Original Article

Polymorphisms of the Renin-Angiotensin System Genes Predict Progression of Subclinical Coronary Atherosclerosis

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Premature coronary artery disease (CAD) in subjects with type 1 diabetes dramatically affects quality of life and morbidity and leads to premature death, but there is still little known about the mechanisms and predictors of this complication. In the present study, we explored the role of genetic variants of angiotensinogen (AGT, M235T), ACE (I/D), and angiotensin type 1 receptor (ATR1, A1166C) as predictors of rapid progression of subclinical coronary atherosclerosis. Five-hundred eighty-five type 1 diabetic patients and 592 similar age and sex control subjects were evaluated for progression of coronary artery calcification (CAC), a marker of subclinical CAD, before and after a 2.5-year follow-up. In logistic regression analysis, CAC progression was dramatically more likely in type 1 diabetic subjects not treated with ACE inhibitor/angiotensin receptor blocker who had the TT-ID-AA/AC genotype combination than in those with other genotypes (odds ratio 11.6 [95%CI 4.5–29.6], $P < 0.0001$) and was even stronger when adjusted for cardiovascular disease risk factors and the mean A1C (37.5 [3.6–388], $P = 0.002$). In conclusion, a combination of genotype variants of the renin-angiotensin system genes is a powerful determinant of subclinical progression of coronary artery atherosclerosis in type 1 diabetic patients and may partially explain accelerated CAD in type 1 diabetes. *Diabetes* 56:863–871, 2007

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ACEI, ACE inhibitor; AER, albumin excretion rate; AGT, angiotensinogen; ARB, angiotensin receptor blocker; ATR1, angiotensin type 1 receptor; CAC, coronary artery calcification; CAD, coronary artery disease; CRP, C reactive protein; CVS, calcium volume score; PAI-1, plasminogen activator inhibitor 1; RAS, renin-angiotensin system; SNP, single nucleotide polymorphism.

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Patients with type 1 diabetes have a dramatically higher risk of coronary artery disease (CAD) compared with the general population (1,2). Coronary artery calcification (CAC), a marker of coronary artery plaque burden, correlates very well with the established CAD risk factor profile in patients with type 1 diabetes (3–5). There is growing evidence that CAC strongly predicts future coronary events in asymptomatic nondiabetic and diabetic subjects (4–6).

Diabetic nephropathy plays an important role in the pathogenesis of advanced CAD in type 1 diabetes (2). However, little is known concerning determinants of subclinical atherosclerosis detected as rapidly progressing CAC. Activation of the renin-angiotensin system (RAS) by hyperglycemia may be the key mechanism (7,8). The RAS involves enzymatic processing of angiotensinogen (AGT) to angiotensin I, further cleaved by ACE to angiotensin II. Treatment with ACE inhibitors lowers the risk of cardiovascular death, myocardial infarction, and coronary revascularization in the general population and in diabetic patients (9–11).

The RAS activity is modified by variants of the genes coding functional proteins of this pathway (12–14). The AGT gene, localized on chromosome 1q41-qter, encodes AGT. There is only one haplotype block at the AGT locus, and all common single nucleotide polymorphisms (SNPs) identified appear to be in complete linkage disequilibrium with the most intensively studied M235T polymorphism (13,15). Although the functional variant has not yet been definitively identified (16), the T235 allele has been consistently associated with cardiovascular disease (13,17) and in some studies (18,19), but not all (20,21), with increased blood levels of AGT.

The ACE gene, localized on chromosome 17q23, is highly polymorphic in the promoter and coding regions, but because of the strong linkage disequilibrium in this region, the functional variant of this gene has not yet been determined (22). The most studied ACE gene polymorphism, the 278-bp insertion (allele I) or deletion (allele D) variant in intron 16, is associated with serum and tissue ACE levels (12,23). Unfortunately, data concerning the role of the I/D polymorphism in CAD risk in different populations are conflicting (24–26).

The ATR1 gene, localized on chromosome 3q21-q25, encodes type 1 angiotensin II receptor, a dominant variant of the receptor in the cardiovascular system. The A1166C

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TABLE 1
Clinical characteristics, frequency of cardiovascular risk factors, and values of CVS markers in type 1 diabetic and control subjects by AGT (MM + MT versus TT), ACE (II + ID versus DD), and ATR1 (AA + AC versus CC) genotypes at baseline visit

