Short Report

Both alleles of the M235T polymorphism of the angiotensinogen gene can be a risk factor for myocardial infarction

Coronary artery disease (CAD) is a multifactorial condition influenced by environmental and genetic factors. Serum cholesterol (CH), smoking (SM), and hypertension (HT) have been established as major risk factors for myocardial infarction (MI) (1). Experimental and clinical data show that the renin-angiotensin system (RAS) plays an important role in cardiovascular physiology. It is known that angiotensin II may have direct toxic effects on myocardial cells, activates the sympathetic nervous system, stimulates fibroblast proliferation, and vasoconstricts coronary vessels (2–5). These findings have led investigations between the risk for CAD and the genetic variants of the RAS genes (6–8). Angiotensinogen (AGT) plasma levels, a key component of this system, correlate with blood pressure (BP) (3, 4). The human AGT gene has been cloned, sequenced, and mapped (9, 10). The T allele of a polymorphism, which encodes threonine instead of methionine at position 235 (M235T), is associated with increased circulating AGT concentrations (8). Recently, this polymorphism has also been found to be associated with an increased risk of essential hypertension (8, 11–13) and ischemic heart disease, and showed opposite results for both M and T alleles (14–16), although other authors have not detected this association (17–23).

There is evidence for a relationship between plasma renin activity (PRA) and the risk of major coronary events. In 1972, Brunner et al. suggested...
that high levels of PRA in hypertensive patients are associated with an increased risk of MI (24). Meade et al. in 1983 reported lower levels of PRA in patients with ischemic heart disease (25). Recently, Alderman et al. found that PRA levels are independent and directly associated with the incidence of MI among hypertensive subjects (26). However, no association between PRA and the incidence of ischemic heart disease among normotensive subjects has yet been reported (27).

The aim of this study was to detect the variations in the frequency of three polymorphisms of the RAS genes (M235T for AGT, I/D for ACE and A1166C for AT1R) between healthy controls and MI patients. We have also studied the role of each genotype as risk factors, comparing the genotype frequencies between MI subjects who presented with the three major risk factors (CH, SM, and HT) and MI subjects without these. The possible influence of these nine genotypes on PRA levels has also been analyzed.

Materials and methods

Subjects

A total of 392 men: 180 healthy > 35 years (56 ± 15), controls; and 212 (54 ± 13) with MI were selected to study the role of three polymorphic genes of the RAS. To determine the differences in PRA levels related to the different genotypes, 100 healthy subjects with a mean age of 22.2 ± 1.46 were also studied. These individuals were not used as controls to compare their genotype frequencies with MI groups. All the individuals were residents of Málaga (southern Spain); their parents and grandparents were Caucasian and born in this region (Andalucia). Healthy individuals were recruited using the Andalusian Health Service identity card. They had normal chest X-ray, electrocardiogram (ECG) and biochemical blood analysis (including cholesterol, triglycerides, and glucose) and did not have a history of CAD.

Subjects with a confirmed diagnosis of MI by specific abnormalities on the ECG and elevated enzyme levels were recruited from different Cardiology Units. To be classified as MI, one of the following had to be present: 1) Dynamic ECG changes typical of MI and lasting more than 24 h, with or without symptoms and/or cardiac enzyme changes, 2) typical (prolonged chest pain) or atypical symptoms (acute congestive heart failure, syncope) and serial cardiac enzyme elevation exceeding twice the upper limit of the reference range.

After the approval by the University Hospital Ethical Committee, all the subjects were contacted and informed by phone, and from those whose consent was obtained, 15 ml of blood was taken. A questionnaire about personal and familial history of cardiovascular diseases, SM habits, CH, and BP was completed. The examination included the measurement of systolic and diastolic BPs with the patient in the sitting position after 4 min rest. We considered an individual as hypertensive if he had systolic or diastolic BP values above 140 or 90 mmHg, respectively (28), after three measurements of BP in basal conditions according with the recommendations of the American Society of Hypertension (29). Total CH was analyzed by the CHOD/PAP method at the laboratory of the University Hospital. Individuals were considered as hypercholesterolemic if they had a previous history of hypercholesterolemia defined as values higher than 220 mg/dl.

The studied MI group consisted of 156 smokers with a mean (± SD) age of 49 ± 10, and 56 non-smokers with a mean age of 60 ± 14. One hundred and twelve subjects were normotensive, mean age 54 ± 13, and 100 were hypertensive, mean age 54 ± 12. One hundred and six subjects had normal cholesterol levels, mean age 57 ± 13, and 106 showed high levels, mean age 51 ± 11. Thirty-nine individuals had all three risk factors (MI subgroup with HT, CH, and SM), mean age of 50 ± 9, and 32 individuals without any risk factor (MI subgroup without HT, CH, and SM), mean age of 58 ± 13.

Identification of ACE genotypes

DNA samples were assayed for the I/D polymorphism using DNA amplification by PCR (30). DD homozygotes were re-amplified using specific primers in order to avoid misclassification of heterozygotes as homozygotes (31).

