusted for age, sex, BMI, and hypertension status.
* P value < .05.
† P value < .004.

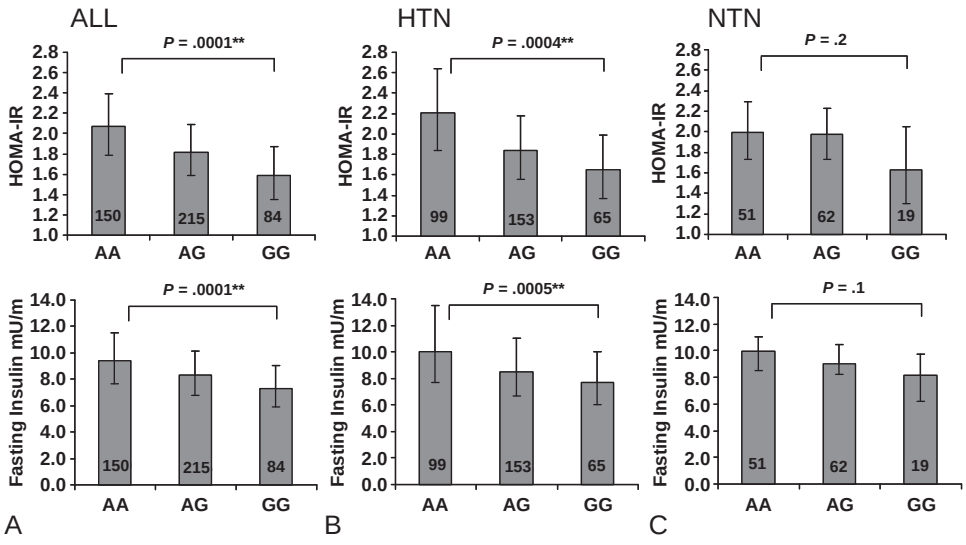

Fig. 2 – Association of rs2493134 with primary and secondary phenotypes. The HOMA-IR and fasting plasma insulin values by SNP rs2493134 in entire population (A) (All), individuals with hypertension only (B) (HTN), and individuals without hypertension (C) (NTN). Point estimates (least-square means), error bars (95% confidence interval), and *P* values were obtained from the mixed model regression accounting for age, sex, BMI, sibling relatedness, and study site. **p<0.004.

mU/mL, AG = 8.50 [6.69-11.02] mU/mL, GG = 7.69 [6.05-9.97] mU/mL; *P* = .0005) existed in the hypertensive population. These results remained significant after accounting for the triglyceride to high-density lipoprotein cholesterol ratio (*P* = .001 HOMA-IR and *P* = .002 fasting insulin), a marker of IR [22]. The normotensive population demonstrated a similar trend, but there was a nonsignificant association between rs2493134 and HOMA-IR (AA = 1.99 [1.73-2.29], AG = 1.97 [1.73-2.23], GG = 1.63 [1.30-2.05]; *P* = .2) and fasting insulin (AA = 9.97 [8.5-11.02] mU/mL, AG = 9.03 [8.25-10.45] mU/mL, GG = 8.17 [6.23-9.78] mU/mL; *P* = .1). Because of the strong influence of hypertension status on the findings, all subsequent exploratory analyses were done with hypertensives only.

3.4. *Exploratory analysis: covariates known to influence AGT genotype: sex and BMI*

Because both sex [23] and obesity [2] are known to interact with SNPs of the AGT gene, we investigated the influence of these covariates on the association between rs2493134 and HOMA-IR. Although the multivariate analysis demonstrated that a significant portion of the variance of HOMA-IR was accounted for by sex, with men having higher HOMA-IR values (*P* = .00001), the interaction between SNP rs2493134 and sex was not significant (*P* = .9), indicating that the SNP's association with HOMA-IR was not influenced by sex.

In contrast, our analysis of the effects of BMI on the association of rs2493134 and HOMA-IR suggested that BMI may be moderating the results. An interaction between BMI as a continuous variable and SNP was not significant (*P* = .6). However, when the population was stratified by obesity status (normal: BMI <25 kg/m^2, overweight: BMI 25-29 kg/m^2, obese: BMI ≥30 kg/m^2) [24], an interaction was close to significant between the obese group and rs2493134 (*P* = .15 additive SNP model; *P* = .06 dominant SNP model). Furthermore, SNP rs2493134 exhibited a greater β estimate (β = –0.20, *P* = .007) for the regression model tested in the obese group compared with the β estimates for the same SNP tested in normal and overweight individuals (β = –0.19, *P* = .06; β = –.10, *P* = .06) (Table 3).

