Genetic associations with human longevity at the *APOE* and *ACE* loci

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In an effort to dissect the genetic components of longevity, we have undertaken case-control studies of populations of centenarians (n=338) and adults aged 20–70 years at several polymorphic candidate gene loci. Here we report results on two genes, chosen for their impact on cardiovascular risk, encoding apolipoprotein E (ApoE), angiotensin-converting enzyme (ACE). We find that the ε4 allele of *APOE*, which promotes premature atherosclerosis, is significantly less frequent in centenarians than in controls ($p<0.001$), while the frequency of the ε2 allele, associated previously with type III and IV hyperlipidemia, is significantly increased ($p<0.01$). A variant of ACE which predisposes to coronary heart disease is surprisingly more frequent in centenarians, with a significant increase of the homozygous genotype ($p<0.01$). These associations provide examples of genetic influences on differential survival and may point to pleiotropic age-dependent effects on longevity.

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Although there is ample evidence supporting the contention that a genetic component contributes to the determination of human longevity[1–4] the only association reported so far was found at the HLA locus in two independent investigations[5,6]. These studies included less than 100 centenarians. Since cardiovascular diseases are among the leading causes of death[7] and their underlying genetic causes have been extensively studied, we decided to investigate possible associations between longevity and alleles of three already characterized candidate genes, coding for apolipoprotein E, apolipoprotein B and angiotensin-converting enzyme (ACE).

APOE has three common alleles[8], designated ε2, ε3 and ε4, which code respectively for the isoforms apoE2, apoE3 and apoE4. These alleles have a major impact on total and LDL-cholesterol levels in the serum, which in turn are highly correlated with risk for atherosclerotic cardiovascular disease[9]. The ε4 allele has been associated with ischaemic heart disease in several studies[9–11]. An insertion (I)/deletion (D) polymorphism in the ACE gene is associated with a major gene effect on the inter-individual variability of plasma ACE concentration[12], and the deletion polymorphism has been identified as a risk factor for myocardial infarction[13,14].

In order to search for genetic influences on human longevity, a population of 300 French centenarians (individuals in their 100th year and beyond) was collected, to be compared with a control group of 160 French adults, aged 20–70. These populations were typed by DNA amplification for alleles of *APOE*[15] and *ACE*[16].

Table 1 Distributions of *APOE* genotypes and alleles in controls and centenarians

E genotype	ε3/ε3	ε2/ε3	ε3/ε4	ε2/ε2	ε4/ε4	ε2/ε4
enarians (n=325)	216	71	30	4	0	4
ols (n=161)	110	18	26	0	3	4

E alleles	Centenarians numbers (frequency)	Controls numbers (frequency)	Other control population (Paris)[22] number (frequency)
	83 (0.128)	22 (0.068)	39 (0.079)
	533 (0.820)	264 (0.820)	399 (0.801)
	34 (0.052)	36 (0.112)	60 (0.120)

ulations are in Hardy-Weinberg equilibrium for *APOE* alleles.
nificantly higher than controls ($p<0.01$ and $p<0.001$ when both control ulations are pooled).
nificantly lower than controls ($p<0.001$ and $p<10^{-5}$ when both control ulations are pooled).

APOE alleles

The ε4 allele frequency was significantly decreased (5.2% versus 11.2%, $p<0.001$) while the ε2 allele frequency was significantly increased (12.8% versus 6.8%, $p<0.01$) in the centenarian group (Table 1). The p[17] values were less than 10^{-5} for ε4 and less than 10^{-3} for ε2 when data for a second control group already genotyped were pooled with the new control population (Table 1). The odds ratios, a measure of the relative chance of becoming a centenarian between subjects with or without a given allele, were 2 and 0.43 for the ε2 and the ε4 alleles, respectively.

ACE alleles and genotypes

The *ACE* allele distribution was shifted in centenarians, with an increase of the DD genotype (39.6% versus 25.6%,

Table 2 Distributions of *ACE* genotypes and alleles in controls and centenarians

ACE genotypes	Centenarians (*n*=310) number (frequency)	Controls (*n*=164) number (frequency)	Other control populations (*n*=553) Lille, Strasbourg, Toulouse[13] number (frequency)
DD	134 (0.396)	42 (0.256)	157 (0.284)
ID	148 (0.438)	91 (0.555)	295 (0.533)
II	56 (0.166)	31 (0.189)	101 (0.183)
ACE alleles	Centenarians number (frequency)	Controls number (frequency)	Other control populations Nancy, Lille, Strasbourg, Toulouse[13,14] number (frequency)
D	416 (0.615)	175 (0.533)	345 (0.555)
I	260 (0.385)	153 (0.467)	276 (0.445)

Statistical analysis of association between longevity and *ACE* polymorphism. *ACE* genotypes (DD genotype more frequent in centenarians): $p = 0.01$ and $p<10^{-3}$ when all genotyped control populations are pooled. *ACE* alleles (D allele more frequent in centenarians than in controls): $p< 0.01$ when all genotyped control populations are pooled. Populations are in Hardy-Weinberg equilibrium for *ACE* alleles.

