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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, LDL 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. Tag single nucleotide polymorphisms (SNPs) in *ACE2* were genotyped in participants of the prospective MORGAM study (N=5 092) 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

Polymorphisms, case-cohort, haplotypes, Renin-angiotensin system, cardiovascular disease

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 (REN) 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. Angiotensin II mediates its effects through promotion of inflammation, production of growth factors[3], thrombosis[4] and LDL oxidation[4] , all of which contribute to the risk of myocardial infarction (MI) and stroke. Additionally, angiotensin II is a potent vasoconstrictor which raises blood pressure and thus plays an important role in the pathogenesis of hypertension[5,6]. Renin-angiotensin system inhibition by the use of angiotensin converting enzyme inhibitors and angiotensin receptor blockers in experimental animal models of atherosclerosis have 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 myocardial infarction and stroke in subjects taking angiotensin converting enzyme inhibitors in comparison to

placebo[10] and also that angiotensin receptor blockers may be as effective as angiotensin converting enzyme inhibitors at lowering risk of cardiovascular disease [11].

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 tested the association of polymorphisms in various components of the renin-angiotensin system, particularly the angiotensinogen, angiotensin converting enzyme 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] and blood pressure variation[19,20].

This study aimed to further examine the associations of genetic variants in *ACE2* with cardiovascular morbidity and mortality by genotyping tag 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[21]. 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 blood pressure (two readings) and previous history of CVD or diabetes. Data on all-cause mortality and on all fatal or non-fatal stroke events (ischemic or hemorrhagic) and coronary heart disease events (definite and possible acute MI or coronary death or unstable angina pectoris), was collected during the follow-up period. Ischemic and haemorrhagic strokes could not be distinguished in all cohorts and so stroke types are pooled in the analysis. The MORGAM cohorts has been described in detail elsewhere[22]. 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.[23] 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[24]. DNA from 20 Irish subjects (10 normotensive and 10 hypertensive) was screened for mutations in the known promoter (-1 224 to +121) and protein coding regions (18 exons, >40bp 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[25]. 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, Jun 2004) with a minor allele frequency of >1% in any ethnic group were selected for genotyping. However, four of the SNPs from dbSNP were found to be monomorphic in the Irish subjects (rs4646179, rs4646116, rs4646114, rs4646113) and assays could not be optimised for the other three (rs4646115, rs4646112, rs2097723).

While rs2097723 has been found at a minor allele frequency of 30% in the HapMap Caucasian population, all other SNPs are monomorphic in Caucasian populations and polymorphic only in African populations according to dbSNP. 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). 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 1-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/)[26] with a haplotype frequency cut-off of 5% in any cohort. Each haplotype was compared to 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.

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[23]. 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 blood pressure (average of the two readings) in all members of the subcohort, adjusted for the same covariates. Subjects who had been taking blood pressure lowering medication within the 2 weeks prior to baseline had a correction factor applied (an additional 15mmHg systolic and 10mmHg diastolic)[27].

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 1 959 cardiovascular disease cases and 2 278 subcohort members, with 387 subjects who were both cases and members of the subcohort. The linear regression analysis was performed on all 2 278 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 (Tables 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 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).

Associations of individual SNPs and haplotypes with BP

There were no significant associations between the SNPs and haplotypes of *ACE2* and blood pressure (Figure 2).

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[28]. Two further SNPs in the *ACE2* gene (rs2106809 and rs6632667) were found to be associated with risk of hypertrophic cardiomyopathy in Chinese males[29]. 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[30].

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%)[28], whereas the frequency of both alleles is around 50% in the

Chinese populations[18,29,31]. All of this data combined indicates a sex- and race-dependent role of variants in the *ACE2* gene on cardiovascular disease risk. Gender differences in the RAS with respect to CVD and blood pressure have been noted previously and have been attributed to hormonal effects [32]. However, the fact that females carry two copies of the *ACE2* gene while men only carry one cannot be ruled out as a factor contributing to such differences. 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. LD data from other studies has indicated that there is strong LD within the *ACE2* gene [18,28]. 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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Titles and legends to figures

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. * indicates results significant at $p<0.05$. ‡ compared to wild type haplotype GG.