3.5. *Haplotype analysis*

The SNP LD plot from our hypertensive population indicated that 3 haplotype blocks existed. Table 4 displays all 3 haplotype blocks and each block's association with HOMA-IR. Haplotype rs2493137A|rs5050A| rs3789678G|rs2493134A in

Table 3 – SNP and HOMA-IR associations in hypertensive population stratified by obesity status

rs2493134	n	HOMA-IR estimates	LCI	UCI	β	*P* value
		Normal: BMI <25				
AA	23	1.67	1.14	2.46	–0.19	0.06
AG	35	1.38	0.96	1.97		
GG	12	1.15	0.73	1.67		
		Overweight: BMI 25-29				
AA	41	2.01	1.70	2.36	–0.10	0.06
AG	74	1.73	1.51	2.01		
GG	29	1.68	1.39	2.05		
		Obese: BMI ≥30				
AA	35	2.89	2.41	3.46	–0.20	0.007*
AG	44	2.25	1.92	2.64		
GG	24	1.95	1.55	2.44		

Point estimates, 95% confidence interval, and β and *P* values were obtained from a mixed model regression accounting for age, sex, sibling relatedness, and study site. LCI indicates lower confidence interval; UCI: upper confidence interval.

* *P* value < .05.

Table 4 – Haplotype analyses for association with HOMA-IR in individuals with hypertension

Block 1	Haplotype	β	P value	Frequency
	CCGG	−0.1477	.09	0.17
	CAGG	−0.1268	.14	0.15
	AAAG	−0.1484	.11	0.14
	AAGA	0.1965	**.0009**†	0.54
rs2493137|rs5050|rs3789678|**rs2493134**				
Block 2	Haplotype	β	P value	Frequency
	AGCA	−0.0869	.31	0.09
	AACA	−0.0968	.16	0.18
	GACA	−0.03961	.63	0.11
	GACG	−0.07393	.47	0.07
	GATG	0.1164	**.02***	0.53
rs2004776|rs11122576|**rs699**|rs6687360				
Block 3	Haplotype	β	P value	Frequency
	CGA	0.02	.64	0.32
	AAC	−0.07	.35	0.14
	AGC	−0.11	.21	0.09
	CGC	0.04	.40	0.45
rs3789671|rs3789670|rs7079				

The primary SNP and allele driving the significant findings are in bold.
* P value < .05.
† P value < .004.

block 1 and haplotype rs2004776G|rs11122576A|rs699T|rs6687360G in block 2 are significantly associated with HOMA-IR ($P = .0009$, $\beta = 0.1965$; $P = .02$, $\beta = 0.1164$). The association of haplotype block 1 with HOMA-IR is significant only when individuals carry the major allele (**A**) for SNP rs2493134 ($P = .0009$ unadjusted; $P = .002$ adjusted for age, sex, and BMI). This is similar for rs699 in block 2. The block 2 haplotype is significant only for individuals carrying the major allele (**T**) ($P = .02$ unadjusted; $P = .03$ adjusted for age, sex, and BMI).

4. Discussion

Our study demonstrates a significant association between SNPs of the AGT gene and insulin sensitivity in a white population. This relationship is robust as evidenced by the numerous significant associations even after multiple comparison adjustment. The current study also demonstrates an association of AGT haplotypes, specifically rs2493137**A**|rs5050**A**|rs3789678**G**|rs2493134**A** and rs2004776**G**|rs11122576**A**|rs699**T**|rs6687360**G**, with HOMA-IR. These haplotypes are driven by the major allele of 2 SNPs in LD: rs2493134 and rs699. These novel SNP associations implicate variations in the AGT gene with increased insulin sensitivity.

The AGT gene, specifically the p.M268T (M235T) polymorphism, was previously associated with essential hypertension, adrenal and renal response to Ang II, and angiotensinogen levels [1-3]; however, an association with this gene and glucose metabolism has been unclear. Sheu et al [9] found no association with p.M268T (M235T) and insulin sensitivity; however, both Guo et al [8] and Takukara et al [10] demonstrated that p.M268T (M235T) was associated with increased IR. Our results suggest otherwise; however, extensive differences between the study designs and populations may explain the contradictory results and are important to highlight for further investigation. First, each study was conducted in a different ethnicity, suggesting that the association of the AGT gene and insulin sensitivity may be ethnically dependent. Furthermore, our analysis accounted for age, sex, BMI, and hypertension status and controlled for medication, diet, and activity, whereas other studies accounted for none or few of these variables. Differences in outcomes between studies suggest that one or all of these variables may be influencing the association observed. Our findings clarify the role of the AGT gene with insulin sensitivity in a white population by capturing the entire AGT gene, as characterized by HapMap, in a well-designed study.

