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RESEARCH ARTICLE

Lack of replication of genetic associations with human longevity

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Abstract The exceptional longevity of centenarians is due in part to inherited genetic factors, as deduced from data that show that first degree relatives of centenarians live longer and have reduced overall mortality. In recent years, a number of groups have performed genetic association studies on long-living individuals (LLI) and young controls to identify alleles that are either positively or negatively selected in the centenarian population as consequence of a demographic pressure. Many of the reported studies have shown genetic loci associated with longevity. Of these, with the exception of APOE, none have been convincingly reproduced. We validated our populations by typing the APOE locus. In addition, we used 749 American Caucasian LLI, organized in two independent tiers and 355 American Caucasian controls in the attempt to replicate previously published findings. We tested Klotho (KL)-VS variant (rs952706), Cholesteryl Ester Transfer Protein (CETP) I405V (rs5882), Paraoxonase 1 (PON1) Q192R (rs662), Apolipoprotein C-III (APOC3) -641C/A (rs2542052), Microsomal Transfer Protein (MTP) -493G/T (rs2866164) and apolipoprotein E (APOE) $\varepsilon 2$ and $\varepsilon 4$ isoforms, (rs7412 and rs429358) haplotypes respectively. Our results show that, at present, except for APOE, none of the selected genes show association with longevity if carefully tested in a large cohort of LLI and their controls, pointing to the need of larger populations for case–control studies in extreme longevity.

Keywords Association studies · Long living individuals · Longevity

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Introduction

The frequency of centenarians in the industrialized world is 1 in 10,000 people and this prevalence is rapidly changing, approaching 1 in 5,000 born in the near future (Perls 2006). The recent reduced mortality in western countries is partly a result of recent changes in food availability and diet (Cordain et al. 2005) and to other environmental changes, such as reduced exposure to infection and consequent reduction in inflammation due the discovery of antibiotics and improvement of health care. The sum of these changes has pushed the female life expectancy in industrialized countries to rise 3 months/year for 160 years by scoring a 4 decade increase in life expectancy in the last 16 decades (Oeppen and

Vaupel 2002). In addition, many other observations point to environmental factors as modulators of human longevity, such as the relationship between lower burden of sickness during childhood (expressed as reduced child mortality), a person's birth order in the family (as index of young age of the mother) and survival to extreme old age (Gavrilova NS and Gavrilov LA 2007).

On the other hand, the role of genetics in exceptional longevity is suggested by the dramatic reduction of centenarian sibling mortality levels (Perls et al. 2002). We recognize that among the phenotype traits analyzed until now, extreme longevity is apparently the most complex of all, being influenced by individual disease susceptibility and the rate of aging. In addition, we do not know which ages should be adopted to observe demographic pressure that is the loss of detrimental alleles and the enrichment of protective ones as the population age. For example, we would not expect to capture loci that influence longevity acting at a very old age if the majority of the individuals chosen for the case arm were still young. We know that genetic case control studies are influenced by many potential bias that are intrinsic in the design. False positive results are mainly due to stratification (admixture in the studied populations), lack of appropriate corrections for multiple testing (due to unpublished negative results) and lack of replications. False negatives are influenced by small sample size and the young ages in the case study. In addition, the identification of extreme longevity genetic determinants relies upon the ability to find the right genes to test among the components of biological pathways related to aging. Following APOE association with longevity, the most scrutinized pathway has been lipid metabolism. Other strategies include the investigation of human homolog of longevity genes in lower eukaryotes such as DAF genes in *C. Elegans* (Christensen et al. 2006) and the SIR2 gene in *S. Cerevisiae* (Gartenberg 2000).

Some non-synonimous SNPs of the genes Insulin Growth Factor-1 Receptor (IGF-1R), apolipoprotein B (APOB), APOC3, CETP, Angiotensin I Converting Enzyme (ACE), Interleukin 6 (IL6), PON1, Sirtuin 3 (SIRT3), Klotho and many others have also been tested and shown to have associations with extreme longevity. However, these associations have not been successfully reproduced. Of the few dozen polymorphisms tested so far by the scientific community, the only locus consistently associated with longevity is the haplotype that determines APOE isoforms $\varepsilon 2$ and $\varepsilon 4$ (Lewis and Brunner. 2004). Apolipoprotein E is a polymorphic protein involved in transport and redistribution of lipids in various tissues (Panza et al. 2003). The two isoforms $\varepsilon 2$ and $\varepsilon 4$ were previously associated with susceptibility to cardiovascular and Alzheimer disease and subsequently were also associated with extreme longevity (Schachter et al. 1994).