Identification of AGT genotypes

PCR was performed according to the method of Russ et al. (32).

Identification of AT1R genotypes

These genotypes were established by PCR using a nesting strategy. After optimizing the protocol, both PCR products were sequenced (16).

PRA

PRA levels were determined in 100 control individuals (22.2 ± 1.46). We selected a very homoge-
neous age group to avoid the known variation of the PRA levels in relation to age and were subjected to a moderate sodium intake around 170 mmol/day (serum NaCl: 140 ± 2.66 mmol/l and mean 24 h urinary Na+ excretion 112 ± 28 mmol).

The different genotype groups were selected and the predominance of any allele of the other two genotypes was avoided to eliminate associated effects. Therefore, we selected 9–13 subjects representing each polymorphism of the three genes. Whole blood was drawn into tubes containing citrate. The plasma was then separated and PRA levels determined by a “Clinical Assays Gamma-Coat Plasma Renin Activity 125RIA” kit (INCASTAR, Stillwater, MN, USA).

Statistical analysis

The chi-squared ($\chi^2$) test with Yates correction was used to assess genotype and allele frequency differences between controls and MI individuals. Fisher’s test was applied when $n$ was $<5$ in any of the cells of the $2 \times 2$ contingency tables. The odds ratios (OR) and 95% confident intervals (CI) were also calculated. Student’s t-test was used to compare the PRA level means of the different genotypes. In order to analyze the association of allele frequencies with each risk factor the logistic regression Forward Stepwise (Wald) was applied to all MI patients.

Results

Polymorphism of the AGT gene

The allele and genotype frequencies of controls and MI groups were compared. Statistically significant differences were: M allele (0.46 vs 0.56, $p < 0.01$), T allele (0.54 vs 0.44, $p < 0.01$), MM genotype (0.19 vs 0.28, $p < 0.05$), and TT genotype (0.27 vs 0.15, $p < 0.01$) (Table 1).

After exclusion of patients with any of the major coronary risk factors (MI subgroup without HT, CH, and SM, $n = 32$), the association of the MM genotype as well as the M allele with MI was even more striking. The MM genotype frequency was 0.50 ($p < 0.001$; OR 4.29; CI 1.95–9.42) and the M allele frequency was 0.69 ($p < 0.01$; OR 2.6; CI 1.46–4.61) (Table 1). It should be pointed out that although the frequency of the TT genotype changed importantly in the group of MI without risk factors (0.12 vs 0.27 in controls), it was not statistically significant because of the small number of individuals ($n = 32$) in this subgroup (OR 0.38; CI 0.11–1.28).

When MI subgroups with and without coronary risk factors were compared, it could be observed
that the T allele frequency increased significantly in the MI subgroup with HT, CH, and SM (0.58 vs 0.31) \( (p < 0.01; \ OR \ 3; \ CI \ 1.45–6.19) \). Furthermore, the TT genotype frequency also increased from 0.12 in the MI subgroup without any risk factor to 0.31 in MI with HT, CH, and SM \( (p = 0.059; \ OR \ 3.11) \). Conversely, the M allele frequency was higher in MI without any risk factor (0.69 vs 0.42) \( (p < 0.01; \ OR \ 3; \ CI \ 1.45–6.19) \) and the MM genotype frequency in MI with the three risk factors, increased from 0.15 to 0.50 in MI without any risk factor \( (p < 0.01; \ OR \ 5.5; \ CI \ 1.72–17.53) \).

Polymorphism of the ACE gene
In controls, genotype frequencies were 0.20, 0.46, and 0.34 for II, ID, and DD respectively, vs 0.16, 0.49, and 0.35 in MI group.

When the genotype frequencies of the ACE polymorphism were analysed in the MI subgroup without HT, CH, and SM (0.18, 0.51, 0.31) and the MI subgroup with HT, CH, and SM (0.21, 0.41, 0.38) for II, ID, and DD respectively, no significant differences were found.

Polymorphism of the AT\(_1\)R gene
No differences in allele frequency between controls and MI group were found. In controls, genotype frequencies were 0.50, 0.45, and 0.05 for aa, ac, and cc, respectively, vs 0.45, 0.42, and 0.13 in MI group. Only significant differences for the cc genotype \( (p < 0.05) \) were observed.

When genotype frequencies of the AT\(_1\)R polymorphism were analysed in MI subgroup without any risk factors (0.44, 0.45, and 0.11) and MI subgroup with three risk factors (0.48, 0.38, and 0.14) for aa, ac, and cc genotypes respectively, no significant differences were found.

PRA levels
Table 2 shows the mean values for PRA levels according to ACE, AT\(_1\)R, and AGT genotypes. No significant differences between the AT\(_1\)R and ACE genotypes were found.

In contrast, PRA levels were significantly different according to the AGT polymorphism. Whereas MM subjects showed a mean value of 2.68 ng/ml/h, the TT genotype was 1.47 ng/ml/h \( (p < 0.001) \). The maximum and minimum values were 4.39 and 2.33 ng/ml/h for MM subjects, and 1.61 and 0.78 ng/ml/h for TT subjects.

