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A polymorphism in *ACE2* is associated with a lower risk for fatal cardiovascular events in females: the MORGAM project

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Abstract

Angiotensin II, a vasoconstrictor and the main effector molecule of the renin–angiotensin system, is known to influence inflammation, thrombosis, low-density lipoprotein oxidation and growth factors, all of which contribute to cardiovascular disease. The associations of polymorphisms in the angiotensin-converting enzyme 2 (*ACE2*) gene with cardiovascular risk have not been fully determined. Single nucleotide polymorphisms (SNPs) in *ACE2* were genotyped in participants of the prospective MORGAM study (n = 5092) from five cohorts: ATBC, FINRISK, Northern Sweden, PRIME/Belfast and PRIME/France. Using a case-cohort design, associations were sought between SNPs and haplotypes with cardiovascular events during follow-up (Cox proportional hazards model). The comparison group were a subset of all MORGAM participants who were selected to ensure similar age and sex distributions among the cases and controls. The A allele of the rs2285666 SNP (HR = 0.3, p = 0.04) was significantly associated with the risk of cardiovascular death in female subjects. These findings complement those found in other studies of SNPs in the *ACE2* gene in relation to cardiovascular disease risk. As females carry two copies of the *ACE2* gene, and given its plausible biological role in cardiovascular disease risk, further studies of *ACE2* should be prioritised.

Keywords

Cardiovascular disease, case-cohort, haplotypes, polymorphisms, renin–angiotensin system

Introduction

The renin–angiotensin system (RAS) plays important roles in the regulation of blood pressure (BP) and electrolyte balance, and also in the pathogenesis of cardiovascular disease (CVD).[1,2] In the first and rate-limiting step of the RAS, renin catalyses the cleavage of angiotensinogen into angiotensin I. Angiotensin I can then be further catalysed to angiotensin II, the main effector molecule of the renin–angiotensin system, by angiotensin-converting enzyme (ACE). Angiotensin II mediates its effects through promotion of inflammation, production of growth factors,[3] thrombosis[4] and low-density lipoprotein oxidation,[4] all of which contribute to the risk of myocardial infarction (MI) and stroke. In addition, angiotensin II is a potent vasoconstrictor which raises BP and thus plays an important role in the pathogenesis of hypertension.[5,6] Renin–angiotensin system inhibition by the use of ACE inhibitors and angiotensin receptor blockers in experimental animal models of atherosclerosis has demonstrated a reduction in vascular lesions[7] and of myocardial damage in both a coronary occlusion reperfusion model[8] and a myocardial stunning model.[9] Clinical trials have shown a reduction in MI and stroke in subjects taking ACE inhibitors in comparison with placebo,[10] and also that angiotensin receptor blockers may be as effective as ACE inhibitors at lowering risk of CVD.[11]

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* Annex 1: Sites and key personnel of the MORGAM Project (see supplementary material)

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More recently, however, some important additional pathways in the RAS have been elucidated. These include the discovery that there is a second angiotensin-converting enzyme (ACE2) which catalyses the conversion of angiotensin I and angiotensin II, to angiotensin(1–9) and angiotensin(1–7), respectively.[12-14] Angiotensin(1–9) is an inactive nonapeptide, while angiotensin(1-7) appears to act as a natural antagonist for angiotensin II, in that it has potent vasodilator, natriuretic, antigrowth and endothelium protective properties.[15]

To date, many candidate gene studies in humans have sought associations of polymorphisms in various components of the renin–angiotensin system, particularly the angiotensinogen, ACE and angiotensin II type 1 receptor genes, with the risk of hypertension and cardiovascular disease.[16] By contrast, just a limited number of association studies have looked at polymorphisms in the ACE2 gene in relation to cardiovascular disease risk,[17,18] BP variation[19,20] and survival after acute coronary syndromes.[21]

This study aimed to further examine the associations of genetic variants in *ACE2* with cardiovascular morbidity and mortality by genotyping tag single nucleotide polymorphisms (SNPs) in participants of the MORGAM project, an international pooling of prospectively collected cardiovascular cohorts.