In this work, we attempted to replicate previous reported associations using: (1) APOE as an internal positive control to validate our populations; (2) populations that in the previous experiments did not show evidence of stratification (data unpublished); (3) two large independent cohorts of LLI.

Among the several polymorphisms that have been previously associated with longevity, we have chosen the association studies that in our opinion needed further clarification.

The SNPs tested in this paper are -493G/T MTP, APOE $\varepsilon 2$ (TT) and $\varepsilon 4$ (CC) haplotypes, CETP I405V, KL-VS, PON1 Q192R and -641 C/A APOC3.

MTP assembles very low density lipoprotein (VLDL) in the liver and regulate the chilomicrons intestinal absorption. A sib-pair analysis strategy was adopted to identify a locus at D4S1564 (Puca et al. 2001) that was subsequently investigated with haplotype based associations on 875 polymorphisms that identified a -493G/T MTP variant. This variant showed a consistent association in a second group of 250 centenarians and 250 controls selected to minimize the mahalanobis distance with respect to the cases to avoid stratification (Geesaman et al. 2003) (Table 1). As for many other studies, the MTP association has not been reproduced in Caucasian European populations (Nebel et al. 2005; Beekman et al. 2006,) (Table 1).

Mice homozygous for severely hypomorphic alleles of the Klotho gene showed features of accelerated aging (Kuro-o et al. 1997), while transgenic mice over-expressing the gene were long-living (Kurosu et al. 2005), making this gene an appealing candidate for human genetic association studies. The heterozygous Klotho variant KL-VS stretch (that contain six polymorphisms) in a Bohemian Czech population was increased in the elderly (protective effect) while the homozygous form was decreased

Table 1 Allele frequencies in reported studies of microsomal triglycerid transfer protein

MTP (-493 G/T) (Ref)[a]	Nationality	LLI[b]	Age[c]	Offspring	Controls	Age	Allele frequency[d] (%)		*P* value
							G	C	
Geesaman et al. (2003)	U.S. Caucasian	175	100.8		183	38.6	26 vs 33	74 vs 67	0.039
	U.S. Caucasian	244	100.8		231	38.6	24 vs 32	76 vs 68	0.029
	French	559	103.1		552	51.2	25 vs 26	75 vs 74	0.54
Nebel et al. (2005)	German	370	102		540	67	28 vs 25	72 vs 75	0.28
	German	1033	98.2		540	67	26 vs 25	73 vs 75	0.34
Beekman et al. (2006)	Caucasian			525	251	59	27 vs 24	73 vs 76	0.41
	Caucasian	379	94		251	59	26 vs 24	74 vs 76	0.60
	Caucasian	655	87		244	31	24.9 vs 24	75.1 vs 76	0.64

Abbreviations: [a]: reference; [b]: long living individuals; [c]: refer to mean age; [d]: %centenarians vs %controls

(detrimental effect) (Arking et al. 2002). The protective effect of the heterozygous state was not corroborated in a second population from the Baltimore WHAS and BWIS studies, stratified by race, while the protective role of the homozygous for VS genotype was confirmed (Arking et al. 2002).

A follow-up study on Ashkenazi-Jewish populations replicated the heterozygous advantage but not the homozygous disadvantage (Arking et al. 2005) (Table 2).

Individuals with exceptional longevity have shown significantly larger low density lipoprotein (LDL) and high density lipoprotein (HDL) particle sizes (Barzilai et al. 2003) and consequently CETP, which determines lipid particle size (Arai et al. 2000), was investigated in the above mentioned Ashkenazi-Jewish populations. Among the few SNPs tested, the homozygous GG genotype of the CETP I405V polymorphism (that correspond to VV), increased in centenarians and centenarian offspring (Barzilai et al. 2003). A follow up study on European centenarians did not replicate the association (Cellini et al. 2005) (Table 2).

APOC3 is a major component of very LDLs and a minor component of HDL. In a recent study, Ashkenazi-Jewish populations were genotyped for 66 SNPs in 33 candidate genes related to cardiovascular diseases. Among these, APOC3 -641CC genotypes were more frequent among long-living individuals. The CC carriers had lower prevalence of hypertension and greater insulin sensitivity (Atzmon et al. 2006) (Table 2).