$p<0.01$ and $p<10^{-3}$ using the pooled controls, Table 2). The odds ratio was 1.9 for individuals with the DD genotype compared to those with an I allele. Taking this value as a measure of relative risk, 18.6% of the centenarians could be attributable to the DD genotype.

Associations in a selected subgroup

A subset of the centenarians included in this study had been independently selected through a different protocol, on the basis of their belonging to long-lived sibships. The long-lived sibships were defined as comprising at least two siblings older than 95 years, if female or older than 90, if male. Thirty-five members of long-lived sibships who all happened to be older than 99 years were pooled with the directly selected population of centenarians. In this subgroup, the *APOE* and *ACE* genotypes displayed the same associations with an even more marked trend (Table 3).

Gender effects and interaction between loci

Our population of centenarians was mainly composed of females, its male to female ratio of 1:8 (see Table 1) reflecting the sex differential in longevity in France.

Table 3 *APOE* and *ACE* alleles in centenarians with long-lived siblings

APOE alleles (*n*=35)	ε2	ε3	ε4
number (frequency)	11 (0.162)	54 (0.794)	3 (0.043)
frequency in P1	0.128	0.820	0.052
frequency in P2	0.068	0.820	0.112
ACE genotypes (*n*=34)	DD	ID	II
number (frequency)	17 (0.486)	14 (0.400)	4 (0.114)
frequency in P1	0.396	0.438	0.166
frequency in P2	0.256	0.555	0.189

The differences reported in Tables 1 and 2 are all enhanced when controls are compared with the selected subgroup of centenarians. However, statistical significance is diminished due to the small sample size. P1, population of all the centenarians. P2, population of controls.

Table 4 Sex-related differences in centenarians

APOE alleles	ε2	ε3	ε4
Females (*n*=282)	0.128	0.823	0.050
Males (*n*=41)	0.122	0.805	0.073
ACE genotypes	DD	DI	II
Females (*n*=294)	0.395	0.442	0.163
Males (*n*=44)	0.409	0.409	0.182

The numbers shown are allele and genotype frequencies. There is no significant difference male and female centenarians for any of these frequencies, at the 5% confidence level or between controls and centenarians of one sex.

However, 41 male centenarians were typed for *APOE* and 44 for *ACE*, which allowed a comparison between sexes: There was no significant sex-related difference in any of the allele frequencies, whether for *APOE* or *ACE* (Table 4). Searching for interactions between loci revealed a trend of negative correlation, that did not reach statistical significance, between the ε2 allele of *APOE* and the deletion allele of *ACE* (Table 5).

APOB polymorphisms

The association of *APOE* alleles with longevity prompted us to investigate polymorphisms in the apolipoprotein B (ApoB) gene, since ApoE is involved in the metabolism of ApoB-containing lipoproteins[9]. We typed two polymorphisms in *APOB* by DNA amplification[18] in our populations. A threonine 2488 neutral substitution detectable by *XbaI* and the ins/del polymorphism in the signal-peptide region, which have been associated respectively with significant effects on total cholesterol, LDL-cholesterol and triglyceride levels (for *XbaI*) and with the severity of global coronary atherosclerosis (for ins/del)[19]. For both of these polymorphisms, the allele frequencies were similar in the centenarian and the control populations (Table 6).

Other polymorphisms

We typed random subsamples of our populations at two other loci. A bi-allelic marker at *D4S95* on 4p16.3 chromosome (the Huntington's disease region), probe 674 with *AccI* (ref. 20), gave a frequency of 0.33 for the rare allele in controls (n=158) and centenarians (n=164) and a dinucleotide repeat within the B-cell leukaemia/lymphoma 2 (*BCL-2*) gene on chromosome 18q21.3 (ref. 21) (GenBank accession number: M15701) with one frequent allele and eight rare alleles gave a frequency of 0.825 and 0.838 for the frequent allele in controls (n=103) and centenarians (n=108), respectively. Thus, for both of these polymorphisms there was no difference between controls and centenarians.