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

	ATBC	FINRISK	Northern Sweden	PRIME/Belfast	PRIME/France
Total, n	1 983	2 146	332	332	299
Men (%)	1 983 (100)	1 487 (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 (%)	1,544 (78)*	537 (25)	67 (20)	98 (30)	78 (26)
Mean BMI, 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 MI (%)	261 (13)	426 (20)	65 (20)	37 (11)	9 (3)
History of stroke (%)	138 (7)	247 (12)	43 (13)	2 (1)	3 (1)

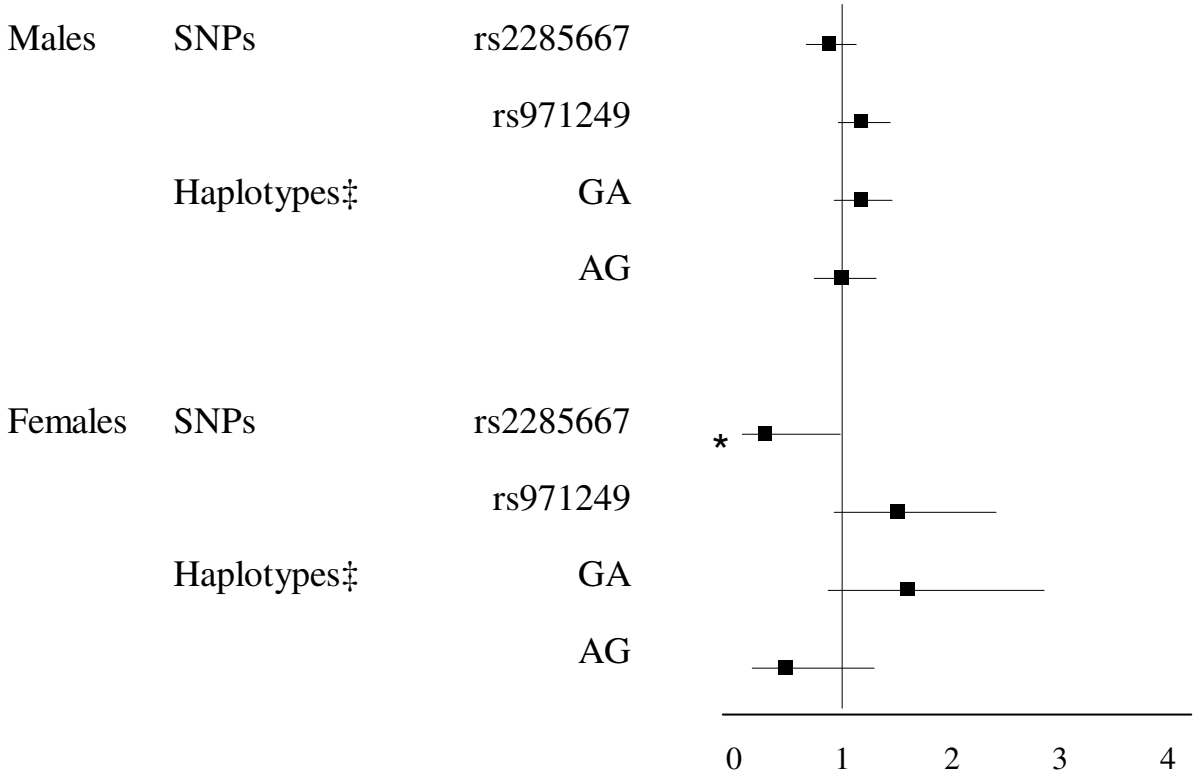
Diabetes (%)	142 (7)	224 (10)	27 (8)	15 (5)*	28 (9)
Mean systolic blood pressure at baseline, mmHg	142.6±19.0	145.6± 21.6*	139.1±21.9	137.7± 23.6	136.8±18.6
Mean diastolic blood pressure at baseline, mmHg	84.9±10.4	85.4±11.6	83.4±12.3	82.9±12.1*	85.3±11.7
Mean HDL cholesterol, mmol/L (SD)	1.2±0.3	1.3±0.4	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