Materials and methods

Study populations

The MORGAM study is a European-wide prospective study of cardiovascular disease risk, consisting of 33,282 participants.[22] This work is based on a subset of MORGAM subjects: the Finnish ATBC study cohort, the FINRISK cohort from Finland, the Northern Sweden cohort, the PRIME cohort from Belfast, Northern Ireland and the PRIME cohort from France. Risk factors measured at baseline in each cohort included sex, height, weight, smoking status, total and high-density lipoprotein (HDL) cholesterol, systolic and diastolic BP (two readings) and previous history of CVD or diabetes. Data on all-cause mortality and on all fatal or non-fatal stroke events (ischaemic or haemorrhagic) and coronary heart disease events (definite and possible acute MI or coronary death or unstable angina pectoris), was collected during the follow-up period. Ischaemic and haemorrhagic strokes could not be distinguished in all cohorts and so stroke types are pooled in the analysis. The MORGAM cohorts have been described in detail elsewhere.[23] The study was approved by local ethics committees and each subject gave written informed consent.

A case-cohort design has been adopted in the MORGAM study whereby a random subset of participants underwent genotyping as well as all additional individuals who experienced CVD events during the follow-up.[24] This random subset of participants, the subcohort, was selected according to population-specific sampling probabilities which were dependent on age and sex distributions in order to ensure that these were similar in the subcohort and the cases.

Identification of gene sequence variants and genotyping

Genomic DNA was extracted from leukocytes by a salting-out procedure.[25] DNA from 20 Irish subjects (10 normotensive and 10 hypertensive) was screened for mutations in the known promoter (-1224 to +121) and protein coding regions (18 exons, > 40 bp of flanking intronic regions) of the *ACE2* gene. As previously described, this was achieved by a combination of ion-pairing reversed-phase partially denaturing high-performance liquid chromatography and direct sequencing.[26] Just three *ACE2* SNPs were detected, and so all of the seven SNPs in *ACE2* that were found in dbSNP (http://www.ncbi.nlm.nih.gov/SNP, build 121, June 2004) with a minor allele frequency of > 1% in any ethnic group were selected for genotyping. However four of these SNPs were found to be monomorphic and there were assay optimisation problems for the other three. Thus, of the three SNPs identified by sequencing, two were in very tight linkage disequilibrium ($r^2 = 0.99$), leaving just two SNPs for further study, eliminating the need to formally identify tagging SNPs.

Hence these two SNPs were genotyped in the MORGAM subjects. Genotyping of SNPs was performed by KBiosciences (Herts, U.K.) using modified TaqMan assays (www.kbiosciences.co.uk).

Statistical analysis

Statistical analyses were performed using the Stata statistical package (version 8.2, StataCorp, College Station, Texas, USA). Phenotypic data were expressed as mean ± SD or as numbers (percentages). Two way ANOVAs and Chi-squared tests were used to compare phenotypic variables across the five cohorts.

Departure from Hardy–Weinberg equilibrium was assessed using Chi-squared tests with one degree of freedom. An additive genetic model was used for all SNP analyses – this assumption was tested through comparison of the fit of an additive model with the fit of a two-degree of freedom pairwise comparison in a likelihood ratio test. Haplotypes of the SNPs in the *ACE2* genes were inferred using the "--hap-phase" function in Plink (version 1.04, http://pngu.mgh.harvard.edu/purcell/plink/)[27] with a haplotype frequency cut-off of 5% in any cohort. Each haplotype was compared with a reference haplotype representing the most frequently occurring haplotype and the analyses were weighted according to haplotype probability. All haplotype analyses were performed in Stata.