	Type 1 diabetic subjects					
	MM + MT	TT	II + ID	DD	AA + AC	CC
n	478	107	427	158	527	58
Age (years)	36.7 ± 9.3	37.4 ± 8.8	36.9 ± 9.2	36.4 ± 9.3	36.9 ± 9.1	35.9 ± 10.1
Sex (% of females)	55.8	53.3	54.6	57.6	54.3	65.5
Follow-up (years)	2.5 ± 0.4	2.5 ± 0.4	2.5 ± 0.4	2.5 ± 0.3	2.5 ± 0.4	2.5 ± 0.3
BMI (kg/m^2)	26.1 ± 4.4	26.1 ± 4	26.1 ± 4.4	26.1 ± 4.1	26.1 ± 4.4	25.9 ± 4.2
Type 1 diabetes duration (years)	23.4 ± 9	23.9 ± 9.1	23.5 ± 9.1	23.5 ± 8.9	23.6 ± 9.1	22.5 ± 8.6
Waist-to-hip ratio	0.82 ± 0.08	0.83 ± 0.08	0.82 ± 0.08	0.81 ± 0.09	0.82 ± 0.08	0.8 ± 0.07
A1C (%)	7.9 ± 1.2	7.8 ± 1.3	7.9 ± 1.3	7.9 ± 1.1	7.9 ± 1.3	7.9 ± 1
HDL (mg/dl)	56 ± 16	60 ± 18*	56 ± 16	57 ± 18	57 ± 16	56 ± 16
LDL (mg/dl)	99 ± 29	105 ± 33	101 ± 30	99 ± 29	100 ± 30	100 ± 29
Triglycerides (mg/dl)	81.7	84.4	83	79.9	82.1	83
AER (μg/min)	9.9	10.8	10.7	8.7	10.1	10.4
Albuminuria (%)	20.7	21.4	21.8	18	20.9	20
Serum creatinine	1.2 ± 0.5	1.2 ± 0.2	1.2 ± 0.5	1.2 ± 0.4	1.2 ± 0.5	1.2 ± 0.3
Smoking (%)	19.4	26	19.9	22.6	20.7	19.3
Blood pressure						
Systolic (mmHg)	116 ± 14	120 ± 13†	118 ± 14	116 ± 12	117 ± 14	115 ± 11†
Diastolic (mmHg)	77 ± 8	79 ± 9§	77 ± 9	77 ± 8	78 ± 9	75 ± 6*
Hypertension (%)	40.1	47.7	41.4	44.6	42.4	41.4
On statins (%)	14.8	20.6	15	18.3	15.6	19
On ACEI/ARB (%)	34.9	32.7	33.5	38	34.7	34.4
CRP (μg/ml)	1.5	1.4	1.5	1.3	1.4	1.7
PAI-1 (ng/ml)	11.1	11.7	11.4	10.5	11.2	11.1
Homocysteine (μmol/l)	8.4 ± 3.9	8.2 ± 2.9	8.3 ± 4	8.3 ± 3	8.4 ± 3.9	8 ± 2.7
Adiponectin (μg/ml)	15.8 ± 8.9	15.2 ± 8.2	15.6 ± 8.7	15.7 ± 9.1	15.7 ± 8.9	15.8 ± 7.5

Data are means ± SD, %, or geometric mean for triglycerides, AER, CRP, and PAI-1. *$P = 0.03$, †$P = 0.01$, ‡$P = 0.016$, §$P = 0.008$, ‖$P = 0.023$; corrected for multiple comparisons, $P_c > 0.05$ for all.

SNP, localized within the haplotype block in the 3′-untranslated region of AGTR1 gene, may be involved in posttranscriptional modification and angiotensin II receptor–mediated cell signaling, which is critical for regulating tissue-specific receptor functions (14,27). There are an increasing number of reports of a possible link between the A1166C allele and cardiovascular disease; however, not all results are consistent (28,29).

We hypothesized that these functional RAS gene polymorphisms predict rapid progression of CAC in initially asymptomatic type 1 diabetic subjects and nondiabetic control subjects of similar age, sex, and ethnicity. The RAS genetic markers chosen, AGT (M235T), ACE (I/D), and ATR1 (1166A/C), have been previously reported to be associated with CAD and show biological effect in functional transcriptional studies or are in a close linkage disequilibrium with putative functional variant, if it has not yet been identified.

RESEARCH DESIGN AND METHODS

Study population. The Coronary Artery Calcification in Type 1 Diabetes study population has been previously described (3). Briefly, 652 men and women with type 1 diabetes and 764 nondiabetic control subjects aged 19–56 years and with no history of CAD were enrolled into a prospective follow-up of the development and progression of CAC. All patients with diabetes had been diagnosed when younger than 35 years, had been treated with insulin within 1 year of diagnosis, and had a history of "long lasting " diabetes (mean duration 23 ± 9 years) on enrollment. Nondiabetic control subjects were generally spouses, friends, or neighbors of the cases. All subjects provided informed consent, and the study was approved by the Colorado Combined Institutional Review Board.

AGT, ACE, and ATR1 genotype data were available in 635 type 1 diabetic and 740 control subjects. Because the number of ethnic minority subjects was small and their genotype distributions significantly different from those in non-Hispanic white participants (Supplemental Table A, online appendix [available at http://dx.doi.org/10.2337/db06-1321]), analyses were limited to 585 type 1 diabetic non-Hispanic white patients and 592 non-Hispanic white control subjects.

Participants were examined at the baseline visit in 2000–2002 and after a mean 2.5 ± 0.4 years of follow-up. Age, race, sex, ethnicity, and data concerning health status and treatment were self-reported. Anthropometric (BMI and waist-to-hip ratio) and blood pressure measurements were obtained at both visits. Insulin resistance was assessed as the inverse of the estimated glucose disposal rate as previously described (3).

Laboratory measurements. Total plasma cholesterol and triglyceride levels were measured with standard enzymatic methods, and LDL cholesterol was calculated by the Friedewald formula. A1C levels were determined by high-performance liquid chromatography (Bio-Rad Variant). Adiponectin, C reactive protein (CRP), and plasminogen activator inhibitor 1 (PAI-1) were measured as described previously (30). Urinary albumin excretion rate (AER) was estimated by averaging AER in two timed overnight samples. Micro- (AER 20–199 μg/min) or macroalbuminuria (AER ≥200 μg/min) was diagnosed based on the American Diabetes Association criteria (31).

CAC. All patients underwent two electron beam tomography scans without contrast at the baseline and follow-up visit. Images were obtained of the entire epicardial system using an Imatron C-150 Ultrafast CT scanner (Imatron, South San Francisco, CA), with a 100-ms exposure. The standard acquisition protocol as previously described was used (32). Images were electrocardiographically triggered at 80% of the R-R interval, and 30–40 contiguous 3-mm slices were acquired.