Table 2: Results of survival analysis showing the hazard ratio's and 95% confidence intervals of the association between each SNP and haplotype with all cardiovascular events, fatal cardiovascular events, MI events and stroke events that occurred during the follow-up period.

		All Cardiovascular events	Fatal cardiovascular events	MI	Stroke
	Males	N=1382	N=453	N=1014	N=448
		HR [95% CI]	HR [95% CI]	HR [95% CI]	HR [95% CI]
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]

HR=Hazard ratio, 95% CI = 95% confidence intervals, *indicates statistically significant association at $p<0.05$, ‡ compared to wild type haplotype GG


Males
SNPs
rs2285667
rs971249
Haplotypes‡
GA
AG
Females
SNPs
rs2285667
*
rs971249
Haplotypes‡
GA
AG
0
1
2
3
4

Supplementary data

Annex 1: Sites and key personnel of contributing MORGAM Centres

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Table S1: Minor allele and haplotype frequencies by cohort

SNPs	rs number	Minor allele	ATBC	FINRISK (M)	FINRISK (F)	Northern Sweden (M)	Northern Sweden (F)	PRIME/ Belfast	PRIME/ France
Int3 9570	rs2285666	A	0.19	0.21	0.20	0.23	0.17	0.15	0.18
Int4 12268	rs971249	A	0.30	0.32	0.29	0.29	0.32	0.39	0.39
	SNPs								
	rs2285666	rs971249							
Haplotypes	G	G	0.49	0.37	0.44	0.49	0.50	0.45	0.42
	G	A	0.29	0.27	0.27	0.29	0.32	0.37	0.37
	A	G	0.18	0.17	0.18	0.22	0.17	0.14	0.18

Int=intron, M=male subjects, F= female subjects, rs number=dbSNP reference number

Table S2: Results of Cox proportional hazard analysis of the association between SNPs and haplotypes with CVD death in each cohort

		ATBC	FINRISK	Northern Sweden	PRIME/Belfast	PRIME/France
	Males	N=277	N=139	N=9	N=22	N=7
		HR[95% CI]	HR[95% CI]	HR[95% CI]	HR[95% CI]	HR[95% CI]
SNPs	rs2285666 (A allele)	0.85 [0.61-1.19]	0.91 [0.56-1.46]	-†	0.26 [0.05-1.36]	2.10 [0.45-9.84]
	rs971249 (A allele)	1.28 [0.98-1.68]	1.03 [0.70-1.52]	9.38 [0.94-92.84]	0.68 [0.21-2.13]	0.23 [0.02-2.06]
Haplotypes‡	GA	1.29 [0.97-1.72]	0.97 [0.61-1.54]	2.80 [0.54-14.93]	0.42 [0.14-1.25]	0.23 [0.02-2.31]
	AG	0.96 [0.67-1.38]	0.99 [0.57-1.70]	-†	0.19 [0.04-1.07]	1.29 [0.24-6.89]
	Females	N=0	N=32	N=1	N=0	N=0
SNPs	rs2285666 (A allele)	-	0.30 [0.08-1.06]	-†	-	-
	rs971249 (A allele)	-	1.51 [0.93-2.44]	-†	-	-
Haplotypes‡	GA	-	1.63 [0.89-2.97]	-†	-	-
	AG	-	0.51 [0.18-1.47]	-†	-	-

HR=Hazard ratio, 95% CI = 95% confidence intervals, † = not enough data to perform analysis due to small sample size, ‡ compared to wild type haplotype GG