Synergistic Interaction of Angiotensinogen and Angiotensin-converting Enzyme Genes in Predicting Coronary Arteriosclerosis

Short title: Synergistic Effect of AGT and ACE Genes on CAD

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Abstract

Angiotensinogen (AGT) and angiotensin-converting enzyme (ACE) gene polymorphisms have been implicated in the pathogenesis of coronary artery disease (CAD) and myocardial infarction (MI). However, conclusions are inconsistent and there are few combination analysis of the AGT and ACE genes in relation to CAD and MI. We therefore study 255 Taiwanese people, including 171 CAD patients and 84 non-CAD controls. A novel stepdown polymerase chain reaction (PCR) was used to genotype the ACE insertion/deletion(I/D) polymorphism and a stepdown PCR-direct sequencing to genotype the M235T and T174M variants of the AGT gene. The results showed that the 235T allele and TT genotype frequencies of the AGT gene were 82% and 65% respectively in the non-CAD group vs. 83% and 68% in the CAD group ($p>0.05$). The 174T allele and TT genotype frequencies were 92% and 86% in the non-CAD group vs. 89% and 80% in the CAD group ($p>0.05$). The D allele and DD genotype frequencies of the ACE gene were 64% and 40% respectively in the non-CAD group vs. 69% and 46% in the CAD group ($p>0.05$). There was no difference of AGT or ACE gene polymorphism in subjects with MI, low risk, or different severities of CAD. However, subjects with double homozygosity of 235TT/DD genotype had a higher prevalence (75%) of CAD than those with 235TT/(ID+II) (52%) or (235MT+MM)/DD (56%). The odds ratio was 2.61 (95% CI 1.88 to 3.61). Subjects with double heterozygosity of (235MT+MM)/(ID+II) also had a higher prevalence (71%) of CAD. The odds ratio was 2.14 (95% CI 1.48 to 3.09). Our data show that no association between each single gene polymorphism of the AGT and ACE and coronary heart disease can be found in the Taiwanese

population. However, there is a synergistic gene effect that confers an increased risk for CAD.

Key words: Gene-gene interaction, Taiwanese, CAD, MI, Heterozygosity

The renin-angiotensin system (RAS) has recently been implicated in the pathogenesis and pathophysiology of coronary artery disease (CAD) and myocardial infarction (MI). Angiotensin II, the endproduct of this enzyme cascade, is a potent vasoconstrictor as well as a growth promoting factor for vascular smooth muscle cells.[1~3] The efficacy of angiotensin-converting enzyme (ACE) inhibition and angiotensin II receptor blockade addresses the important role of the RAS in the genesis of coronary arteriosclerosis and its related disorders.[4,5] Thus, looking beyond the classical risk factors, i.e., hypercholesterolemia, hypertension, diabetes, smoking, etc, many investigators have recently pursued the study of the genetic factors of RAS to predict the development of CAD and MI.

The first step of this enzyme cascade, which is rate-limiting, is the reaction of angiotensinogen (AGT) with renin. Molecular variant M235T of the AGT gene has been found to be capable of determining the plasma AGT level and subsequently the angiotensin II level.[6] Recently, this variant of the AGT gene has been found to be associated with CAD.[7,8] The second key enzyme in this cascade is the ACE. The insertion/deletion (I/D) polymorphism at intron 16 of the ACE gene determines plasma ACE activity,[9] which has been reported to be associated with CAD and MI.[10,11]

Despite some positive findings in studies of the association between the AGT or ACE gene and CAD, overwhelmingly negative results have recently appeared,[12~14] including some of our own observations. Previously, we could not find any association between ACE I/D polymorphism and CAD, MI, or essential hypertension in a Taiwanese population,[15,16] although we did find a positive association of AGT 235T with essential hypertension in the Taiwanese.[17] Allover, the role of the AGT or ACE gene alone in predicting the development of CAD appears to be a minor one.

Because CAD is a multifactorial disease with polygenetic traits involving both genetic and environmental factors, the gene-environment and gene-gene interactions must be clarified before fully understand the development of coronary arteriosclerosis.

Since there are few studies regarding the association of the AGT gene with CAD in the Taiwanese population, and few reports dealing with the combined effect of the AGT and ACE genes on CAD,[18~20] we conducted this single center, case-control study to address those question. Additionally, in this study, we used a more efficient genotyping strategy to type the AGT and ACE genes, which was different than that used in previous studies.

