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Gene/longevity association studies at four autosomal loci (*REN, THO, PARP, SOD2*)

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The possibility that four loci (*REN*, *THO*, *PARP*, *SOD2*) are associated with longevity was explored by comparing the genotypic pools of subjects older than 100 years with those of younger subjects matched for sex and geographic area (northern and southern Italy). The markers (all located within the respective gene) were HUMREN4; HUMTHO1; HUMPARP $_{(gt)845nt}$; SOD2(C/T)$_{401nt}$. In order to reduce the number of genotypes, multiallelic polymorphisms were recoded as diallelic according to allele size and frequency patterns (small: S, and large: L, alleles). A significant loss of LL homozygous genotypes was found at the *THO* locus in male but not in female centenarians with respect to matched controls. On the other hand no significant difference was found between case/control genotypic frequencies at *REN*, *PARP*, *SOD2* loci. The latter loci therefore do not affect inter-individual variability in life expectancy (at least in terms of qualitative variants associated with the tested markers). However, the data is consistent with an association between the *THO* locus and longevity.

Keywords: human longevity; *REN*; *THO*; *PARP*; *SOD2*

Introduction

The number of genes which could affect inter-individual variability in human life-span is expected to be high[1] and probably the majority affect longevity by altering the risk of death at various ages.[2] One approach to identifying some of these genes is a comparative analysis at candidate loci between gene pools of extremely elderly individuals and younger people from the same population.[3] However, in this approach, special attention must be paid to case/control matching. This is particularly important when studies on Italian people are carried out, not only because different genetic components are present in the Italian gene pool,[4] but also because migrations from southern

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to northern Italy due to social and economic factors occurred in the last century. We have therefore planned a research project in which polymorphic markers at candidate loci were compared between centenarians and younger people, whose ancestry in the specific geographic area was checked back as far as the grandparents' generation.

Four autosomal genes, which code for proteins involved in fundamental metabolic pathways, were chosen as candidate loci. The genes were renin (*REN*), tyrosine hydroxylase (*THO*), poly(ADP-ribose) polymerase (*PARP*) and superexidodismutase 2 (*SOD2*).

The *REN* gene (9 exons; 1q32 chromosome) codes for renin, an aspartylprotease which catalyses the first step of the biosynthetic cascade leading to angiotensin II. An (*acag*)*n* repeat polymorphic region[5] lies in intron 7 (HUMREN4 marker). The five known alleles, designated from 8 to 12 according to the repeat number (allele 9 is absent in Caucasians), show bimodal frequency distribution, with peaks at 8 and 11 repeats.[6]

The *THO* gene (14 exons; 11p15.5 chromosome) codes for tyrosine hydroxylase, the rate-limiting enzyme for synthesis of catecholamines, aminoacid-derived molecules which act both as hormone (adrenalin) and neurotransmitters (dopamine and noradrenalin). By alternative splicing of the THO transcript, four types of mRNA are produced, which differ in the inclusion or exclusion of exon 2 in the spliced products.[7] Specifically in intron 1 an (*aagt*)*n* repeat polymorphism (HUMTHO1) is located,[8], with 8 alleles varying from 5 to 11 repeats (two alleles have 10 repeats; one of them, 10-1 allele, lacks a single *a*). Allele frequency distribution is bimodal, with peaks at 6 and 10-1 repeats.[9] HUMTHO1 alleles are in linkage disequilibrium with INS-VNTR alleles[10,11] which affect the levels of insulin transcription differently in pancreas[12,13] and thymus.[14,15]

The *PARP* gene (23 exons, 1q41–42 chromosome) codes for a zinc finger nuclear enzyme which is strongly activated by DNA breaks and promotes DNA repair.[16] *PARP* activity is positively correlated with species-specific life span in mammalian species.[17,18,19] A *(gt)n* repeat polymorphism lies in exon 1.[20] The known six alleles, designated according to size (85 bp–99 bp size range), show bimodal frequency distribution, with peaks at 85 bp and 93 bp alleles.

