Association of Genetic Polymorphisms in the ACE, ApoE, and TGF β Genes with Early Onset Ischemic Heart Disease

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Summary

Background: The genetic factors that contribute to ischemic heart disease (IHD) are poorly understood, and it is likely that multiple genes acting independently or synergistically contribute to the risk of IHD and outcome. The genes for angiotensin-converting enzyme (ACE) and apolipoprotein E (ApoE) have been implicated independently in the risk of IHD.

Hypothesis: This study examined whether genetic polymorphisms in the ACE and ApoE genes are associated with early onset IHD. Polymorphisms in a third gene, transforming growth factor β 2 (TGF β 2), with a known role in wound repair and cardiac development, are also examined with respect to early onset IHD.

Methods: In all, 101 patients with IHD and onset of disease before 55 years for men and 60 years for women, and 100 controls with angiographically confirmed normal coronary arteries were recruited for this study. The ACE, ApoE, and TGF β 2 genotypes were determined by polymerase chain reaction amplification or Southern blotting and were compared with the patient's clinical and family histories.

Results and Conclusion: The frequency of the ACE D allele was significantly lower in the patient group (0.475) than

in the control group (0.59, $p = 0.03$), which was attributed to a reduction in the number of patients with the DD genotype (patients: 24% DD, controls: 33% DD). Sudden cardiac death was also associated with the DD genotype. These data are consistent with the ACE D allele contributing to a fatal outcome. No association between the DD genotype and risk of myocardial infarction, presenting age, extent of vessel disease, family history, hypertension, or hypercholesterolemia was seen. Analysis of the ApoE genotype showed no association with early onset IHD. There was no evidence for a synergistic effect between the ACE and ApoE genotypes on the risk of early onset IHD. A polymorphism in the TGF β 2 gene was rare and not associated with early onset IHD.

Key words: angiotensin-converting enzyme, apolipoprotein E, transforming growth factor β 2, DNA polymorphisms, ischemic heart disease

Introduction

The genetic factors that predispose toward disease are poorly understood, and it is likely that the risk of ischemic heart disease (IHD) is influenced by many genes acting both in concert and independently. Considerable attention has focused on the gene for angiotensin-converting enzyme (ACE) and its potential role in IHD. There are two common genetic variants of ACE, which differ in the presence (insertion = I) or absence (deletion = D) of 287 base pair (bp) of DNA in intron 16.¹ The known role of ACE in the angiotensin-bradykinin system and the effect of the insertion/deletion polymorphism on serum ACE levels has led to a number of investigations exploring the effect of these polymorphisms on the risk of IHD.^{1–6} The outcome of these studies has been ambiguous, and in some cases the data were consistent with the ACE D allele being a potent risk factor for IHD.^{1,2} In other studies, no effect of the ACE genotype was seen.⁷ The interpretation of these data is often confounded by the different selection criteria for entry into the study, ethnic variation, and the presence of other risk factors.

This work was supported in part by the Brighton Heart Support Trust and the RM Phillips Charitable Foundation. LVM is an RM Phillips Senior Research Fellow into Aging.

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Received: March 12, 1998

Accepted with revision: July 20, 1998

Recent studies have begun to look at the interaction of several genetic loci on the risk of IHD. Tiret *et al.*⁸ demonstrated a synergistic effect on the risk of myocardial infarction (MI) between the ACE genotype and a polymorphism in the angiotensin II type I receptor (AT1R) gene. In another study, however, polymorphisms in the ACE, angiotensinogen, and AT1R gene failed to correlate with MI.⁹

The aim of this study was to examine whether genetic polymorphisms in the ACE, apolipoprotein E (ApoE), and transforming growth factor β 2 (TGF β 2) genes, either alone or in combination, predisposed individuals to early onset IHD. Genetic polymorphisms in the ApoE gene were previously described to be associated with hyperlipidemia and an increased risk of coronary artery disease, and TGF β has a well-described role in cardiac development.¹⁰⁻¹³

Methods

Subjects

Early onset IHD was defined as the onset of IHD before the age of 55 years in men and 60 years in women, and was determined by the presence of one or more of the following: a coronary stenosis > 50% in one or more coronary arteries at coronary arteriography, previous MI based on the World Health Organization (WHO) criteria,¹⁴ or signs of ischemia on standard exercise testing.