Molecular genetics and physiology studies provide insight into a possible mechanism underlying our finding of an association of the AGT gene with insulin sensitivity. First, molecular genetic studies demonstrate a relationship between variants of the AGT gene, AGT gene expression, and plasma AGT levels. An AGT promoter variant, rs5051 (c.−44G>A [−6G>A]), a SNP in complete LD with rs699 and rs2493134, has been shown to increase AGT gene expression in vitro [25,26]. Subsequent studies demonstrated that increased AGT gene expression leads to increased plasma AGT levels in a mouse model [27]. More recently, AGT gene variants were found to be associated with increased plasma AGT levels in humans [1]. Together, these studies support the hypothesis that variations in the AGT gene lead to increased AGT expression and subsequently to increased plasma AGT levels in humans [21]. Important to our study is the following question: How may increases in AGT levels contribute to processes of insulin sensitivity in humans? Human studies of RAAS physiology provide insight into possible answers to this question.

Physiology studies demonstrate a relationship between components of the RAAS and glucose metabolism, potentially linking AGT levels with insulin sensitivity. Infusion of AngII in humans has been shown to improve insulin sensitivity in normotensive individuals with and without type 2 diabetes mellitus [28-31]. Subpressor doses of AngII exhibit a similar effect without an increase in BP, demonstrating that hemodynamic alterations are not the sole mechanism for improved insulin sensitivity [28]. Studies in animal and cell culture further these findings, with AngII (2 μg/100 g body weight) increasing insulin-stimulated glucose uptake in rat adipocytes [32]. In the above in vivo studies, additional AngII was administered exogenously. The same relationships may not exist when the activity of the RAAS is modified physiologically. For example, a recent study demonstrated that consumption of a low-salt diet, a state that physiologically increases RAAS activity, resulted in higher HOMA-IR levels [33], supporting the hypothesis that AngII levels per se may not necessarily produce a consistent metabolic effect. The type of effect may be dependent more on the physiologic "appropriateness" of the AngII level. Thus, it is possible that increased plasma AGT levels, a known effect of AGT gene variants, increase AngII levels physiologically inappropriately and thereby affect glucose homeostasis via the mechanisms outlined above.

Our results suggest an influence of obesity, albeit not significant on the study results. An interaction between the

AGT gene and BMI has been shown in previous studies [2]; and it is possible that, with a larger sample size, the interaction suggested in our analyses would become significant. AGT gene expression has been shown to be increased in both a high-fat diet in human visceral adipocytes [34] and in a hyperinsulinemic state in human 3T3-L1 adipocytes [35]. Furthermore, in humans, plasma ANGII levels have been shown to positively correlate with body weight [36]. Further analyses in larger cohorts are necessary to confirm possible interactions between the AGT gene and BMI on IR. Our data suggest that, with obesity, the effect of the p.M268T (M235T) AGT gene variant on insulin sensitivity is enhanced.

The results of our haplotype analysis are consistent with the single SNP analyses, similar to other AGT haplotype studies [37]. The association is primarily driven by rs2493134 and rs699 in haplotype block 1 and block 2. The results demonstrate that major allele carriers are most likely to have elevated HOMA-IR values and are insulin resistant or, as described in our SNP results, the minor allele is associated with insulin sensitivity. Interestingly, an extensive haplotype that includes AGT p.M268T (M235T) has been found to be more strongly associated with AGT levels than the SNP alone [1]. This may explain why some individuals known to have an increased frequency of the minor allele of this SNP, African Americans, have an increased risk of IR when our data suggest that the SNP should be protective from altered glucose metabolism. Further studies are necessary to assess the association of the AGT gene with insulin sensitivity in an African American population, specifically; whether extensive haplotype analyses provide the most relevant information.

There are several limitations to this study. First, this gene association study has a relatively small sample size when compared with the "classic" genomewide association approach. However, our study was conducted in a well-phenotyped population where medication, diet, and activity were tightly controlled within an inpatient setting. Our results suggest that studying individuals within a carefully controlled setting enables the use of smaller sample sizes to identify genotype/phenotype associations. Functional data are not present in this study, however; previous studies, including one from the HyperPATH cohort, demonstrate that AGT p.M268T (M235T) is associated with increased AGT levels and functional differences in aldosterone secretion, renal blood flow, salt handling, and salt sensitivity of BP [1-3,8,37]. However, although this SNP is located in AGT's coding region, no direct functional differences have been reported based on genetic variation at this site. Furthermore, although this SNP is in nearly complete LD with –6 in the upstream regulatory region, no interactions or effects of other molecular genetic factors (eg, microRNAs, variation in methylation function either in the histone or DNA) have been reported. However, intriguingly, a recent report raises the possibility that the results of this study may not be related through an effect of AngII, but rather an effect of AGT interacting with its receptor (AGTR1). MicroRNA encoded by intronic SNPs in the AGTR1 has been shown to increase AGTR1 gene expression [38]. It is possible that intronic SNPs of the AGT gene could act in a similar manner, through microRNA, to affect AGT gene expression. No data are available presently to assess this possibility.