The threshold for CAC was set at computed tomography density of 130 Hounsfield units in at least three contiguous pixels. A region of interest was encircled within each coronary artery, and computer-driven measurement of the lesion area and maximum density were recorded. A coronary artery calcium score (CAC) for each region was calculated by multiplying the area by the density score according to the standard Agatston method (33). A total calcium score was calculated by adding up scores for all slices and separately for left main, left anterior descending, circumflex, and right coronary arteries. Total calcium volume scores (CVSs) were calculated using the volumetric method, which is based on isotropic interpolation as previously described

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TABLE 1
Continued

Control subjects					
MM + MT	TT	II + ID	DD	AA + AC	CC
503	89	421	171	544	48
39.1 ± 8.9	39.9 ± 9.1	39 ± 9	39.8 ± 8.6	39.3 ± 9	38.3 ± 7.1
48.4	41.6	48.5	44.7	47.9	41.7
2.4 ± 0.4	2.4 ± 0.3	2.4 ± 0.4	2.4 ± 0.3	2.4 ± 0.4	2.3 ± 0.2
25.9 ± 4.9	26.6 ± 4.8	25.8 ± 4.8	26.5 ± 5.2	26 ± 5	26.1 ± 4
—	—	—	—	—	—
0.83 ± 0.09	0.84 ± 0.09	0.83 ± 0.09	0.84 ± 0.09	0.83 ± 0.09	0.85 ± 0.09
5.5 ± 0.4	5.5 ± 0.4	5.5 ± 0.4	5.5 ± 0.4	5.5 ± 0.4	5.4 ± 0.4
50 ± 14	50 ± 13	50 ± 14	49 ± 13	50 ± 14	49 ± 15
113 ± 32	120 ± 34	113 ± 32	115 ± 32	114 ± 33	112 ± 28
110.3	110.6	106.7	119.9*	109.2	124.9†
4.4	4.3	4.3	4.6	4.4	4.3
2.9	1.1	2.2	3.7	2.9	0
1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2
23.9	18.4	21.7	26.6	22.7	27.7
113 ± 12	115 ± 11§	114 ± 12	114 ± 12	114 ± 12	113 ± 11
79 ± 8	75 ± 6	79 ± 8	79 ± 8	79 ± 8	79 ± 7
13.4	19.3	14.1	14.1	14.9	6.4
4.5	4.6	3.6	6.6	4.7	2.1
3.2	1.1	2.1	4.7	3.1	0
1.3	1.4	1.3	1.4	1.3	1.6‖
19.1	20.6	18.6	21.3	19.3	19.5
8.4 ± 2.2	9 ± 4	8.5 ± 2.2	8.4 ± 3.3	8.5 ± 2.6	8 ± 2
10.8 ± 6.3	10.4 ± 7.2	10.8 ± 6.2	10.7 ± 6.6	10.6 ± 6.4	9.6 ± 4.8

(33). CVS progression was defined according to the method of Hokanson et al. (32) as an increase of 2.5 or greater in square root–transformed CVSs.

Genotyping. Genomic DNA was extracted from leukocytes by the salting out method. Polymorphism of AGT gene encoding amino acid M253T substitution, deletion/insertion polymorphism of ACE gene, and the 1166A/C polymorphism of AGTR1 gene were determined by amplification with biotinylated primers and hybridization to immobilized sequence-specific oligonucleotides (34).

Statistical analysis. AGT M/T, ACE I/D, and ATR1 A/C genotype frequencies were estimated by gene counting, and the differences between the studied groups were evaluated by Pearson's χ^2 test or Fisher's exact test when appropriate. Hardy-Weinberg equilibrium expectations were tested using a χ^2 goodness-of-fit test.

Variables were examined for a linear relationship with progression of coronary calcium and were categorized if necessary. Demographic and other variables of interest were evaluated in a univariate logistic regression model to determine their relationship to progression of coronary calcium. For continuous variables, differences between groups were evaluated with the Mann-Whitney *U* test.

Multiple logistic regression models were used to assess the relationship between the progression of CVS and AGT, ACE, and ATR1 gene polymorphisms. Maximum likelihood estimates of the odds of having coronary calcium in relation to AGT M/T, ACE I/D, and ATR1 A/C genotype combinations were obtained from models with and without cardiovascular risk factors (Table 4).

For all comparisons, statistical significance was defined as $P < 0.05$. P corrected ($P_c = P * n$) values for multiple comparisons (n) were calculated when appropriate. All analyses were carried out in SAS 9.0 (SAS Institute).

RESULTS

Genotype frequencies. The frequencies of polymorphic variants of AGT, ACE, and ATR1 genes did not differ significantly between 585 non-Hispanic white patients with type 1 diabetes and 592 non-Hispanic white control subjects included in the analyses, respectively (AGT MM/MT/TT, 34.2/47.5/18.3% vs. 37.2/47.8/15%, $P = 0.27$; ACE II/ID/DD, 20/53/27% vs. 21.1/50/28.9%, $P = 0.59$; and AT1R AA/AC/CC, 44.6/45.5/9.9% vs. 51.5/40.4/8.1%, $P = 0.056$). The genotypes were distributed according to the Hardy-Weinberg equilibrium in the studied populations.

Baseline patient characteristics in relation to the AGT, ACE, and ATR1 genotypes. The baseline clinical characteristics of patients with type 1 diabetes and nondiabetic control subjects included in the analyses are presented in Table 1 by AGT, ACE, and ATR1 genotype status. The AGT TT genotype was associated with higher average systolic and diastolic (in type 1 diabetic patients only) blood pressure, compared with the MM or MT genotypes. Only in type 1 diabetic subjects, the ATR1 CC genotype was associated with lower systolic and diastolic blood pressure than the AA and AC genotypes (Table 1). However, these and other minor differences in lipids and CRP levels were not statistically significant, when corrected for multiple comparisons.