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A Cox proportional hazards model was used to test for the associations of each of the SNPs and haplotypes with all prospective stroke or MI events, all fatal prospective stroke or MI events, all prospective MI events and all prospective stroke events using the time-to-event in days. Each of these models was stratified by history of stroke or MI at baseline, and adjusted for age, smoking, MORGAM cohort, body mass index (BMI), history of cardiovascular events (stroke or MI) at baseline, history of diabetes and ratio of total cholesterol to HDL. Due to the case-cohort design, a robust variance estimator was applied to account for the fact that some of the members of the subcohort were also cases.[24]

The subject identifiers were used as the cluster variable. Cases outside the subcohort were given a weight of one, non-cases in the subcohort were weighted with the inverse of the subcohort sampling probability, while cases in the subcohort require two records – one censored observation for the time before the event with inverse sampling probability weight, and one uncensored observation from the time before the event with weight one. These weights were included as an offset in the analysis.

Linear regression analysis was used to look for associations between the SNPs and haplotypes with systolic and diastolic baseline BP (average of the two readings) in all members of the subcohort, adjusted for the same covariates. Subjects who had been taking BP-lowering medication within the 2 weeks prior to baseline had a correction factor applied (an additional 15 mmHg systolic and 10 mmHg diastolic).[28]

The Cox proportional hazards and the linear regression analyses were also performed in each of the five cohorts separately. As the *ACE2* gene is on the X chromosome, separate analyses were performed for males and females. Associations with $p < 0.05$ were considered to be statistically significant.

Results

Population description

The baseline characteristics of each of the cohorts are described in Table 1. Only the FINRISK and the Northern Sweden cohorts included women. For the Cox proportional hazards analysis, there were 1959 cardiovascular disease cases and 2278 subcohort members, with 387 subjects who were both cases and members of the subcohort. The linear regression analysis was performed on all 2278 subcohort members.

Genetic data

The overall genotyping success rate was 96% and the rate of discrepancies between blinded duplicate samples was 0.07%. Both SNPs were found to be in Hardy–Weinberg equilibrium in all cohorts. In general, the three Scandinavian cohorts had similar minor allele and haplotype frequencies, often differing from that of the two PRIME cohorts (see supplementary material Table S1).

Associations of individual SNPs and haplotypes with CVD events

There were no significant associations between any of the SNPs or haplotypes with all prospective MI and stroke events, prospective MI events only or prospective stroke events only (Table 2 and supplementary material Table S2). The A allele of rs2285666 was found to be associated with a decreased risk of fatal stroke and MI events in female subjects (HR = 0.3, $p = 0.04$) (Figure 1). However once the Bonferroni correction for multiple testing is applied, this association is no longer statistically significant.

Table 1. Baseline characteristics of the MORGAM case-cohort subjects

	ATBC	FINRISK	Northern Sweden	PRIME/Belfast	PRIME/France
Total, *n*	1983	2146	332	332	299
Men (%)	1983 (100)	1487 (69)	219 (66)	332 (100)	299 (100)
Mean age at baseline, years	63.6 ± 5.0*	57.3 ± 9.9	57.7 ± 10.2	55.0 ± 2.9	55.3 ± 3.0
Current daily smoker (%)	1544 (78)*	537 (25)	67 (20)	98 (30)	78 (26)
Mean body mass index, kg/m^2	26.8 ± 4.3	28.1 ± 4.4*	27.1 ± 4.0	26.4 ± 3.3	26.9 ± 3.7
Drug treatment for high cholesterol (%)	0 (0)	175 (8)	11 (3)	5 (2)	44 (15)*
Drug treatment for high blood pressure (%)	0 (0)*	548 (26)	77 (23)	34 (10)*	69 (23)
History of myocardial infarction (%)	261 (13)	426 (20)	65 (20)	37 (11)	9 (3)
History of stroke (%)	138 (7)	247 (12)	43 (13)	2 (1)	3 (1)
Diabetes (%)	142 (7)	224 (10)	27 (8)	15 (5)*	28 (9)
Mean systolic blood pressure at baseline, mmHg	142.6 ± 19.0	145.6 ± 21.6*	139.1 ± 21.9	137.7 ± 23.6	136.8 ± 18.6
Mean diastolic blood pressure at baseline, mmHg	84.9 ± 10.4	85.4 ± 11.6	83.4 ± 12.3	82.9 ± 12.1*	85.3 ± 11.7
Mean high-density lipoprotein cholesterol, mmol/l (SD)	1.2 ± 0.3	1.3 ± 0.4	1.3 ± 0.4	1.1 ± 0.3*	1.2 ± 0.3
Mean total cholesterol, mmol/l (SD)	5.9 ± 1.1	5.8 ± 1.1	6.6 ± 1.2*	6.0 ± 1.1	5.7 ± 1.0