In conclusion, this study identifies that SNPs of the AGT gene are associated with insulin sensitivity in white persons. Haplotype analysis extends this finding and implicates SNPs rs2493134 and rs699, variants known to affect plasma AGT levels, as the most influential SNPs. Our results indicate that both hypertension status and BMI may be influencing the association, with the genotype effect being the strongest in hypertensive, obese individuals. These results demonstrate a potential role for the AGT gene to explain why some individuals, even with an abnormal cardiometabolic profile, are insulin sensitive. As clinicians attempt to use AGT genotype as a genomic marker for individualized hypertension treatment, the effects of this gene on glucose metabolism should be considered.

Acknowledgment

The project described was supported in part by the following grants: U54LM008748, from the National Library of Medicine; UL1RR025758 Harvard Clinical and Translational Science Center; from the National Center for Research Resources and M01-RR02635; Brigham & Women's Hospital, General Clinical Research Center; from the National Center for Research Resources, as well as NIH grants HL47651, HL59424, F31 NR011108 (PCU), K23 HL084236 (JSW), Specialized Center of Research (SCOR) in Molecular Genetics of Hypertension P50HL055000.

We thank all other investigators and staff of the HyperPath Protocol including Nancy Brown Vanderbilt University, Nashville TN, as well as the General Clinical Research Center staff and participants of all protocol sites.

Appendix

List of principle investigators of each study site:

Boston: Gordon H. Williams
Salt Lake City: Paul N. Hopkins
Paris: Xavier Jeunemaitre
Vanderbilt: Nancy Brown

REFERENCES

[1] Watkins WS, Hunt SC, Williams GH, et al. Genotype-phenotype analysis of angiotensinogen polymorphisms and essential hypertension: the importance of haplotypes. J Hypertens 2010;28:65-75.
[2] Hopkins PN, Lifton RP, Hollenberg NK, et al. Blunted renal vascular response to angiotensin II is associated with a common variant of the angiotensinogen gene and obesity. J Hypertens 1996;14:199-207.
[3] Hopkins PN, Hunt SC, Jeunemaitre X, et al. Angiotensinogen genotype affects renal and adrenal responses to angiotensin II in essential hypertension. Circulation 2002;105:1921-7.
[4] Abuissa H, Jones PG, Marso SP, et al. Angiotensin-converting enzyme inhibitors or angiotensin receptor blockers for prevention of type 2 diabetes: a meta-analysis of randomized clinical trials. J Am Coll Cardiol 2005;46:821-6.
[5] The NAVIGATOR Study Group. Effect of valsartan on the incidence of diabetes and cardiovascular events. N Engl J Med 2010;362:1477-90.