Association between AGT, ACE, and ATR1 genotypes and prevalence of CAC at baseline. At the baseline examination, the prevalence of positive CAC scores was higher in subjects with type 1 diabetes, compared with control subjects (37.3 vs. 27.2%, $P = 0.0002$). At the baseline, 247 (42.3%) of type 1 diabetic patients had hypertension, 113 (19.3%) had micro- or macroalbuminuria, and 270 (46.2%) had either. Among the nondiabetic control subjects, the rates were, respectively, 85 (14.3%), 16 (2.7%), and 96 (16.2%). Among type 1 diabetic patients, prevalence of coronary calcification was higher among hypertensive versus normotensive subjects (50.6 vs. 27.6%, $P < 0.0001$) and in those with albuminuria compared with patients free of albuminuria (57.5 vs. 34.5%, $P < 0.0001$). Given the facts that genetic variants of RAS have been associated with diabetic nephropathy/albuminuria (35–38) and/or hypertension (13,16,28) and that both nephropathy

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TABLE 2
The frequencies of CAC at baseline visit in relation to AGT, ACE, and ATR1 genotypes by albuminuria/hypertension status and presence of ACEI/ARB treatment in subjects with type 1 diabetes* (n = 550) and control subjects (n = 592)

	AGT				ACE				AT1R			
	MM	MT	TT	P	II	ID	DD	P	AA	AC	CC	P
Type 1 diabetes without albuminuria and hypertension at baseline visit												
Baseline (n)	93	137	50		56	153	71		126	129	25	
CAC frequency (%)	33.3	21.2	36	0.048†	26.8	30.7	22.5	0.44	27	29.5	24	0.82
Type 1 diabetes with albuminuria and/or hypertension at baseline visit												
Baseline (n)	89	125	56		54	142	74		120	123	27	
CAC frequency (%)	47.2	53.6	50	0.65	53.7	50	50	0.88	47.5	54.5	48.2	0.53
Controls												
Baseline (n)	220	283	89		125	296	171		305	239	48	
CAC frequency (%)	31.4	25.8	21.4	0.15	26.4	26	29.8	0.66	24.9	29.7	29.2	0.44

*Five type 1 diabetic subjects were excluded from this analysis because of unavailable albuminuria data at baseline visit. †$P_c > 0.05$.

and hypertension are the key risk factors for CAD and CAC development, type 1 diabetic patients were stratified by hypertension/albuminuria status to reveal a potential confounder effect of hypertension/albuminuria on RAS gene association with CAC progression (Table 2).

The prevalence of CAC was higher in subjects carrying the AGT 235TT genotype, compared with those with MT or MM genotypes among subjects without albuminuria and/or hypertension (respectively, 36 vs. 21.2 vs. 33.3%, P = 0.048) but not among type 1 diabetic subjects with albuminuria and/or hypertension at the baseline visit (50 vs. 53.6 vs. 47.2%, P = 0.65) or in healthy control subjects (21.4 vs. 25.8 vs. 31.4%, P = 0.15).

In both populations, subjects with type 1 diabetes and control subjects, there were no significant differences in the frequency of baseline CAC with regard to the studied genotypes of ACE and ATR1 genes.

AGT, ACE, and ATR1 genotypes as predictors of CAC progression during prospective follow-up. Data concerning CAC progression were available in 490 subjects with type 1 diabetes and 506 control subjects, who completed both the baseline and the follow-up examinations (Supplemental Fig. A, online appendix).

CAC progression was significantly higher in carriers of the TT genotype, compared with those having MT or MM genotypes among patients without albuminuria/hypertension (28.2 vs. 7.1 vs. 6.7%, P = 0.001) and with albuminuria or hypertension and not treated with ACE inhibitor (ACEI)/angiotensin receptor blocker (ARB) during the follow up (57.1 vs. 6.3 vs. 25%, P = 0.028) (Table 3). In contrast, no effect of the studied AGT gene variants on CAC progression was observed in type 1 diabetic subjects on ACEI/ARB treatment during the follow up (32.4 vs. 38.1 vs. 40.3%, P = 0.72).

There was no statistically significant association of any particular ACE or ATR1 genotype with CAC progression in subjects with type 1 diabetes and control subjects (Table 2). However, when the combinations of AGT-ACE, AGT-ATR1, and AGT-ACE-ATR1 genotypes were analyzed, some genotype combinations were shown to have an additive effect on CAC risk progression in comparison with the effect of TT genotypes only (Table 4).

In type 1 diabetic subjects without albuminuria and without hypertension, the frequency of CAC progression was significantly higher among carriers of AGT(TT)-ATR1(AC) (40 vs. 8.9%, P = 0.0003), AGT(TT)-ATR1(AA) (27.8 vs. 9.6%, P = 0.02), and AGT(TT)-ACE(ID) (50 vs. 7.2%, $P < 0.0001$) combinations, when compared with subjects with the remaining genotype combinations. When the combinations of three studied genes were constructed, the highest frequency of CAC progression was observed in type 1 diabetic subjects carrying AGT(TT)-ACE(ID)-ATR1(AA/AC) in comparison with those with other genetic variants among type 1 diabetic subjects without albuminuria and without hypertension at second visit (56.3 vs. 7.1%, $P < 0.0001$).

A similar tendency for higher CAC progression was observed in carriers of AGT(TT)-ATR1(AA), AGT(TT)-ATR1(AC), and AGT(TT)-ACE(ID)-ATR1(AA/AC) combinations among type 1 diabetic subjects with albuminuria and/or hypertension at second visit but not treated with ACEI/ARB during the follow-up; however, because of a small number of subjects, these differences (respectively, 66.7 vs. 19.4%, P = 0.13; 100 vs. 18.9%, P = 0.048; and 66.7 vs. 19.4%, P = 0.13) were not statistically significant or lost significance after correction for multiple comparisons (Table 4).