Discussion

The potential overlap between cases and controls (th frequency of controls that might become centenarians), small enough — less than 2.4% and 0.5% of women an men respectively at age 70, based on 1980 estimates taken from a Caucasian population[22] — as to remain negligible. The frequencies found in our control group for *APOE* and *ACE* alleles agree well with those reported in French populations from Paris[17], Lille and Strasbourg[13] and (for

Table 5 Interaction between *APOE* and *ACE* in centenarians

	DD	ID	II
ε2	29 (0.115)	35 (0.132)	19 (0.182)
ε3	210 (0.833)	219 (0.823)	78 (0.750)
ε4	13 (0.052)	12 (0.045)	7 (0.067)

The frequencies of *APOE* alleles are shown in parenthesis for each genotype at the *ACE* locus. The visible trend of negative correlation between the ε2 and D alleles does not reach significance (p=0.09 for a comparison of the ε2 allele frequency between DD and II genotypes).

APOE) non French Caucasian populations from Framingham (USA)[23] and Münster (West Germany)[24].

Although a decrease in the ε4 allele frequency was found in Canadian octogenarians[9], it was not as marked as in our centenarians and there was no significant change in the ε2 allele frequency; on the other hand, the increase of the ε3 allele frequency in females was less pronounced in our group of centenarians. Another study[25] reported a decreased frequency of ε4 in older female diabetic patients. The well documented impact of ApoE-ε4 on cardiovascular risk is commonly attributed to its hypercholesterolaemic effect; the ε2 allele has opposite effects on total and LDL-cholesterol, which has led to the suggestion that it might protect against atherosclerosis, however it also has a hypertriglyceridaemic effect and may be a risk factor for ischaemic heart disease in certain environments[11]. Thus, while the lower frequency of ApoE-ε4 in centenarians is consistent with its risk factor status for heart disease, the increase in ε2 allele frequency is a more unexpected result. If ε2 and ε3 were neutral, with respect to survival, the decrease in ε4 frequency would result in an evenly distributed increase in the two other allele frequencies, whereas in fact the entire increase is borne by ε2, adding to the evidence for a protective effect of this allele.

More surprising is the increased frequency of *ACE*/DD genotypes found in centenarians, in view of its reported association with myocardial infarction. Perhaps the risk conferred by the *ACE*/D allele is offset by some unknown long-term protective effect. Although this *ACE* polymorphism is associated with a major gene effect on the plasma concentration of the enzyme, with a higher concentration for the DD genotype, it is unrelated to blood pressure and hypertension in adult populations[13,26]. However, such a correlation may arise at a very old age, which might then relate to higher blood pressure appearing to be a positive survival factor in centenarians (M. Allard, personal communication). ACE has other biological functions besides its role on the renin-angiotensin and kallikrein-kinin systems. Its ability to cleave neuropeptides such as enkephalin, substance P and LHRH[27,28] and its regional distribution in the brain[29] point to neuroendocrine functions. Furthermore, an increase, with age, in brain cortical ACE activity[29] suggests that an adaptive response to increasing needs may occur during ageing. ACE may also function as an immunomodulator by helping to process endogenous peptides within the MHC class I complex in cytotoxic T lymphocytes[30], and its level is also associated with the I/D polymorphism in these cells[28]. These data indicate potential roles of ACE, outside the cardiovascular system, that may well influence survival. Insofar as the quantity of ACE enzyme is a significant parameter in these other functions, they provide a rationale for understanding the differential influence on survival of the *ACE*-I/D polymorphism. However, the association with longevity may also stem from linkage disequilibrium with another influential polymorphism in a nearby gene. In this regard, it is notable that the gene for human growth hormone, which may have an important role in senescence[31], shows strong linkage to *ACE*, on chromosome 17q23 (ref. 26).

Table 6 Comparison of *APOB* allele frequencies between centenarians and controls

APOB alleles	Centenarians numbers (frequency)	Controls numbers (frequency)
X^+	297 (0.518)	166 (0.494)
X^-	275 (0.482)	170 (0.506)
ins	294 (0.671)	220 (0.659)
del	144 (0.329)	114 (0.341)

X^+/X^-, Presence/absence of the *Xba*I site at codon 2488. ins/del, Insertion/deletion allele in the signal peptide region (ref. 19).