[6] DREAM Trial Investigators. Effect of ramipril on the incidence of diabetes. N Engl J Med 2006;355:1551-62.
[7] Bonnet F, Patel S, Laville M, et al. Influence of the ACE gene insertion/deletion polymorphism on insulin sensitivity and impaired glucose tolerance in healthy subjects. Diabetes Care 2008;31:789-94.
[8] Guo X, Cheng S, Taylor KD, et al. Hypertension genes are genetic markers for insulin sensitivity and resistance. Hypertension 2005;45:799-803.
[9] Sheu WH, Lee WJ, Jeng CY, et al. Angiotensinogen gene polymorphism is associated with insulin resistance in nondiabetic men with or without coronary heart disease. Am Heart J 1998;136:125-31.
[10] Takakura Y, Yoshida T, Yoshioka K, et al. Angiotensinogen gene polymorphism (Met235Thr) influences visceral obesity and insulin resistance in obese japanese women. Metabolism 2006;55:819-24.
[11] Raji A, Williams GH, Jeunemaitre X, et al. Insulin resistance in hypertensives: effect of salt sensitivity, renin status and sodium intake. J Hypertens 2001;19:99–105.
[12] Chamarthi B, Kolatkar NS, Hunt SC, et al. Urinary free cortisol: an intermediate phenotype and a potential genetic marker for a salt-resistant subset of essential hypertension. J Clin Endocrinol Metab 2007;92:1340-6.
[13] Williams JS, Williams GH, Jeunemaitre X, et al. Influence of dietary sodium on the renin-angiotensin-aldosterone system and prevalence of left ventricular hypertrophy by EKG criteria. J Hum Hypertens 2005;19:133-8.
[14] Donovan DS, Soloman CG, Seely EW, et al. Effect of sodium intake on salt sensitivity. Am J Physiol 1993;264:730-4.
[15] Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-9.
[16] International HAPMAP Consortium. The International HAPMAP Project. Nature 2003;426:789-96.
[17] den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 2000;15:7–12.
[18] Halangk J, Berg T, Neumann K, et al. Evaluation of angiotensinogen c. 1-44G>A and p.M268T variants as risk factors for fibrosis progression in chronic hepatitis C and liver disease of various etiologies. Gen Test Mol Bioma 2009;13:407-14.
[19] Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81:559-75.
[20] American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care 2010;33:S62-9.
[21] Lalouel JM, Rohrwasser A, Terreros D, et al. Angiotensinogen in essential hypertension: from genetics to nephrology. J Am Soc Nephrol 2001;12:606-15.
[22] McLauglin T, Abbasi F, Cheal K, et al. Use of metabolic markers to identify overweight individuals who are insulin resistant. Ann Intern Med 2003;139:802-9.
[23] Tsai CT, Hwang JJ, Lai LP, et al. Interaction of gender, hypertension, and the angiotensinogen gene haplotypes on the risk of coronary artery disease in a large angiographic cohort. Atherosclerosis 2009;203:249-56.
[24] Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults—the evidence report. national institutes of health. Obes Res 1998;6: 51S–209S.
[25] Inoue I, Nakajima J, Williams CS, et al. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. J Clin Invest 1997;99:1786-97.
[26] Jeunemaitre X, Soubrier F, Kotelevtsev YV, et al. Molecular basis of human hypertension: role of angiotensinogen. Cell 1992;71:169-80.
[27] Kim HS, Krege J, Kluckman KD, et al. Genetic control of blood pressure and angiotensinogen locus. Proc Natl Acad Sci 1995;92:2735-9.
[28] Fliser D, Arnold U, Kohl B, et al. Angiotensin II enhances insulin sensitivity in healthy volunteers under euglycemic conditions. J Hypertens 1993;11:983-8.
[29] Morris AD, Petrie JR, Ueda S, et al. Pressor and subpressor doses of angiotensin II increase insulin sensitivity in NIDDM. dissociation of metabolic and blood pressure effects. Diabetes 1994;43:1445-9.
[30] Jonk AM, Houben AJ, Schaper NC, et al. Angiotensin II enhances insulin-stimulated whole-body glucose disposal but impairs insulin-induced capillary recruitment in healthy volunteers. J Clin Endocrinol Metab 2010;95:3901-8.
[31] Buchanan TA, Thawani H, Kades W, et al. Angiotensin II increases glucose utilization during acute hyperinsulinemia via a hemodynamic mechanism. J Clin Invest 1993;92:720-6.
[32] Juan CC, Chien Y, Wu LY, et al. Angiotensin II enhances insulin sensitivity in vitro and in vivo. Endocrinology 2005;146:2246-54.
[33] Garg R, Williams GH, Hurwitz S, et al. Low-salt diet increases insulin resistance in healthy subjects. Metabolism 2010 [October 29, Epub ahead of print].
[34] Rahmouni K, Mark AL, Haynes WG, et al. Adipose depot-specific modulation of angiotensinogen gene expression in diet-induced obesity. Am J Physiol Endocrinol Metab 2004;286: E891-5.
[35] Jones BH, Standridge MK, Taylor JW. Angiotensinogen gene expression in adipose tissue: analysis of obese models and hormonal and nutritional control. Am J Physiol 1997;273: R236-42.
[36] Saiki A, Ohira M, Endo K, et al. Circulating angiotensin II is associated with body fat accumulation and insulin resistance in obese subjects with type 2 diabetes mellitus. Metabolism 2009;58:708-13.
[37] Jeunemaitre X, Inoue I, Williams CS, et al. Haplotypes of angiotensinogen in essential hypertension. Am J Hum Genet 1997;60:1448-60.
[38] Sethupathy P, Borel C, Gagnebin M, et al. Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3′ untranslated regions: a mechanism for functional single-nucleotide polymorphisms related to phenotypes. Am J Hum Genet 2007;81:405-13.