Discussion
The T allele of the M235T polymorphism of the AGT gene has been functionally related to increased AGT plasma levels \( (8) \). Although some authors have found the T allele to be a risk factor for MI \( (14, 15) \), many others found no association \( (19–23) \). On the contrary, in our population the M allele, and more clearly the MM homozygous genotype, showed a higher frequency in MI groups than in controls. This difference increases progressively when subjects with other risk factors such as hypercholesterolemia, hypertension, and SM are excluded. The frequency of the MM genotype rises from 15% in MI individuals who present with the three risk factors (HT, CH, and SM) up to 50% in MI individuals without them. The opposite effect is observed for the TT genotype, its frequency decreases from 31% in individuals with HT, CH, and SM down to 12% in subjects without the three risk factors \( (Table \ 1) \). It should be emphasized that the logistic regression gave to hypercholesterolemia/MM the most potent negative correlation between genotype frequencies and risk factors. This means that, in MI populations, to be MM and hypercholesterolemic are inversely related. Perhaps it is due to the potency and independence of both risk factors. These variations on the frequencies related to the risk factors are not observed for the other studied polymorphisms \( (I/D \ for \ ACE \ and a1166c \ for \ AT\(_1\)R genes) \). It has been observed that when the M allele frequency related to other risk factors are studied, its real meaning as a risk factor can be hidden. The contrary effect is found for the T allele when MI individuals with three risk factors are studied. This clear dependency between allele

| Table 2. PRA levels according to the RAS gene (genotypes in healthy men) |
|---------------------------|----------------|---------------|
| Gene | Genotype | n  | PRA levels (ng/ml/h) |
| | | | mean ± SD |
| | | | (SD) |
| AGT | | | |
| MM | 10 | 2.68 ± 0.78* |
| T T | 11 | 1.47 ± 0.48* |
| ACE | | | |
| II | 10 | 1.86 ± 0.74 |
| ID | 12 | 1.86 ± 1.08 |
| DD | 11 | 1.87 ± 1.00 |
| AT\(_1\)R | | | |
| a a | 12 | 1.36 ± 0.89 |
| a c | 12 | 2.09 ± 1.31 |
| c c | 9 | 1.78 ± 1.36 |

ACE: angiotensin-converting enzyme; AGT: angiotensinogen; AT\(_1\)R: angiotensin II type 1 receptor; PRA: plasma renin activity; RAS: renin-angiotensin system. Significance between MM and TT genotypes: \( p < 0.001 \).
and genotype frequencies and the major risk factors could be an explanation for the contrary results obtained by different authors (16–23).

Analysis of PRA levels in selected control individuals representing each polymorphism of the three genes only showed significant differences between the MM and TT genotypes of the AGT gene. Physiologically, it could mean that higher AGT levels lead to lower PRA levels in TT than MM subjects. This is consistent with the known low renin profiles in women and black population (25). The black Nigerian population shows a 91% frequency for the T allele (81% in Afro-American) (33) and women have also higher AGT levels due to the estrogen effect (34). Black populations, as well as other ethnic groups with a high T allele frequency, have a lower rate of MI if epidemiological data are referred to populations in their original habitat (33, 35).

Paradoxically, we can consider the two MM and TT homozygous genotypes as a coronary risk depending on the presence of other major coronary risk factors. In individuals with none of the other risk factors, TT subjects are highly protected against MI, whereas MM subjects have an increased risk of MI. However, when TT subjects present with the other three major factors, this protection become a risk. The molecular explanation to this dual possibility could be referred to the AGT as a limiting factor of renin activity. Perhaps angiotensin II is the main regulator of the RAS and its levels depend on renin activity and AGT synthesis. Renin is negatively regulated by angiotensin II and limited by the AGT level, which means that the AGT, as a renin substrate, is in a lower molar concentration than renin itself. Therefore, variations in the AGT levels (TT and MM subjects) should produce changes on PRA that will be regulated by the increase or decrease of the angiotensin II levels. We have found the TT subjects to have lower levels of PRA than MM individuals in basal conditions. However, it can be inferred that TT subjects have potentially a higher capacity to produce angiotensin II than MM individuals, because of their higher AGT basal levels, and therefore, a higher vascular effect mediated by the angiotensin II. On the other hand, it is known that hypertension, raised cholesterol, and cigarette SM, directly or indirectly, increase PRA levels (25, 26). In this way, a high AGT concentration with a stimulated PRA can induce a sustained vascular response.

We can conclude that the M allele is an independent risk factor for MI in the studied population and this can be better observed when none of the other coronary risk factors are present. We consider TT subjects with lower PRA levels to have a potential higher capacity to elevate PRA due to higher AGT levels, the renin-limiting factor. This higher capacity can be facilitated by other factors that, directly or indirectly, stimulate renin activity. Therefore, the TT genotype can be a dependent factor, as a risk when added to the major risk factors or as a protector when none of the three major risk factors are present. Finally, these findings should be corroborated by futher studies including a large number of subjects.

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References