Q192R variant of PON1 was selected for its role in regulating LDL oxidation. This polymorphism showed a controversial association with longevity, being sometimes reported as increased (protective) in centenarian populations, and sometimes decreased (detrimental) (Rea et al. 2004) (Table 2).

The results reported in this study hopefully clarify the need of using large populations to avoid false positives and identify true associations with longevity, like APOE.

Methods

Subjects

Group I: 381 US LLI (309 females and 79 males, mean age 101.7 years [age range 93–111; Confidence interval ±9.3]); group II: 368 US LLI (174 females and 194 males, mean age 95.9 years [age range 93–105; Confidence interval ±8.7]) and 355 anonymous controls, self-identified as "Caucasian" and <35 years of age (191 females and 164 males, mean age 27.4 years [age range 0–35; Confidence interval ±7.6]). LLI data collected included health and socio-demographic histories, proof of age, usually in the form of a birth certificate, a three-generation pedigree, and measures to assess functional independence and cognitive status.

The subjects were selected among the general population by different strategies, contacted by mail or telephonically, and properly consented.

The institutional review board granted approval for this study and all subjects involved signed an informed consent form. This study was conducted in accordance with the ethical principles that have their origins in the Declaration of Helsinki. Individuals were identified and recruited by a variety of methods

Table 2 Genotype and allele frequencies in published studies

Ref[a]	Rs	Nationality	LLI[b]	Age	Co[c]	Controls	Mean age	Genotype frequency[d] (%)			*P* value	Allele frequency[d] (%)		*P* value
CETP								II	IV	VV		I	V	
Arai et al. (2000)	rs5882	Ashk[e]	213	98	216	258	68.3			25 vs 21 vs 9	0.001	54 vs 57 vs 71	46 vs 43 vs 29	
Cellini et al. (2005)	rs5882	Italian	175	>100		189	70	47 vs 40	44 vs 50	9 vs 10	0.63	69 vs 65	31 vs 35	0.18
APOC3								AA	AC	CC				
Atzmon et al. (2006)	rs2542052	Ashk[e]	213	95–107	216	258	71.3			25 vs 20 vs 10	0.0001[f] 0.001[g]			
KLOTHO								FF	FV	VV				
Arking et al. (2002)	rs9527026	Bohemian Czech	435	>75		611	nb[h]	74 vs 79	25 vs18	1 vs 3	0.08			
		Baltimore Caucasian	723	>75		420	nb[h]	73 vs 74	26 vs 23	1 vs 3	0.05			
		Baltimore African-American	242	>75		226	nb[h]	70 vs 69	28 vs 26	2 vs 5	0.06			
Arking et al. (2005)	rs9527026	Ashk.[e]	216	>95		309	51–94				0.004[i]			
PON1								QQ	QR	RR		Q	R	
Rea et al. (2004)	rs662	Italian	308	101				46 vs 55	45 vs 37	9 vs 8	0.03	68 vs 73	32 vs 27	0.02
		Ireland	296	90		296	41	47 vs 52	44 vs 43	9 vs 5	0.14	69 vs 74	31 vs 26	0.09

Abbreviations: [a]: reference; [b]: long living individuals; [c]: centenarian offspring; [d]: %centenarians vs %centenarians offspring vs %controls; [e]: Ashkenazi Jewish population; [f]: *P* value referred to centenarians vs controls; [g]: *P* value referred to Offspring vs Controls; [h]: newborn; [i]: In Ashkenazi populations a significant increase in the frequency of KL-VS heterozygous individuals is observed for ages >79 years ($p < 0{,}004$)

including institutional web sites, direct mailings, and advertisements in newspapers targeting potential participants or organizations involved with the aging community.

DNA isolation and genotyping

Centenarian and control DNA was isolated from whole blood using a Qiamp DNA blood Midikit (Qiagen) according to the manufacturer's protocol.

Genotyping was performed using Taqman Assay (Applied Biosystems). PCRs and and post-PCR fluorescence measurements were carried out on an ABI7900 (Applied Biosystems). For genotyping, cluster plots were made of the fluorescent labels using SDS software (v2.2 Applied Biosystems). Spots falling outside a cluster were labelled as undetermined. Standard quality control procedures were applied such us duplications of the DNA extractions and comparisons of the new aliquots with plated DNA by SNP typing.