The control group consisted of 100 individuals with normal coronary arteries who presented for coronary arteriography for one of the following: typical or atypical chest pain, valvular heart disease, idiopathic cardiomyopathy, or congenital heart disease.

Both the control group and the study population were interviewed. The interview was based on a questionnaire aimed at risk factors for IHD and included a detailed family history. All 201 questionnaires were completed.

Risk Factors

Hypertension: The definition and diagnosis of hypertension were based on the criteria proposed by the WHO/International Society of Hypertension (ISH) guidelines for the management of hypertension.¹⁵ We collected previous medical records of raised blood pressure, with and without treatment. For patients with no history of hypertension, 24-h ambulatory blood pressure monitoring was performed to exclude this diagnosis in the remaining patients.

Hyperlipidemia: Patients were divided into three groups on the basis of their cholesterol levels: <5.2 mmol/l, 5.2–6.5 mmol/l, and >6.5 mmol/l. All cholesterol measurements were preceded by a fast of at least 12 h and were undertaken before lipid-lowering treatments were initiated.

Diabetes: We recorded both insulin-dependent and noninsulin-dependent diabetes in accordance with standard criteria.¹⁶

Family history: A detailed family history was taken from each member in the study. Specific targeting of a family history for both early and late onset IHD, hypertension, hyperlipidemia, and diabetes mellitus was made. The family history included grandparents, parents, siblings, and children.

General Methods

Sample preparation: Fifty μ l aliquots of whole blood were centrifuged and washed three times in 10 mM Tris HCl pH 7.5, 1 mM EDTA. The final pellet was resuspended in polymerase chain reaction (PCR) buffer (see below) with 0.5% Tween 20 and 100 μ g/ml proteinase K. Tubes were incubated at 56°C for 1 h followed by a 10 min incubation at 95°C. DNA samples were stored at –20°C until use.

Molecular biology techniques: Standard molecular biology techniques were as described.¹⁷

Angiotensin-Converting Enzyme Insertion/Deletion Polymorphism

Primers and reaction conditions were as described.^{18, 19}

ApoE Restriction Fragment Length Polymorphism

A 295 bp fragment from the ApoE gene was amplified by PCR. PCR reactions contained 10 μ l DNA, 60 mM Tris HCl, pH 8.5, 15 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 0.25 mM dNTPs, 5% DMSO and 12.5 pM of each primer in 100 μ l. Primer sequences were as follows:

P1A: 5'-GAACAACTGACCCCGGTGGCGG-3' and
P2A: 5'-GGATGGCGCTGAGGCCGCGCTC-3'.

Tubes were incubated for 18 min at 97°C, and 2.5 units Amplitaq DNA polymerase (Perkin Elmer, Norwalk, Conn., USA) were added. The cycling reaction was 96°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 18 cycles. The PCR reaction was then repeated with fresh polymerase.

Polymerase chain reaction-amplified DNA was digested overnight with Hin6I (IGI) and resolved on 12% polyacrylamide gels in Tris Borate Buffer. Gels were stained in SYBR Green I (Molecular Probes, Inc., Eugene, Ore., USA) and visualized under ultraviolet irradiation.

Southern Analysis

Angiotensin-converting enzyme gene: To confirm PCR data, 10 μ g HindIII digested genomic DNA were resolved on 0.7% agarose gels and used for Southern blotting. Membranes were probed with an ACE gene fragment produced by EcoRI/SP1 digestion of pB35.¹⁹

Transforming Growth Factor β 2 Gene: Fifteen μ g purified genomic DNA was digested overnight with SacI and used for Southern blotting. Membranes were probed with either the 3' or 5' end of the TGF β 2 gene, isolated following digestion of pPC21 with HindIII and EcoRI.

Results

Clinical Details and Risk Factors

Patients and controls are described in Table I. In the patient group, men outnumbered women nearly five to one, consistent with previous observations on the increased risk of IHD in men, but there was no significant difference in the number of men and women in the control group. Other clinical features that differed between the two groups included a higher incidence of heart failure and cerebral vascular accidents (CVA) in the control group. This is most likely a secondary consequence of the high number of valve abnormalities in this group (ratio of chest pain:valve abnormalities 1:1). Over half the patients in the patient group had suffered an MI, and four individuals had multiple MIs.