SOD2 is a manganese-containing enzyme which is coded by a nuclear gene (6q25 chromosom) and is post-translationally imported into the mitochondrial matrix.[21] Here SOD2 rapidly converts superoxide radicals into H_2O_2 which is further detoxified by catalase and glutathione peroxidase. A structural *t* to *c* substitution in the mitochondrial targeting domain has recently been described[22,23] which changes valine$_{16}$ to alanine$_{16}$ thus modulating the efficiency of mitochondrial transport.[23]

Materials and Methods

Samples

Both centenarians and controls were clinically healthy unrelated subjects from various socio-economic backgrounds. Birth and residence of each individual in the specific geographic area (northern or southern Italy) was checked back as far as the grandparents' generation. All individuals gave informed consent prior to their inclusion in the study.

Centenarians Only individuals who were over 100 years old on the day the blood was taken were included in the study. The oldest subject was a 109-year-old woman; 109 subjects (83 females and 26 males) were from northern Italy (Lombardia, Veneto, Liguria, Emilia-Romagna) and 87 subjects (60 females and 27 males) were from southern Italy (Sicilia, Calabria, Campania, Puglia).

Controls The ages of the control group ranged from 10 to 85 years (median ages 41 and 31 years in northern and southern Italian samples, respectively); 119 subjects (70 females and 49 males) were from northern Italy and 239 subjects (126 females and 113 males) were from southern Italy, from the regions listed above. The individuals aged from 10 to 18 years were children from primary and secondary schools, whilst the others were volunteer donors (chiefly undergraduate students and University staff) invited to take part in the study by an appropriate campaign. A total of eight sub-samples were obtained, arranged by age (centenarians/controls), sex (males/females) and geographic area (northern/southern Italy).

Genotypic Typing

DNAs were extracted from blood buffy-coats following standard procedures.

HUMREN4 and HUMTHO1 typings were carried out by PCR co-amplification of the DNA target sequences.[9] Alleles were detected by silver staining. Home-made allele ladders were used as size markers. The quality of the ladder was tested by comparison with a standard ladder kindly donated by Dr C Previderè, University of Pavia. Alleles were designed according to the repeat number.[9] PARP$_{(gt)n845nt}$ polymorphism was typed on PCR-amplified DNA products[20] stained by silver. Home-made allele ladders were used as size markers.

(t/c)$_{401nt}$ SOD2 polymorphism was investigated by an original ARMS-PCR assay. PCR reaction was carried out in a 25 μl volume containing 80 ng genomic DNA, 0.5 μM of a sense primer specific for either *c* (5'gcaggcagctggctccgac3') or *t* (5'gcaggcagctggctccgat3') at nucleotide +401 and an antisense primer (5'acgcctcctggtacttctcc3'), 200 μM of each dNTP, 1.5U Taq polymerase, 1 × Taq polymerase buffer under the

Table 1 REN locus. Genotypic numbers and relative frequencies of the HUMREN4 polymorphism recoded as diallelic

North Italy				Males			
Genotypes		*Controls (n=50)*			*Centenarians (n=24)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	21	0.420	0.070	15	0.625	0.100	0.137
SL	28	0.560	0.070	7	0.292	0.093	0.046
LL	1	0.020	0.020	2	0.083	0.056	0.213
				Females			
Genotypes		*Controls (n=76)*			*Centenarians (n=83)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	44	0.579	0.057	54	0.651	0.052	0.328
SL	28	0.368	0.055	25	0.301	0.050	0.406
LL	4	0.053	0.026	4	0.048	0.023	0.711
South Italy				Males			
Genotypes		*Controls (n=38)*			*Centenarians (n=14)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	20	0.526	0.081	10	0.714	0.121	0.344
SL	16	0.421	0.080	4	0.286	0.121	0.524
LL	2	0.053	0.036	0	0	0	1
				Females			
Genotypes		*Controls (n=54)*			*Centenarians (n=36)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	27	0.500	0.068	20	0.556	0.083	0.670
SL	24	0.444	0.068	15	0.417	0.082	0.831
LL	3	0.056	0.031	1	0.028	0.027	0.624

S: alleles 7 and 8; L: alleles 10, 11, 12. *P*: probability of falsely rejecting the hypothesis of equal genotype frequency (SS or SL or LL) in centenarians and controls $\alpha = 0.017$.

following conditions. 1 min at 94°C, 21 cycles at 94°C for 25 s, 60°C for 45 s, 72°C for 30 s, 9 cycles at 94°C for 25 s, 55°C for 1 min, 72°C for 2 min. Amplification products (15 µl) were detected by ethidium bromide staining after 2% agarose gel electrophoresis.