Statistical analysis

The chi-square test was performed using the statistical package Stata ve 9.1 for a comparison of the allele and genotype frequencies between the groups.

We used a subroutine GENHW to estimate allele, genotype frequencies and disequilibrium coefficients which performs asymptotic Hardy-Weinberg (HW) equilibrium tests.

For the case–control genetic studies was used GENCC that calculates the *p*-value and the confidence interval (95%) of the odds ratio. A *p*-value less than 0.05 was considered the threshold for statistical significance. The subroutine TABULATE was used to produce one-way tables of frequency counts along with measures of association. The haplotype association test between cases and controls was performed using Haploview 3.2 software. (http://www.broad.mit.edu/personal/jcbarret/haploview/index.php) (Barret et al. 2005).

Results

In the present study we have genotyped 749 LLI (divided in two groups of samples) and 355 controls from USA for seven SNP in the genes supposedly associated with longevity.

The analysis of APOE ε2 (TT haplotype, Cys112-Cys158) and ε4 (CC haplotype, Arg112-Arg158) isoforms (rs7412 and rs429358) showed, as expected, that isoform ε2 is enriched and isoform ε4 is depleted in centenarians compared to controls (Table 3).

-493G/T MTP polymorphism was genotyped using the perfectly correlated SNP rs2866164 (Geesaman et al. 2003). The first comparison (LLI group I versus controls) shows a statistically significant decrease in the frequency of the minor allele and the genotype GG in centenarians compared to control population, similar to our previous report (Geesaman et al. 2003) (Table 4). In the second comparison (LLI group II versus controls) the association did not approach significance. Due to other reports that show a difference in control allele frequency, we genotyped an additional 156 controls (mean age 30.2 years). By including these controls the association disappeared (Table 4).

The homozygous GG (that corresponds to VV) for CETP I405V (rs5882) shows no differences in frequency among cases and controls (Table 5). This is contrary to the original data published (Arai et al. 2000), but is in accordance with a subsequent attempt to reproduce the results in Italian populations (Cellini et al. 2005) (Table 2).

KL-VS variant (rs952706), PON1 Q192R (rs662), and -641C/A APOC3 (rs2542052) showed the same distributions of allele and genotype frequencies between cases and controls (Table 5), not supporting previous reports (Table 2). In addition, we combined the analysis of group I and II (Table 5) to see if a modest effect could be detected by a larger population set. None of the association tests supported the original findings, including -493G/T MTP, and despite a nominal p value of $p = 0.04$ ($p = 0.08/2$ being the hypothesis tested one-way: cases minor allele frequency < control minor allele frequency),

Table 3 APOE ε4 haplotype frequencies

	ApoE isoforms	LLI[a] vs controls frequencies	Chi-square	*P* value
US group I	ε4	0.055 vs 0.118	19.419	0.0000105
US group II	ε4	0.067 vs 0.122	12.968	0.0003

Abbreviations: [a]: long living individuals

Table 4 Genotype and allele frequencies of MTP

MTP	LLI* I group (318)	Controls I group (350)	Controls II group (506)	LLI* II group (376)	Controls I group (350)	Controls II group (506)	LLI* I+ II group (694)	Controls II group (506)
Genotype frequency								
CC (%)	57.5	50.8	53.1	55.8	50.8	53.1	56.6	53.1
CG (%)	37.1	38.4	37.8	36.7	38.4	37.8	36.8	37.8
GG (%)	5.3	10.7	9.0	7.4	10.7	9.0	6.4	9.0
P value genotype		0.024	0.111		0.201	0.557		0.177
Odds ratio (95% C.I.)**		1.3 (1.06–1.73)	1.2 (0.98–1.55)		1.2 (0.97–1.54)	1.1 (0.9–1.3)		0.85 (0.71–1.02)
Allele frequency								
C (%)	76.1	70.1	71.5	74.2	70.1	71.5	75.1	71.5
G (%)	23.9	29.9	28.5	25.8	29.9	28.5	24.9	28.5
P value allele		0.012	0.062		0.07	0.27		0.084

Abbreviations: *: long living individuals; **: confidence interval

the association test did not pass the level of significance after Bonferroni correction for multiple testing.