The prevalence of risk factors for IHD is presented for both groups in Table II. As expected, there is a significantly higher incidence of each risk factor in the patient group. Of this patient group, 38% were hypertensive and 81% had elevated

lipid levels. The prevalence of diabetes in the patient group (16 compared with 7 in the control group) was consistent with the increased risk of IHD associated with diabetes. A family history of IHD was reported in 74% of the patient group compared with only 42% in the control group, suggesting a strong genetic contribution to disease in the patient group. Smoking levels were high in both groups, with only 12 (11.8%) individuals who had never smoked in the patient group and 29 (29%) individuals in the control group. Although the prevalence of risk factors is lower in the control group, the values are generally higher than the population average (e.g., for the age range 35–74 years in England, 41.5% have never smoked, 31.5% are ex-smokers, and 27% are current smokers).²⁰ This is likely to reflect a selection bias to the control group as the criteria for an angiogram would include both atypical chest pain and the presence of one or more risk factors.

The data for family history of disease are shown in Table II. The familial incidence of early onset IHD in the study group was three-fold that of the control group. The incidence of late onset IHD was also significantly elevated (1.8-fold higher) in

TABLE I Clinical details of patients and controls

	Patients—IHD		Controls	
Number	101		100	
Male	84		53	
Female	17		47	
Ave. presenting age (years)	46.4 (6.3 SD)		51.0 (16.6 SD)	
	47.8 F (8.1 SD)		50.1 F (16.4 SD)	
	46.0 M (5.9 SD)		51.9 M (16.9 SD)	
Diagnostic criteria				
Angiographic groups	Multivessel disease	67) 86	Normal coronary arteries	95
	Single-vessel disease	19)	Minimal atheroma	5
Clinical data	Myocardial infarction	10)	Chest pain	41
	Sestamibi scan	1) 15	Valvular heart disease	49
	Exercise test	4)	Rhythm abnormalities	7
			Myocarditis	2
			Congenital heart disease	1
Other clinical features				
Ventricular arrhythmia	7		8	
Sudden cardiac death	8		5	
Congestive cardiac failure	6		20	
Aneurism	3		1	
Dilated cardiomyopathy	1		0	
Cerebrovascular accident	3		9	
Peripheral vascular disease	6		1	
Myocardial infarction	MI = 0 38			
	MI = 1 59			
	MI = 2 3			
	MI = 3 1			
Interventions				
CABG ≥ 1	51			
PTCA ≥ 1	11			

Abbreviations: IHD = ischemic heart disease, SD = standard deviation, MI = myocardial infarction, CABG = coronary artery bypass grafting, PTCA = percutaneous transluminal coronary angiography.

TABLE II Risk factors and family history of disease

	Patients—IHD n = 101	Controls n = 100
Risk factors		
Hypertension	38	29
Hypercholesterolemia		
>5.2 mMol/l	81	64
>6.5 mMol/l	57	25
Diabetes	16	7
Family history IHD	74	42
Smoking history		
Never smoked	12	29
Current smoker	15	17
Former smoker	74	54
Family history of	(n)	(n)
Premature IHD	46	15
IHD	74	42
Other		
Hypertension	4	6
Hypercholesterolemia	11	2
Diabetes mellitus	3	9
None	83	83

Abbreviation: IHD = ischemic heart disease.

the study group. The incidence of a family history for hypercholesterolemia was low in both groups, with only 11 individuals reporting a positive family history in the study group and 2 in the control group. The history of hypertension was very similar between the two groups. Despite the high incidence of diabetes in the patient group, only three individuals reported a family history of diabetes compared with nine in the control group. If IHD is excluded, 83 individuals in both groups reported no other family history of disease. These data are consistent with a genetic predisposition to both early and late onset IHD in the patient group.

Genetic Polymorphisms and Ischemic Heart Disease

The frequency of the ACE D allele (Table III) was significantly higher in the control group (0.590, $p = 0.03$), free of coronary artery disease, than in the IHD patient group (0.475). However, the control frequency falls within the previously reported normal range (0.51–0.60),^{5,6,21,22} suggesting that the apparent difference in allele frequency is most likely due to a loss of the D allele in the patient group. In particular, there was a significant decrease in the number of D homozygotes. We detected no significant differences in the overall frequency of the D allele between men and women (data not shown).