Most interestingly, the effect of AGT(TT)-ACE(ID)-ATR1(AA/AC) combination on CAC progression was statistically significant in type 1 diabetic subjects with mean A1C ≥7% (53.1 vs. 23.4%, P = 0.0003) but not in those with A1C <7% (28.6 vs. 20.2%, P = 0.63).

Presence of particular AGT-ACE, AGT-ATR1, or AGT/ACE/ATR1 genotype combinations did not influence the risk of CAC progression either in type 1 diabetic subjects treated with ACEI/ARB (Table 4) or in the control group (data not shown).

Logistic regression analysis of CAC progression. In logistic regression analysis, CAC progression was predicted by type 1 diabetes (odds ratio [OR] 2.3, P = 0.065), AGT (TT) genotype (OR 2.1, P = 0.0008), ATR1 (AA+AC) genotype (OR 3.5, P = 0.007), interaction between 235TT AGT genotype and albuminuria status ($OR_{TT\_AGT* \text{ albuminuria}}$ 0.05, P = 0.003), presence of CAC at the first visit (OR 3.9, $P < 0.0001$), and age (OR 2.5/10 years, $P < 0.0001$) in a model adjusted for ACE (ID) genotype, sex, BMI, waist-to-

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TABLE 3
The prevalence of CAC progression over a 2.5-year follow-up in relationship to the AGT, ACE, and ATR1 genotypes by diabetes status and presence of ACE inhibition or angiotensin receptor blocking treatment

	AGT				ACE				AT1R			
	MM	MT	TT	*P*	II	ID	DD	*P*	AA	AC	CC	*P*
Type 1 diabetes without albuminuria and/or hypertension at second visit												
n	60	85	39		42	93	49		87	79	18	
CAC progression (%)	6.7	7.1	28.2	0.001	11.9	14	6.1	0.37	10.3	13.9	5.6	0.55
Type 1 diabetes with albuminuria and/or hypertension at second visit without ACEI/ARB treatment												
n	16	16	7		4	24	11		16	17	6	
CAC progression (%)	25	6.3	57.2	0.028*	50	16.7	27.3	0.32	31.3	23.5	0	0.30
Type 1 diabetes with albuminuria and/or hypertension at second visit on ACEI/ARB treatment												
n	77	113	37		46	120	61		91	114	22	
CAC progression (%)	40.3	38.1	32.4	0.72	39.1	40.8	31.2	0.44	40.1	50.2	9.7	0.55
Controls												
n	173	202	77		96	227	129		231	180	41	
CAC progression (%)	15	11.9	10.4	0.52	15.6	12.3	11.6	0.64	12.1	16.1	2.4	0.055

*$P_c > 0.05$.

hip ratio, estimated glucose disposal rate, mean levels of A1C, HDL, LDL, triglycerides, fibrinogen, PAI-1, adiponectin, CRP, homocysteine, apolipoprotein B, creatinine, smoking, mean systolic/diastolic blood pressure, ACEI/ARB treatment, treatment with statins, and duration of follow-up.

After stratification by diabetes status, AGT(TT) genotype (OR 6.5, $P = 0.0005$), ATR1 (AA+AC) genotype (OR 3.7, $P = 0.031$), presence of CAC at the first visit (OR 3.3, $P = 0.001$), age (OR 1.94/10 years, $P = 0.008$), diabetes duration (OR 1.94/10 years, $P = 0.0016$), and the interaction between AGT (TT) genotype and ACEI/ARB treatment status ($OR_{TT\_AGT^* \ ACEI/ARB}$ 0.14, $P = 0.007$) significantly predicted CAC progression in subjects with type 1 diabetes.

When the interaction between AGT(TT) and ACE(ID) genotypes had been added to the last model, this interaction was found to have a significant effect on CAC progression ($OR_{AGT(TT)^* \ ACE(ID)}$ 3.6, $P = 0.037$) with the suggestive deviance of the last two studied models ($\chi^2 = 4.094$, $P = 0.0430$). Unfortunately, we were unable to perform a test of the two-way interaction between AGT(TT) and ATR1(AA/AC) and a test of three-way interaction because of complete separation of the data.

The risk of CAC progression in nondiabetic control subjects was associated with the presence of baseline CAC (OR 4.2, $P = 0.0006$), age (OR 2.5/10 years, $P = 0.0027$), BMI (OR 4.7, $P = 0.0021$), and fibrinogen (OR 1.7, $P = 0.013$). In contrast to type 1 diabetic subjects, in nondiabetic control subjects AGT, ACE, and ATR genetic variants were not significant predictors of CAC progression.

Because significant interactions between an ACEI/ARB treatment status and 235TT AGT genotype were observed, further stratification according to ACEI/ARB treatment status was performed in type 1 diabetic patients (Table 5).

Type 1 diabetic patients not treated with ACEI/ARB carrying TT AGT genotype had an approximately fivefold greater risk of CAC progression compared with those not carrying this genotype (Table 5). This relationship was twofold greater after adjustment for age, sex, BMI, waist-to-hip ratio, diabetes duration, duration of follow-up, smoking, treatment with statins, baseline CAC, LDL, HDL, triglycerides, serum creatinine, fibrinogen, PAI-1, homocysteine, and adiponectin (model 2) (OR 10.7, $P = 0.0008$). The adjustment for the presence of albuminuria (model 3) had only slight influence on this relationship (OR 13, $P = 0.004$). In contrast, adjustment for mean A1C levels (model 4) significantly increased the strength of this association (OR 21.8, $P = 0.003$) (Table 5).