Methods

Subjects. From August 1996 to January 1997, 310 patients who were admitted to National Taiwan University Hospital for the evaluation of coronary or other various heart diseases were consecutively recruited for this study. Informed consent had been obtained for diagnostic procedures and the usage of genetic materials. Cardiac catheterization and coronary angiography were performed according to the standard procedures. Coronary angiograms were obtained from multiple projections and the degree of stenosis was quantitatively analyzed. A stenosis of >50% of the luminal diameter of any of the three major branches was defined as coronary artery disease. A stenosis of <10% was classified as a normal coronary artery (non-CAD). Myocardial infarction subjects were defined as those who presented typical clinical manifestations with a pathological Q wave on ECG or abnormal wall motion by left ventriculography. Low risk CAD subjects were defined as those with only one or none of the classical risk factors for CAD, i.e., hypertension, hypercholesterolemia, smoking, and diabetes. The extent or severity of CAD was defined by the number of diseased coronary arteries i.e. one-vessel disease (1VD), two-vessel disease (2VD) or three-vessel disease (3VD).

Demographic and clinical data were obtained from the patient's record curing admission. Patients who were current drinkers or smokers were considered as having a positive history of drinking or smoking. Since most of the patients had had their blood pressure, blood lipids, or blood glucose modified, a positive history of hypertension, diabetes or hypercholesterolemia was based on the treatment history, regardless of the actual value on admission. A positive family history was considered when one of the first-degree relatives had CAD or MI based on the history of coronary angiography, PTCA or death certificate.

Patient were excluded for criteria including failure of coronary angiography and inadequate blood sampling or DNA extraction. Finally, 171 CAD subjects and 84 age-adjusted non-CAD subjects were enrolled for the genotyping of both AGT and ACE genes.

AGT genotyping. This was carried out using a stepdown polymerase chain reaction (PCR) and direct sequencing according to our previous report.[17] Briefly, genomic DNA was extracted by a non-enzymatic method.[21] DNA fragments, including the M235T and T174M variants, were amplified by PCR. Forward and reverse primers were selected from the genomic sequence of AGT. The forward primer sequence form +921 to +940 in exon 2 of the AGT gene was 5'-GATGCGCACAAGGTCCTGTC-3'; the reverse primer from +1255 to +1274 was 5'-GCCAGCAGAGAGGTTTGCCT-3'. The PCR mixture consisted of 0.5 μg DNA, 25 pmoles of each primer, 0.15 nM dNTP and 1 U Tag polymerase in a final volume

of 50 μl. The PCR was carried out in a Perkin-Elmers thermal cycler (Model 480, Norwalk, CT, USA). The reaction condition was achieved by denaturing first for 3 min, and then repeating the following cycle: denaturing at 95 ℃ for 1 min, annealing at 70℃ for 45 sec, and extension at 72℃ for 1 min. This cycle was repeated five times, then the annealing temperature reduced to 65℃ for five cycles and then to 60℃ for 25 cycles with a final extension for 10 min. The 394 base-pairs PCR product was resolved on a 2% ethidium bromide-stained gel and purified by centrifugation through a paper slurry for sequencing.

DNA sequencing. Sequencing for molecular variants M235T and T174M was conducted using a dye-terminator cycle sequencing method from Applied Biosystem Division (ABD, Perkin-Elmers-Cetus, Foster city, CA, USA). Single-strand sequencing primer. The PCR reagents and cycling conditions were the same as described above, except with dye-labeled ddNTP replacing unlabeled ddNTP and using AmpliTag-FS enzyme. The product was run in an automatic sequencing apparatus (ABI 373A sequencer, Perkin-Elmers-Cetus) in a 6% denatured polyacrylamide gel at 1500 volts and 40℃. The results were analyzed using incorporated sequencing analysis software (Version 2.01, ABD, Perkin-Elmers-Cetus). The molecular variant M235T and T174M of the AGT gene was designated as the TT homozygoite (235T and 174T), MT heterozygoite (235TM and 174TM) and MM homozygoite (235M and 174M).