Discussion

Many case control studies for exceptional longevity have been published in the recent years, and, with the exception of APOE, none of the initial findings have been consistently reproduced. In an attempt to shed light on longevity variants, we selected some of the SNPs previously reported to be associated with longevity. To avoid false positive associations we decided to genotype two tiers of centenarians and one of controls, to minimize eventual admixture among the centenarians. To address the problem of false negative results (lack of power), we have run the study on large set of samples (380) with average ages of cases above 95 years to capture eventual alleles that confer advantage or disadvantage at very old ages. The results of our analysis show a consistent association of APOE $\varepsilon 4$ with longevity while the analysis of Klotho KL-VS variant (rs952706), CETP I405V (rs5882), PON1 Q192R (rs662), -641C/A APOC3 (rs2542052) and -493G/T MTP (rs2866164) showed no associations with exceptional longevity.

To be noted, we had to run additional 156 controls for -493G/T MTP to disentangle the incongruent reports of the other groups that showed no association.

Overall, these results indicate that, for the exception of APOE, some of the previous attempts to identify genes influencing longevity are not replicable.

An explanation on why so many findings are not replicated by other studies could be that reported associations, due to stratification by age between cases and controls, instead of pointing to longevity enabling polymorphisms, select polymorphisms that have changed in frequency in younger generations due to recent immigration. This could be especially true in the case of polymorphisms that show a great difference in allele frequencies among ethnicities and so more sensitive to undetected mild stratification. It has to be said that, despite the above mentioned reasonable theories, our initial analysis of 450 nonagenarians and 450 young controls with 317 thousand SNPs did not show any stratification

Table 5 Genotype and allele frequencies of PON1, Klotho, CETP and ApoC3

	LLI[a]		LLI[a]	Controls
	Group I	Group II	Group I + II	
PON1 (Q192R)	(350)[b]	(360)[b]	(710)[b]	(351)[b]
GG (%)	9.8	10.2	10	11
AG (%)	40.8	39.7	40.2	39.1
AA (%)	49.3	50	49.6	49.8
P value genotype	0.477	0.933	0.849	
G (%)	30.3	30.1	30.2	30.7
A (%)	69.7	69.9	69.8	69.3
P value allele	0.861	0.816	0.814	
Odds ratio (95% C.I.[c])	1.0 (0.81–1.27)	0.97 (0.77–1.21)	0.97 (0.80–1.18)	
KLOTHO (KL-VS)	(345)[b]	(363)[b]	(708)[b]	(332)[b]
GG (%)	67.8	76.4	73	72.5
AG (%)	28.1	21.7	24	25.6
AA (%)	4.1	1.8	3	1.8
P value genotype	0.146	0.235	0.350	
G (%)	81.9	88.1	85.1	85.4
A (%)	18.1	11.9	14.9	14.6
P value allele	0.081	0.2437	0.721	
Odds ratio (95% C.I.[c])	1.29 (0.96–1.72)	0.79 (0.58–1.08)	1.04 (0.80–1.36)	
CETP (I405V)	(361)[b]	(361)[b]	(722)[b]	(348)[b]
GG (%)	9.1	11.3	10.2	11.4
AG (%)	40.4	41.2	40.8	47.4
AA (%)	50.4	47.4	48.9	41
P value genotype	0.041	0.19	0.050	
G (%)	29.4	32	30.6	35.2
A (%)	70.6	68	69.4	64.8
P value allele	0.018	0.188	0.0335	
Odds ratio (95% C.I.[c])	1.3 (1.04–1.63)	1.15 (0.93–1.43)	0.81 (0.67–0.98)	
APOC3 (–641 C/A)	(356)[b]	(357)[b]	(713)[b]	(354)[b]
CC (%)	11.5	13.7	12.7	16.1
AC (%)	48	46.5	47.3	44
AA (%)	40.4	39.6	39.9	39.9
P value genotype	0.189	0.638	0.289	
A (%)	64.5	63	63.6	61.9
C (%)	35.5	37	36.4	38.1
P value allele	0.309	0.672	0.4103	
Odds ratio (95% C.I.[c])	0.89 (0.72–1.10)	0.95 (0.77–1.18)	0.92 (0.76–1.11)	

Abbreviations: [a]: long living individuals [b]: number of samples; [c]: confidence interval

(unpublished data), in agreement with other studies (Cardon and Palmer 2003). Our data confirm the difficulty in replicating previous associations with human longevity. The use of small sample sizes and over interpretation of marginal results in the initial findings, the interpopulation heterogeneity of gene–environment and gene–gene interactions and the subtle differences in the selected phenotypes are possible explanations for such discrepancies (Cardon and Palmer 2003).

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