Further analysis confirmed that genetic variants of ACE and ATR1 genes have an additional influence on the strength of the association between the TT AGT genotype and the risk of CAC progression (Table 5). Among type 1 diabetic patients not treated with ACEI/ARB, CAC progression was dramatically more likely in subjects carrying the AGT(TT)-ACE(ID)-ATR1(AA/AC) genotype combination than in those with other genotypes (OR 37.5, $P = 0.002$, model adjusted for other CAD risk factors) (Table 5).

In contrast, the presence of any particular studied genotype or genotype combinations had no significant effect on the risk of CAC progression in type 1 diabetic subjects treated with ACEI/ARB during the follow-up (Table 5).

DISCUSSION

To our knowledge, this is the first study in subjects with type 1 diabetes that has shown a significant association between AGT TT genotype and progression of CAC, a marker of subclinical coronary atherosclerosis. The risk of CAC progression was significantly higher for the combina-

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TABLE 4
The frequencies of CAC progression in type 1 diabetes carriers of the AGT-ACE, AGT-ATR1, or AGT-ACE-ATR1 genotype combinations versus remaining genotype combinations by albuminuria and/or hypertension status and ACEI/ARB treatment during the follow-up

	Type 1 diabetes without albuminuria and/or hypertension at second visit (n = 184)		Type 1 diabetes with albuminuria and/or hypertension at second visit without ACEI/ARB during the follow-up (n = 39)		Type 1 diabetes with albuminuria or hypertension at second visit on ACEI/ARB during the follow-up (n = 227)	
Genotype combinations	CAC progression in carriers of studied versus remaining genotype combinations	P	CAC progression in carriers of studied versus remaining genotype combinations	P	CAC progression in carriers of studied versus remaining genotype combinations	P
AGT-AGTR1						
TT-AA	27.8 (5 of 18) vs. 9.6 (16 of 166)	0.02*	66.7 (2 of 3) vs. 19.4 (7 of 36)	0.13	38.9 (7 of 18) vs. 37.8 (79 of 209)	0.92
TT-AC	40 (6 of 15) vs. 8.9 (15 of 169)	0.0003	100 (2 of 2) vs. 18.9 (7 of 37)	0.048*	29.4 (5 of 17) vs. 38.6 (81 of 210)	0.45
TT-CC	0 (0 of 6) vs. 11.8 (21 of 178)	1	0 (0 of 2) vs. 24.3 (9 of 37)	1	0 (0 of 2) vs. 38.2 (86 of 225)	0.53
MT-AA	7.5 (3 of 40) vs. 12.5 (18 of 144)	0.57	0 (0 of 4) vs. 25.7 (9 of 35)	0.56	31 (13 of 42) vs. 39.5 (73 of 185)	0.30
MT-AC	5.1 (2 of 39) vs. 13.1 (19 of 145)	0.26	11.1 (1 of 9) vs. 26.7 (8 of 30)	0.65	46.4 (26 of 56) vs. 35.1 (60 of 171)	0.13
MT-CC	16.7 (1 of 6) vs. 11.2 (20 of 178)	0.52	0 (0 of 3) vs. 25 (9 of 36)	1	26.7 (4 of 15) vs. 38.7 (82 of 212)	0.35
MM-AA	3.5 (1 of 29) vs. 12.9 (20 of 155)	0.21	33.3 (3 of 9) vs. 20 (6 of 30)	0.41	48.4 (15 of 31) vs. 36.2 (71 of 196)	0.19
MM-AC	12 (3 of 25) vs. 11.3 (18 of 159)	1	16.7 (1 of 6) vs. 24.2 (8 of 33)	1	34.2 (14 of 41) vs. 38.7 (72 of 186)	0.59
MM-CC	0 (0 of 6) vs. 11.8 (21 of 178)	1	0 (0 of 1) vs. 23.7 (9 of 38)	1	40 (2 of 5) vs. 37.8 (84 of 222)	1
AGT-ACE						
TT-II	8.3 (1 of 12) vs. 11.6 (20 of 172)	1	0 (0 of 0) vs. 23.1 (9 of 39)	—	40 (4 of 10) vs. 37.8 (82 of 217)	1
TT-ID	50 (9 of 18) vs. 7.2 (12 of 166)	<0.0001	40 (2 of 5) vs. 20.6 (7 of 34)	0.34	38.9 (7 of 18) vs. 37.8 (79 of 209)	0.92
TT-DD	11.1 (1 of 9) vs. 11.4 (20 of 175)	1	100 (2 of 2) vs. 18.9 (7 of 37)	0.048*	11.1 (1 of 9) vs. 39 (85 of 218)	0.16
MT-II	22.2 (4 of 18) vs. 10.2 (17 of 166)	0.13	0 (0 of 1) vs. 23.7 (9 of 38)	0.58	36.4 (8 of 22) vs. 38.1 (78 of 205)	0.88
MT-ID	4.9 (2 of 41) vs. 13.3 (19 of 143)	0.17	0 (0 of 11) vs. 32.1 (9 of 28)	0.04	42.4 (25 of 59) vs. 36.3 (61 of 168)	0.41
MT-DD	0 (0 of 26) vs. 13.3 (21 of 158)	0.048*	25 (1 of 4) vs. 22.9 (8 of 35)	1	31.3 (10 of 32) vs. 39 (76 of 195)	0.40
MM-II	0 (0 of 12) vs. 12.2 (21 of 172)	0.37	66.7 (2 of 3) vs. 19.4 (7 of 36)	0.06	42.9 (6 of 14) vs. 37.6 (80 of 213)	0.78
MM-ID	6.9 (2 of 34) vs. 12.7 (19 of 158)	0.38	25 (2 of 8) vs. 22.6 (7 of 31)	1	39.5 (17 of 43) vs. 37.5 (69 of 184)	0.80
MM-DD	14.3 (2 of 14) vs. 11.2 (19 of 170)	0.66	0 (0 of 5) vs. 26.5 (9 of 34)	0.32	40 (8 of 20) vs. 37.7 (78 of 207)	0.83
AGT-ACE-AGTR						
TT-ID-(AA or AC)	56.3 (9 of 16) vs. 7.1 (12 of 168)	<0.0001	66.7 (2 of 3) vs. 19.4 (7 of 36)	0.13	41.2 (7 of 17) vs. 37.6 (79 of 210)	0.77