Data expressed as mean ± SD or as number (%), * indicates characteristics of a particular cohort that is statistically significantly different ($p < 0.05$) from the other groups

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Table 2. Results of survival analysis showing the hazard ratios and 95% confidence intervals of the association between each single nucleotide polymorphism and haplotype with all cardiovascular events, fatal cardiovascular events, myocardial infarction events and stroke events that occurred during the follow-up period

		All CVD events HR [95% CI]	Fatal CVD events HR [95% CI]	MI HR [95% CI]	Stroke HR [95% CI]
Males		*n* = 1382	*n* = 453	*n* = 1014	*n* = 448
SNPs	rs2285666 (A allele)	0.97 [0.83–1.12]	0.87 [0.67–1.13]	0.93 [0.78–1.11]	1.12 [0.88–1.44]
	rs971249 (A allele)	1.10 [0.97–1.25]	1.17 [0.95–1.44]	1.17 [1.01–1.36]	0.92 [0.73–1.14]
Haplotypes	GA	1.09 [0.95–1.26]	1.16 [0.92–1.46]	1.16 [0.98–1.37]	0.93 [0.73–1.1]
	AG	1.04 [0.88–1.22]	0.98 [0.74–1.31]	1.04 [0.86–1.26]	0.93 [0.73–1.18]
Females		*n* = 183	*n* = 33	*n* = 93	*n* = 96
SNPs	rs2285666 (A allele)	0.86 [0.60–1.25]	0.27 [0.08–0.97]*	0.87 [0.53–1.42]	0.98 [0.62–1.55]
	rs971249 (A allele)	1.20 [0.92–1.55]	1.49 [0.92–2.41]	1.18 [0.85–1.63]	1.19 [0.85–1.67]
Haplotypes	GA	1.27 [0.94–1.70]	1.58 [0.87–2.86]	1.31 [0.90–1.91]	1.27 [0.86–1.87]
	AG	0.96 [0.66–1.41]	0.46 [0.16–1.29]	0.97 [0.61–1.56]	1.09 [0.64–1.84]

CVD, cardiovascular disease; HR, hazard ratio; MI, myocardial infarction; SNP, single nucleotide polymorphism; 95% CI, 95% confidence intervals; *indicates statistically significant association at $p = 0.04$, ‡ compared with wild-type haplotype GG.

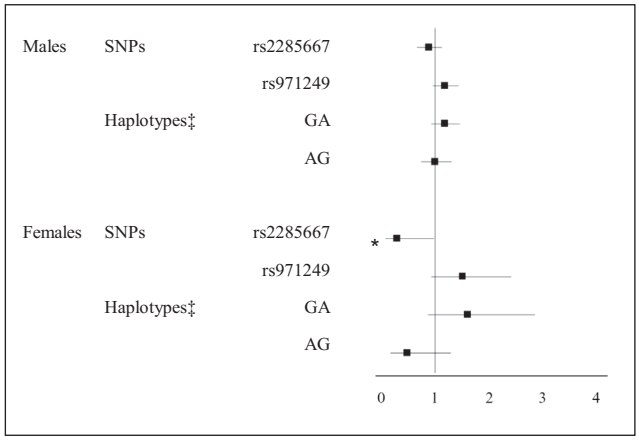

Figure 1. Results of association between fatal cardiovascular disease events during follow-up (hazard ratio, 95% confidence intervals) with each SNP and haplotype in the *ACE2* gene.
*Results significant at $p < 0.05$. ‡Compared with wild-type haplotype GG.