Genotyping of ACE. This was performed by using a similar stepdown PCR strategy which was developed in this laboratory. This typing method was proved to be more efficient and accurate than the conventional method,[22] and similar to the confirmatory method.[23] Briefly, the PCR condition was similar to that for the AGT genotyping, optimized at a magnesium concentration of 2 mM. The PCR primers were the same as those used by Rigat et al.[22] The salt concentration from 0.1 to 50 mM of NaCl did not affect the amplification efficiency. All the amplifications were carried out in the same thermal cycler (Model 480, Perkins-Elmer-Cetus, Foster City, CA, USA). The reaction condition was achieved by denaturing first for 5 min, and then repeating the following cycle: denaturing at 95℃ for 1 min, annealing at 70℃ for 1 min, and extension at 72℃ for 1 min. This cycle was repeated for five times, then the annealing temperature was reduced to 65℃ and 60℃ each for five cycles each and then to 60℃ for 25 cycles with a final extension for 10 min. Each sample was amplified in duplicate by the same person. The PCR products were resolved using 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The ACE genotypes were designated as DD homozygoite, ID heterozygoite and II homozygoite.

Statistics. Parametric data between groups were analyzed by unpaired t test

and ANOVA, while non-parametric data were analyzed by chi-square test, allele frequencies were counted from the genotypes. The odds ratios with 95% confidence interval (CI) were calculated as the relative risk of disease versus control by Woolf's method. A p value of <0.05 was considered statistically significant.

Results

Demographic data. The baseline characteristics of the CAD and non-CAD patients are shown in Tables 1 and 2. The CAD group had significantly more men, heavier weights, more smoking, higher blood pressures, more diabetes, and more hyperlipidemia than the control group. These findings represented the typical characteristics of CAD. Family history and history of myocardial infarction were more prevalent in the CAD group. As for different severities of CAD, there were more men, and greater incidence of hypertension and myocardial infarction in the groups with 2VD or 3VD.

Allele frequencies and genotype distribution of the AGT gene. The distribution of the AGT gene polymorphism in the CAD and non-CAD subjects are shown in Tables 3 and 4. The allele and genotype frequencies of the M235T and T174M were in Hardy-Weinberg equilibrium for the non-CAD and CAD groups. The 235T allele frequency was 82% in the non-CAD group, while it was 83% in the CAD group ($p>0.05$). The TT genotype was 65% in the non-CAD group vs. 68% in the CAD group ($p>0.05$). In subjects with MI, low risk, or different severities of CAD, the 235T or TT frequency did not differ. The 174T allele was 92% in the non-CAD group vs. 89% in the CAD group ($p>0.05$). The 174TT genotype was 86% in the non-CAD group vs. 80% in the CAD group ($p>0.05$). There was no difference in 174T or 174TT frequencies in subjects with MI, with low risk, or with different severities of CAD.

Allele frequencies and genotype distribution of the ACE gene. These are shown in Table 5. The allele frequencies were in Hardy-Weinberg equilibrium for both the CAD and non-CAD groups. The D allele frequency in the non-CAD group was 64%, while it was 69% in the CAD group ($p>0.05$). The DD genotype was 40% in the non-CAD group, while it was 46% in the CAD group ($p>0.05$). There was also no significant difference of D allele or DD genotype in subjects with MI, with low risk, or with different severities of CAD.

Combined effect of the AGT and ACE gene. The gene-gene interactions were evaluated by combining the AGT and ACE gene polymorphism for each subject, and the differences among the four combinations are shown in Table 6. Subjects with

double homozygosity of 235TT/DD genotype had a higher prevalence (75%) of CAD than subjects with 235TT/(ID+II) (52%) or (235MT+MM)/DD (56%). The odds ratio was 2.61 (95% CI 1.88 to 3.61). Interestingly, subjects with double heterozygosity of (235MT+MM)/(ID+II) also revealed a higher prevalence (71%) of CAD. The odds ratio was 2.14 (95% CI 1.48 to 3.09). Subjects with single homozygosity of 235TT of the AGT gene or single DD homozygosity of the ACE gene appeared to have protection from CAD.

Discussion

In this study, we used a stepdown PCR to genotype the ACE I/D polymorphism and PCR-direct sequencing for the M235T and T174M variant of the AGT gene. This PCR strategy was developed in our laboratory and it had been found to be more efficient than the conventional method reported by Rigat el al [22](?). The stepdown PCR rarely overamplifies D allele and thus, does not overestimate the frequency of DD genotype of the ACE gene. The efficiency of amplification by stepdown PCR is equivalent to the confirmatory PCR reported by Shangmugam.[23] The D allele frequency in the non-CAD group in this study was 64%, a much higher frequency compared to that in Caucasian populations. There was no association of ACE I/D polymorphism with CAD or MI in this Taiwanese population. Our results agreed with some investigations, especially the recent larger cohort studies,[13,14] although there has been controversy over whether or not there is a true association between ACE I/D polymorphism and coronary heart disease.