Data are %. *$P_c > 0.05$.

tion of AGT (TT) and ACE (ID) and/or ATR1 (AA or AC) genotypes, which suggests the additive role of other genes (ACE and ATR1) coding the proteins of the RAS pathway in the process of coronary atherosclerosis in type 1 diabetic subjects. Most interestingly, the effect of the studied genetic variants on CAC progression was observed only in type 1 diabetic subjects with poor metabolic control (A1C ≥7%) and not treated with ACEI/ARB but not among patients with mean A1C <7% and/or on ACEI/ARB treatment. No significant association between the risk of progression of subclinical atherosclerosis and the studied SNPs of RAS genes has been found in nondiabetic control subjects.

The role of genetic factors associated with premature coronary atherosclerosis in subjects with type 1 diabetes has not been intensively studied yet. To our knowledge, there is only one published study that analyzed the same genetic variants of RAS as evaluated in the present study (39). Apparently contrary to the present observations, no associations of AGT 235M/T polymorphism or interactions

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TABLE 5
Multivariate logistic regression analysis of CAC progression in type 1 diabetic subjects with the studied AGT/ACE/ATR1 genotype combination versus remaining genotype combinations by ACEI/ARB treatment during the follow-up

Genotype combinations	Model 1 (not adjusted) OR (95% CI)	*P*	Model 2 (adjusted for CAD risk factors)* OR (95% CI)	*P*	Model 3 (model 2 adjusted for albuminuria status) OR (95% CI)	*P*	Model 4 (model 3 adjusted for mean A1C) OR (95% CI)	*P*
Type 1 diabetes without ACEI/ARB treatment during follow-up								
AGT (TT)	5.1 (2.4–10.8)	<0.0001	10.7 (2.7–42.4)	0.0008	13 (2.3–73.5)	0.004	21.8 (3–161)	0.003
AGT (TT)-ACE (ID)	8.1 (3.3–19.6)	<0.0001	15.4 (3.5–68.6)	0.0003	16 (2.1–124)	0.008	36.9 (3.6–380)	0.002
AGT (TT)-ATR1 (AA/AC)	6.9 (3.2–15.2)	<0.0001	12.1 (3–49.8)	0.0005	13.9 (2.5–78.4)	0.003	21.9 (3–159)	0.002
AGT (TT)-ACE (ID)-ATR1 (AA/AC)	11.6 (4.5–29.6)	<0.0001	17 (3.7–76.9)	0.0002	17.2 (2.2–136)	0.007	37.5 (3.6–388)	0.002
Type 1 diabetes on ACEI/ARB during follow-up								
AGT (TT)	0.8 (0.4–1.6)	0.56	0.8 (0.3–2.1)	0.68	0.9 (0.3–2.5)	0.83	1.1 (0.4–3.1)	0.92
AGT (TT)-ACE (ID)	1 (0.4–2.8)	0.93	1.3 (0.4–4.5)	0.72	1.1 (0.3–4.3)	0.92	0.9 (0.3–4.6)	0.90
AGT (TT)-ATR1 (AA/AC)	0.8 (0.4–1.8)	0.63	1 (0.4–2.7)	0.94	1.2 (0.4–3.3)	0.77	1.4 (0.5–4.3)	0.53
AGT (TT)-ACE (ID)-ATR1 (AA/AC)	1.2 (0.4–3.2)	0.77	1.6 (0.4–5.9)	0.46	1.4 (0.3–6)	0.62	1.5 (0.3–6.6)	0.58

*Adjusted for age, sex, BMI, waist-to-hip ratio, mean systolic blood pressure, mean diastolic blood pressure, diabetes duration, duration of follow-up, smoking, treatment with statins, baseline CAC, LDL, HDL, triglycerides, serum creatinine, fibrinogen, PAI-1, apolipoprotein B, homocysteine, and adiponectin.

between AGT 235M/T, I/D ACE, or/and 1166A/C polymorphisms with regard to cardiovascular disease were found by van Ittersum et al. (39) in the cross-sectional Dutch study comprising 257 subjects with type 1 diabetes. Surprisingly, in this study, the use of ACEIs was not included as a covariate in logistic regression analysis of CAD risk (39). Because in our study, the effect of RAS gene variants is highly related to ACEI/ARB treatment, we cannot be sure that the results of two studies are different. Moreover, because the type 1 diabetic population from the study of van Ittersum et al. had significantly shorter disease duration (17.1 ± 11.4 years) in comparison with our cohort (23.5 ± 9 years at baseline) and had a limited number of cardiovascular events (38 of 257), it is also possible that their study did not have sufficient power to detect differences in CAD risk between the carriers of different RAS genotypes or genotypes combinations.