Associations of individual SNPs and haplotypes with BP

There were no significant associations between the SNPs and haplotypes of *ACE2* with BP or any of the other baseline measures common to the cohorts.

Discussion

This study demonstrates that the A allele of the rs2285666 polymorphism in the *ACE2* gene influences the risk of fatal CVD events in female participants of the MORGAM study. As the *ACE2* gene is located on the X chromosome, this study has investigated the risk of the SNPs independently in males and females, who have one and two gene copies, respectively.

In a study of a Chinese Han population, the rs2285666 SNP was found to increase the risk of MI in females, though the result was not significant (p = 0.06).[18] The same study showed that two other SNPs in the *ACE2* gene (rs1978124 and rs4646142) were significantly associated with risk of MI in females.[18] A study of left ventricular mass and septal wall thickness in German males did not find any association with rs2285666, but found significant associations between other SNPs in the *ACE2* gene (rs4646156, rs879922, rs4240157 and rs233575) and these measures.[29] Two further SNPs in the *ACE2* gene (rs2106809 and rs6632667) were found to be associated with risk of hypertrophic cardiomyopathy in Chinese males.[30] Another study in a Chinese population revealed that male carriers of the A allele of rs2285666 had lower interventricular septal end-diastolic thickness and lower left ventricular mass than G allele carriers in patients with type II diabetes.[31] Conversely, it was found in another study that there was a significant association between the rs1978124 SNP and mortality in males following acute coronary syndromes but not in females, although the direction of the effect was the same in females.[21] Given these gender and ethnic-group differences, it is likely that there is differing linkage disequilibrium between Europeans and Asians in the *ACE2* gene. Interestingly, A is the minor allele of this SNP in our study (frequency = 19%) and in the study of German subjects (frequency = 23%),[29] whereas the frequency of both alleles is around 50% in the Chinese populations.[18,30,32] All of these data combined indicate a sex- and race-dependent role of variants in the *ACE2* gene on CVD risk. Gender

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differences in the RAS with respect to CVD and BP have been noted previously, and have been attributed to hormonal effects.[33] However, the fact that females carry two copies of the *ACE2* gene while men only carry one copy may account for the larger effect of rs2285666 in women, assuming a dose effect of the variant on the ACE2 protein. However this does not help to explain why the study by Palmer *et al*.[21] found that a SNP in ACE2 was associated with the risk of CVD mortality in men but to a lesser degree in women. This apparent contradiction may be accounted for by differing effects of the two SNPs on the ACE2 protein, which are currently unknown. The likely role for *ACE2* in CVD is via its effects on levels of both angiotensin II and angiotensin(1–7).

One of the strengths of the prospective case-cohort study design in the MORGAM project above that of retrospective studies is that it allows for the study of fatal events that occur during the follow-up period. Genetic variants that predispose to the more acute events that cause mortality cannot be determined from retrospective studies.

The tag SNPs selected for this study were chosen based on sequencing and genotyping in an Irish population. This approach was used before the release of HapMap data, which indicates that five tag SNPs in *ACE2* would be necessary to represent 85% of the genetic variation in that gene (www.hapmap.org, release #27). Rs2285666 has not been genotyped as part of the HapMap project, however. Linkage disequilibrium data from other studies has indicated that rs2285666 is located within a large linkage disequilibrium block,[34,35] and other SNPs from within this block have been found to be associated with CVD risk.[18,21] Nevertheless, it is difficult to say how well the SNPs we have selected accounted for the genetic variation in the MORGAM cohorts.

Conclusions

The analyses of these tag SNPs in this study have indicated some interesting findings, and have replicated the findings of other studies. Therefore further investigation of the *ACE2* gene in relation to CVD risk in other populations should be prioritised.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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