A molecular variant of the AGT gene has been found to be associated with essential hypertension [6,17,24,25]and most likely to be pathogenetically linked to hypertension.[26,27] Most of theses studies obtained positive results, especially with the M235T variant of the AGT gene. They utilized conventional PCR and restriction fragment length polymorphism or to identify the M235T or T174M variant. Those methods had inherent error in the enzyme cutting or oligonucleotide hybridization.[25] We developed an efficient stepdown PCR and direct mini-sequencing method to simultaneously detect these two variants of the AGT gene. By this method, the 235T allele and 174T allele were found in 82% and 92% of the non-CAD group respectively, which again were frequencies much higher than those found in the Caucasian population. These two polymorphisms of the AGT gene were not associated with coronary heart disease in the Taiwanese population. Our results disagreed with some of the previous investigations,[7,8] but were in agreement with others.[12]

The discrepancies regarding the association of AGT and ACE gene polymorphism with coronary heart disease cannot be well-explained. Several possible factors may contribute to the diversities, i.e., sample bias, different sample sizes, ethnic differences, and various methodologies for genotype screening. Coronary artery disease is a complex disorder with polygenetic traits. Several lipoprotein genes have been reported to be associated with hypertension and coronary heart disease.[28] The gene-gene and gene-environment interactions of these lipoprotein genes have been well-described.[28] However, most of the association studies between CAD and the renin-angiotensin system have only been at the level of single-gene effect. The effects of a combination of different genes in the renin-angiotensin system have been rarely reported.[18~20] Jeunemaitre et al could not find any synergistic effect of AGT and ACE gene polymorphism on the development of CAD, and concluded that the renin-angiotensin system might not play a significant role in the genesis of CAD.[18] Ludwig et al reported that an association of the combined AGT and ACE gene polymorphism with CAD could only be seen in a subgroup of patients with low risk.[19] Our data showed that subjects with combined double homozygosity of ACE DD and AGT 235TT polymorphism had a higher risk of CAD, although the single-gene effect of ACE or AGT was not significant. The odds ratio was 2.61 (95% CI 1.88 to 3.61) and the risk was independent of the classical risk factors.

Our hypothesis is that subjects with ACE DD polymorphism have higher plasma ACE levels and activity, and subjects with M235T variants had an elevated plasma AGT concentration. The combination of these two genotypes leads to more of the end product of the renin cascade, angiotensin II, which may then promote coronary arteriosclerosis. Although no single gene effect was observed in our data or those of other studies, combination of the two genes may enhance the effect on the development of CAD, as was demonstrated in this Taiwanese population. Kamitani et al also found a combination effect of the AGT and ACE genes on the development of MI.[20] Having only DD polymorphism of the ACE or 235TT genotype of the AGT gene may not have a strong enough effect on the product of the renin cascade to reveal a significant association with CAD.

In regards to the subjects with neither DD polymorphism of the ACE nor 235TT genotype of AGT, they still had a higher prevalence of CAD than . The reason for this result is not fully understood. Probably there are other atherogenic factors attributable to CAD in this patient group, for example, genes for hyperhomocysteinemia, high Lip(a) orApoE, etc. Nevertheless, our data show that loss of single homozygosity of the ACE/DD or AGT/235TT appears that ? have resulted in increased risk of CAD, irrespective of the classical risk factors.

There are several limitations to this study. First, the non-CAD subjects with

normal coronary angiography, were probably not truly normal ones, as they might have had other cardiovascular disorders. Second, many numerical clinical parameters, i.e., blood glucose, lipids, or blood pressure, had been modified by medications, so that the parameters were only expressed qualitatively by a positive history of treatment. This might not have accurately reflected degree of disrangement. Whether our observations are true or by chance is not yet known. Confirmatory studies, especially in a larger cohort, are needed.

In summary, we used a stepdown PCR and direct sequencing to genotype AGT and ACE gene polymorphism. No association between each single gene polymorphism and coronary heart disease could be found in this Taiwanese population. However, there was a combined synergistic gene effect, in which subjects with both or neither ACE DD polymorphism and 235TT genotype of the AGT gene were conferred an increased risk for CAD, this risk was independent of the classical risk factors.