The same questions arise for ApoE, which lies in the apolipoprotein cluster in chromosome 19q13.2 (ref. 32). There is compelling evidence for a direct effect of the ε4 allele: ApoE plays a role in nerve development and repair; it is expressed in large quantities in the brain, where it binds to β-amyloid with an affinity that is highest for the ε4 isozyme; and the ε4 allele has an increased frequency in late-onset Alzheimer's disease (AD) patients[33]. It was shown recently that ε4 has a dose-effect on risk of AD, age at onset and survival, in both familial and sporadic late-onset forms of the disease[34]. The ratio of ε4 carriers between late onset AD patients and control subjects was 2 and 2.6 respectively for the sporadic and familial cases. According to a Swedish population-based study of dementia[35], 13% of the 85-year-olds had AD, with a 42.2% three year mortality rate. This population will have virtually disappeared before reaching 100 years of age, which suggests that there may be a steep negative selection differential of ε4 carriers due to AD after age 85. It would help explain a decrease in ε4 allele frequency between nonagenarians and centenarians. Furthermore, ε4 gene dose is inversely related to age of onset and survival, which strengthens the notion of its important AD-related impact on survival. Thus, ε4 appears as a risk factor for both heart disease and Alzheimer's disease, two major causes of mortality and morbidity at advanced ages.

We have reported the first non-HLA genetic associations with human longevity in centenarians, implicating two genes that, each in its own way, are simultaneously involved in the cardiovascular, central nervous and immune systems. The effects found provide mixed clues to underlying pleiotropic interactions[36]. ApoE-ε4 illustrates the idea that deleterious alleles are not selected against if they act late in life, when the effects of natural selection are weaker. However, its deleterious effects are probably spread out over the last decades of life. Perhaps these are counterbalanced by earlier beneficial influences in order to account for a stable frequency around 15% in Caucasian populations. Conversely, the ε2 variant, although coding for a metabolically defective enzyme and present at a lower frequency in adult populations, appears to have a long-term protective effect. The high frequency of the

ACE/D allele in normal populations, in spite of it being a risk factor for a leading cause of mortality, suggests that it may confer some early selective advantage. The unexpected association with longevity may indicate a late reversal of its purported negative survival value. Linkage disequilibrium with polymorphisms in neighbouring genes may complicate the picture. Finally, as longevity is the net outcome of life-long interactions between genotypes and environment, it will be important to check for such associations in populations of different ethnic backgrounds living in different environments.

Methodology

Populations. Centenarians were recruited from all over France (regional distribution: 40% Paris region, 18% central, 4% northern, 5% eastern, 15% western, 18% southern), at their home or in institutions, through media advertising and retirement organisations. They were all Caucasians residing in France. The criterium for inclusion in the study was to be at least 99 years old on the day of blood collection. The population of 338 centenarians is composed of 11% men and 89% women with a mean age of 100.71 ± 0.13. Controls were chosen among unrelated normal individuals from French CEPH pedigrees.

Genotypes. 1, The *APOE* genotypes were analyzed by *Hha*I digestion of a 244 bp PCR-amplified fragment containing the two polymorphic sites, with the restriction fragments resolved on a 10% non denaturing polyacrylamide gel[15]. 2, The *ACE* diallelic polymorphism was genotyped by amplification of the variable segment and resolution of the 190 and 490 bp alleles on a 1.5% agarose gel[16]. 3, *APOB* polymorphisms were analyzed as described previously[22] by amplification of the two DNA segments, direct visualization of the amplified product on a 10% non-denaturing polyacrylamide gel for ins/del and visualization of the *Xba*I digestion products on a 1.5% agarose gel for the Thr_{2488} substitution. 4, The polymorphism at *D4S95* was revealed by Southern hybridization with probe 674 and *Acc*I digested samples[20]. 5, The *BCL-2* microsatellite alleles were typed as described by Fougerousse *et al.* (personal communicaton). Primers 5'-TGTGTGTGTGTAGCGCGTGT-3' and 5'-CTGGCCGTGTGAGTGTGTGT-3' were used for amplification in a reaction mix containing 200 ng genomic DNA, 10 pmoles of each primer and 0.75 U of *Taq* polymerase in a final volume of 50 µl. Samples were subjected to 30 cycles consisting of 40 s at 92 °C, 30 s at 62 °C and 30 s at 72 °C. The alleles were revealed by Southern hybridization with a $(CA)_n$ probe after resolution of the fragments on a 10% denaturing polyacrylamide gel.

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