Table IV compares the clinical histories by genotype. The average presenting age for patients with early onset IHD as a group was 46.4 years [± 6.3 standard deviation (SD)] and the average presenting age for each genotype was very similar. The risk of MI and the extent of vessel disease appeared to be largely independent of the ACE genotype. Similarly, the D

allele was not associated with a greater risk of hypertension or hypercholesterolemia in either the patient or control groups, although patients with hypertension had a lower frequency of the D allele (data not shown). The D allele was also not associated with an increased family history of IHD. Within the limits of this study, there was no evidence for an effect of the ACE genotype on any of these factors.

There are three common ApoE alleles in the population—ApoE2, ApoE3, and ApoE4—which can result in six genotypes (E2:E2, E3:E3, E4:E4, E2:E3, E2:E4, and E3:E4). In contrast to the frequency of the ACE D allele, the frequency of the ApoE genotypes was very similar between the patient and control groups (Table III). The ApoE 2:2 genotype is relatively rare in the general population and was not found in our study or control groups. The ApoE3:E3 genotype is the most common in the general population and accounted for over 50% of both the patient and control groups. The overall frequency of the three ApoE alleles was also similar between the two groups and resembled previously published data.¹⁰

The presenting age (Table IV) for the patients with the E2:E3, E3:E3, and E3:E4 genotypes was similar, but individuals with the E2:E4 and E4:E4 genotype all presented at a younger age (37, 41, 37, and 34 years) below the group average (46.4 years ± 6.3 SD), suggesting that the E2 and E4 alleles predisposed to earlier onset of disease. However, the number of individuals with these genotypes was small and we cannot rule out that this result happened by chance.

Cholesterol levels were high (> 6.5 $\mu\text{mol/l}$) for all ApoE genotypes except E2:E4 (average 5.60 ± 0.85 SD), obscuring

TABLE III Angiotensin-converting enzyme and apolipoprotein E genotypes

	Patients—IHD n = 101	Controls n = 99 ^a
ACE genotype		
DD	0.25	0.33
ID	0.45	0.52
II	0.30	0.15
Allele frequency		
D	0.475 ^b	0.59 ^b
ApoE genotype	n = 100 ^a	n = 100
2.2	0	0
2.3	0.11	0.17
2.4	0.02	0.04
3.3	0.57	0.52
3.4	0.28	0.24
4.4	0.02	0.03
Allele frequency		
E2	0.065	0.105
E3	0.765	0.725
E4	0.170	0.170

^a One sample could not be amplified by PCR.

^b $p = 0.03$.

Abbreviations: IHD = ischemic heart disease, PCR = polymerase chain reaction.

TABLE IV Comparison of clinical histories by genotypes

	Presenting age	MI = 0 n = 38	MI ≥ 1 n = 63	MVD ^a n = 67	SVD ^b n = 19
ACE genotype					
DD n = 25	46.7	0.26	0.24	0.24	0.26
ID n = 45	45.6	0.50	0.42	0.46	0.42
II n = 31	47.1	0.24	0.35	0.30	0.32
ApoE genotype					
E2.E3 n = 11	46	0.13	0.10	0.16 n = 11	0
E2.E4 n = 2	39	0.05	0.04	0.015 n = 1	0
E3.E3 n = 57	46.5	0.60	0.55	0.52 n = 35	0.79 n = 15
E3.E4 n = 28	47.6	0.18	0.34	0.28 n = 19	0.16 n = 3
E4.E4 n = 2	35.5	0.03	0.02	0.015 n = 1	0.05 n = 1

^{a/b} Angiography was performed on 86 patients to determine the number of affected arteries.

Abbreviations: MI = myocardial infarction, MVD = multivessel disease, SVD = single-vessel disease.

any potential effects due to differences in ApoE genotype. Similarly, triglyceride levels were raised and did not correlate directly with ApoE genotype. The highest triglyceride levels were associated with the Apo E2:E3 genotype (3.80 ± 1.93 SD) in the patient group.

The incidence of MI was high in the patient group and only 38 patients had not suffered an MI at the time of this study. The frequency of ApoE genotypes for individuals with MI (Table IV) is similar to those without MI, except for the E3.E4 genotype, where there is nearly a twofold increase in the number of individuals with MI.