In fact, there is increasing evidence that the T235 variant is associated with myocardial infarction and other forms of clinically overt coronary heart disease in the general population (17,40). Recently, the RAS has been thought to play a critical role also in the development of CACs (41). Angiotensin II activates transcription of "calcification-related" genes: parathyroid hormone receptor, bone morphogenic protein 2, and bone-liver-kidney-alkaline phosphatase genes in the coronary artery vascular smooth muscle cells (41). These effects seem to be parallel to RAS influence on the other mechanisms of coronary atherosclerosis development, including vascular smooth muscle proliferation, intimal fibrosis, chemoattraction of inflammatory cells, increase of plasminogen activator inhibitor-1 expression, and lipid accumulation/atherogenecity (42–44).

In the present study, we did not find a separate association of ACE genotypes with coronary atherosclerosis development in type 1 diabetic subjects or the control group, which is in line with most large studies and meta-analysis in nondiabetic populations (26,45). Our data are in line with the results of a Danish study, reported by Tarnow et al. (46), which have shown a lack of association of ACE genotypes with the risk of CAD development among type 1 diabetic patients without nephropathy.

Because only 41 type 1 diabetic subjects in our study had macroalbuminuria and/or reduced renal function (increased creatinine levels), we were not able to confirm results of two previous studies among type 1 diabetic subjects with diabetic nephropathy: the GENEDIAB Study and the previously mentioned study of Tarnow et al. (46), showing that individuals carrying ACE ID or DD genotypes have significantly higher frequencies of myocardial infarction and other forms of coronary heart disease in comparison with those with the II genotype (respectively, 10.6 vs. 6.6 vs. 1.1% and 19 vs. 24 vs. 7.5%) (46,47). Because type 1 diabetic subjects with diabetic nephropathy have significantly higher risk of CAD and there is a growing evidence that I/D ACE polymorphism is associated with the presence and severity of diabetic nephropathy (35,36), the coexistence of myocardial infarction and diabetic nephropathy can be a source of confusion for assessing the role of ACE gene polymorphism in susceptibility to CAD in subjects with type 1 diabetes (47).

In the present study, we have found a significant interaction between the AGT M235T polymorphism and ACE ID genotype with regard to CAC progression risk. This additive effect of ACE ID heterozygote (but not ACE DD homozygote) seems rather surprising, and we cannot exclude possibilities of by chance association. Interestingly, the same interaction between AGT TT and ACE ID genotypes has been previously observed among nondiabetic patients with clinically diagnosed CAD (37). These findings are in line with the growing evidence that phenotypic effects of one locus could be altered or masked by effects of genetic variants of other loci that encode proteins involved in the same biochemical pathway (16,48). This epistatic effect appears to be one of the reasons that results of studies evaluating the associations of single SNPs with complex diseases (like CAD) are not consistent (48).

Our present data confirm that the effect of genetic variants on clinical phenotypes is dependent on gene-

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environment (gene-metabolic) interactions. The risk of subclinical CAD progression associated with RAS genetic variants was related to the presence of an unfavorable diabetic milieu (hyperglycemia). The effect of the AGT(TT) genotype or AGT(TT)-ACE(ID)-ATR1(AA/AC) combination on CAC progression was statistically significant in type 1 diabetic subjects with mean A1C $\geq$7% but not in those with A1C $<$7%.

The effect of hyperglycemia on the activation of circulating and tissue-specific RAS is well documented and could be the key mechanism of increased risk of early atherosclerosis development in subjects with diabetes (7,8). Moreover, because the magnitude of the RAS response to hyperglycemia is variable among type 1 diabetic subjects (7,8), one can hypothesize that this variability is related to the genetic variants coding the proteins of the RAS pathway, but this hypothesis needs to be confirmed in future studies.

The observed tendencies for the association of the TT AGT genotype with higher blood pressure and CC ATR1 genotype with lower blood pressure confirm the results of some previous studies but not all (20,21,28). We do not believe that the presently reported small difference in the mean systolic or diastolic blood pressure might be responsible for the alteration of the risk of subclinical CAD in our study. However, because the direction of the blood pressure changes are in line with the effect on CAC progression, it seems to support the functional role of studied RAS genetic variants.

It is highly probable that the common mechanism of accelerated progression of subclinical CAD in type 1 diabetic patients is associated with pathologically increased RAS activity, which seems to be particularly high in subjects with the studied variants of the RAS genes, who were exposed to prolonged hyperglycemia and were not treated with ACEI/ARB.

In the present study, we did not measure the activity of ACE and protein plasma levels of other components of the RAS, because it is broadly accepted that activity of the renin-angiotensin tissue system rather then systemic effects are responsible for the structural and functional perturbations that occur in CAD (42,43).

We believe that our present study has advantages over the previous studies, which evaluated the role of RAS genes in CAD development in type 1 diabetes in terms of the higher number of studied subjects, the presence of nondiabetic control group, the prospective design of the study, the well-defined measurable marker of subclinical CAC, and the multivariate analysis, including other CAD risk factors. It is probable that the inconsistencies with previous studies can be explained by lack of analysis of metabolic/treatment-gene and gene-gene interactions.

In summary, the present study shows the evidence that the RAS genetic markers are very strong predictors of rapid progression of subclinical coronary atherosclerosis in type 1 diabetic subjects. Importantly, the results of our study suggest that in type 1 diabetic subjects with RAS genetic predisposition, the rapid atherosclerosis progression could be diminished by ACEI/ARB treatment and good metabolic control.

We believe that further confirmation of the association of RAS gene polymorphisms with early progression of subclinical coronary atherosclerosis in type 1 diabetes would provide the rationale for clinical trials to investigate the use of ACEI or angiotensin II receptor antagonist in the early prevention in type 1 diabetic subjects who are at high risk of subclinical atherosclerosis progression based on RAS genetic markers.

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