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Table 1. Baseline characteristics of CAD and normal controls

	CAD	Normal	p
n	171	84	
Age(years)	62±11	59±10	0.093
Sex(male/female)	138/33	48/36	0.000
BMI(Kg/M^2)	25.4±3.5	24.3±3.5	0.041
Drinking(yes)	68(40)	20(24)	0.230
Smoking(yes)	88(51)	24(29)	0.000
Hypertension (yes)	114(67)	32(38)	0.000
Diabetes(yes)	49(29)	3(4)	0.000
Lipid (yes)	75(44)	13(15)	0.000
Family history(yes)	25(15)	0(0)	0.000
MI(yes)	67(39)	0(0)	0.000

BMI = body mass index; MI = myocardial infarction; ( ) indicates percentage

Table 2. Baseline data of CAD with different severity

	1VD	2VD	3VD	p
n	49	59	63	
Age(years)	61±11	62±11	63±1	0.721
Sex(male/female)	35/14	46/13	57/6	0.032
BMI(Kg/M^2)	25.0±2.7	25.2 ±4.2	25.8±3.5	0.539
Drinking(yes)	21(43)	22(37)	25(40)	0.841
Smoking(yes)	27(55)	28(47)	33(52)	0.719
Hypertension (yes)	23(47)	47(80)	44(70)	0.001
Diabetes(yes)	10(20)	18(31)	21(33)	0.219
Lipid(yes)	18(37)	29(49)	28(44)	0.430
Family history(yes)	7(14)	9(15)	9(14)	0.986
MI(yes)	9(18)	21(36)	37(59)	0.000

BMI = body mass index; MI = myocardial infarction.

Table 3. Allele frequencies and genotype distribution of AGT M235T

in CAD and normal control

	Genotypes			Alleles	
	235M	**M235T**	**235T**	**M**	**T**
CAD	4	51	116	59(17)	283(83)
normal	1	28	55	30(18)	138(82)
p		0.757			
with MI	1	18	45		
without MI	3	33	71		
p		0.796			
≦1 risk CAD	0	9	21		
＞1 risk CAD	4	42	95		
p		0.657			
1VD	0	15	34		
2VD	2	19	38		
3VD	2	17	44		
p		0.724			

Table 4. Allele frequencies and genotype distribution of AGT T174M

in CAD and normal control

	Genotypes			Alleles	
	174T	**T174M**	**174T**	**T**	**M**
CAD	3	31	137	37(11)	305(89)
normal	.2	10	72	14(8)	154(92)
p		0.431			
with MI	0	15	49		
without MI	3	16	88		
p		0.170			
≦1 risk CAD	0	4	26		
＞1 risk CAD	3	27	111		
p		0.523			
1VD	0	10	39		
2VD	2	7	50		
3VD	1	14	48		
p		0.399			

Table 5. Allele frequencies and genotype distribution of ACE in CAD and normal control

	Genotypes			Alleles	
	II	**ID**	**DD**	**I**	**D**
CAD	16	77	78	109(32)	233(69)
normal	10	40	34	60(36)	108(64)
p		0.440		0.298	
with MI	6	33	28		
without MI	5	44	50		
p		0.414			
≦1 risk CAD	5	24	24		
＞1 risk CAD	6	53	54		
p		0.608			
1VD	3	20	26		
2VD	7	30	22		
3VD	6	27	30		
p		0.525			

Table 6. Differences among the 4 combinations of AGT M235T

and ACE I/D polymorphism

	235TT/DD	235TT/ID+II	235MT+MM/DD	235MT+MM/ID+I	p
n	68	103	39	45	
Age(years)	61±10	59±10	64±9	63±11	0.027
Sex(M/F)	53/15	70/33	25/14	34/11	0.448
BMI(Kg/M^2)	24.7 ±3.3	25.1±3.4	24.8±3.6	25.1±3.5	0.868
Drinking(yes)	19(28)	34(33)	9(23)	14(31)	0.934
Smoking(yes)	35(51)	39(38)	14(36)	17(38)	0.336
HT(yes)	38(56)	51(50)	25(64)	24(53)	0.638
Diabetes(yes)	16(24)	13(13)	12(31)	9(20)	0.097
Lipid(yes)	20(29)	32(31)	14(36)	14(31)	1.000
CAD(yes)	51(75)	54(52	22(56)	32(71)	0.015
Fx	7(10)	14(14	1(3)	2(4)	0.174
MI	14(21)	24(23)	6(15)	13(29)	0.680

HT = hypertension history; Lipid = hyperlipidemia; Fx = family history of CAD; MI = myocardial infarction history; parenthesis indicates percentage