Multivessel disease (Table IV) occurred more frequently than single-vessel disease in our patients with early onset IHD, and the low number of patients with single-vessel disease made direct comparisons with the ApoE genotypes between the two groups difficult. However, all patients in our study with the E2:E3 genotype ($n = 11$) had multivessel disease and none of the patients with single-vessel disease carried the E2 allele. As triglyceride levels were exceptionally high in these patients, this may be an important factor in multivessel disease. While numbers are too small to determine the statistical significance of these findings, it suggests that the E2 allele may predispose toward multivessel disease.

A novel genetic polymorphism in the TGF β 2 gene was examined by Southern blotting. SacI digestion of DNA produced two bands of approximately 12 and 4.2 kb (Fig. 1, lanes 1–4) for most individuals. One individual demonstrated the SacI polymorphism (lane 5), producing an additional band of 1.7 kb. The rarity of this genetic variant suggests that it is unlikely to have a significant impact on the risk of IHD in the population as a whole. The patient was heterozygous for the ACE gene polymorphism, homozygous for the ApoE3 allele, and his clinical presentation was unremarkable.

Discussion

Lifestyle factors contribute significantly to the risk of IHD and are expected to have an accumulative effect with age.

Therefore, individuals with early onset heart disease may reflect a genetic predisposition, which is manifested before such factors can act. The high incidence of a positive family history for IHD in our patient group supports this view. We therefore examined the association between genetic polymorphisms in the ACE, ApoE, and TGF β 2 genes with early onset IHD.

Analysis of the ACE genotype showed no association between the D allele and early onset IHD, the risk of MI, presenting age, extent of vessel disease, hypertension, or hyper-

FIG. 1 Southern blotting was used to visualize the DNA polymorphism in the TGF β 2 gene. DNA was digested with SacI, fragments were resolved on agarose gels and transferred to nylon membranes. The membrane was probed with the TGF β 2 gene. Lanes 1–5: DNA samples from five different patients. Lanes 1–4: Normal DNA digest producing bands of approximately 12 and 4.2 kb. Lane 5: Rare TGF β 2 polymorphism producing an additional 1.7 kb band.

cholesterolemia. The frequency of the D allele in the patient group (0.475) was significantly lower than that of the control group in this study (0.59 $p = 0.03$) and was lower than that in previously published studies.^{5,6,21,22} Similarly, the frequency of the DD homozygotes was reduced. Our results are similar to those of Bohn *et al.*,³ who demonstrated a D allele frequency of 0.48 in men under the age of 56 years and women under the age of 61 years, who had survived an MI, with a control frequency of 0.57.

Our study and that of Bohn *et al.*³ differs from other studies in selecting for younger patients. This suggests that in young people the DD genotype was more likely to lead to death, or that the I allele in young people is a predisposing factor to disease, and that early onset heart disease may occur by different mechanisms than late onset. The findings of Evans *et al.*⁶ support the hypothesis that the D allele is a risk factor for fatal MI and sudden cardiac death (SCD). In their study, the frequency of the DD and ID genotypes was significantly elevated in autopsy cases, in which death was due to coronary heart disease; however, this effect appeared to be independent of age. Eight patients in our study suffered SCD, six of these were DD homozygotes, while the remaining two were II and ID, respectively. While these numbers are low, they are consistent with the D allele predisposing to a fatal outcome.

The known association of ApoE variants with hyperlipidemia and IHD¹⁰⁻¹² led us to examine the ApoE genotype of our study group. In contrast to the ACE genotype, the ApoE genotype of patients and controls was very similar and not consistent with an association between the ApoE genotype and risk of early onset IHD in this group.

The known role of TGF β in smooth muscle growth, wound repair, and cardiac development led us to explore the potential role of a novel polymorphism in the TGF β 2 gene. This polymorphism was rare, however, and was detected in only one individual in this study. The clinical history of this patient is unremarkable, and it is unlikely that this polymorphism is associated with an increased risk of IHD.

Acknowledgments

The authors are grateful to Dr. F. Soubrier for the plasmid containing the ACE gene (pB35.19), to Dr. Purchio for the TGF β 2 containing plasmid (pPC21), and to Terry Ayers for his help with collecting blood samples.

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