Statistical Analysis

Allele frequencies were computed by counting genes from the observed genotypes and allele frequency distributions were examined. According to allele size and major frequency peaks, each multiallelic system (REN, THO, PARP) was recoded as a diallelic system by grouping the alleles into two classes (small: S and large: L alleles). All the following analyses were carried out on such recoded systems.

Exact tests were applied on each polymorphism to verify Hardy-Weinberg equilibrium[24] in each of the eight subsamples.

The presence of interactions among the three factors – genotype/sex/geographic area – within each sample (centenarians; controls) was tested by log-linear analysis of the respective three-way tables.[25] The frequency of each genotype was compared between centenarians and controls matched for sex and geographic area by Fisher exact tests. To reject at level $\alpha' = 0.05$ the null hypothesis that no genotypic frequency difference existed between centenarians and controls of specified sex and geographic area, the p values of the three individual tests were compared against $\alpha = 1-(1-\alpha')^{1/3} = 0.017$.

Results

Both centenarians and controls showed the known bimodal allele frequency patterns at (*REN*, *THO* and *PARP* loci. According to these patterns, alleles were

Table 2 THO locus. Genotypic numbers and relative frequencies of the HUMTHO1 polymorphism recoded as diallelic

North Italy							
				Males			
Genotypes	*Controls (n = 49)*			*Centenarians (n = 26)*			*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	17	0.347	0.068	10	0.385	0.095	0.803
SL	20	0.408	0.070	15	0.577	0.097	0.225
LL	12	0.245	0.061	1	0.038	0.038	0.027
				Females			
Genotypes	*Controls (n = 70)*			*Centenarians (n = 83)*			*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	15	0.214	0.049	14	0.169	0.041	0.537
SL	38	0.543	0.059	48	0.578	0.054	0.744
LL	17	0.243	0.051	21	0.253	0.048	0.994
South Italy							
				Males			
Genotypes	*Controls (n = 113)*			*Centenarians (n = 27)*			*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	33	0.292	0.043	10	0.370	0.093	0.488
SL	56	0.496	0.047	17	0.630	0.093	0.284
LL	24	0.212	0.038	0	0	0	0.004
				Females			
Genotypes	*Controls (n = 126)*			*Centenarians (n = 60)*			*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	48	0.381	0.043	20	0.333	0.061	0.626
SL	64	0.508	0.044	26	0.433	0.064	0.352
LL	14	0.111	0.028	14	0.233	0.055	0.047

S: alleles 6, 7 and 8; L: alleles 9, 10–1, and 10. *P*: probability of falsely rejecting the hypothesis of equal genotype frequency (SS or SL or LL) in centenarians and controls $\alpha = 0.017$.

grouped as follows. Locus *REN*: 7, 8 small (S) alleles; 10,11,12 large (L) alleles. Locus *THO*: 6, 7, 8 small (S) alleles; 9, 10-1, 10 large (L) alleles. Locus *PARP*: 85, 87, 89 small (S) alleles; 93, 95, 97, 99 large (L) alleles.

The observed genotypes of the recoded systems, as well as those of the SOD2 diallelic system, are reported in Table 1, Table 2, Table 3, Table 4. They were in agreement ($P > 0.05$) with those expected at Hardy-Weinberg equilibrium in every sub-sample at each locus, except for male centenarians at the *THO* locus ($P = 0.028$) in southern Italy. Direct comparisons between genotypes in centenarians and controls matched for sex and geographic area (last column of Table 1, Table 2, Table 3, Table 4) showed sex-specific *THO* locus/longevity association. A loss of homozygous LL genotypes was observed in male centenarians (Table 2). The loss was statistically significant ($P = 0.004$ with $\alpha = 0.017$) in southern Italy and had borderline significance ($P = 0.027$) in northern Italy. On the contrary, no significant case/control difference was found at loci *REN* (Table 1), *PARP* (Table 3) and *SOD2* (Table 4). An analysis of interactions between genotypes/sex/geographic area within both the sample selected for longevity and the controls confirmed the peculiarity observed at the *THO* locus. The geographic factor interacted with genotypic frequencies in controls ($P = 0.040$) but not in centenarians ($P = 0.142$), while sex strongly interacted with the genotypic frequencies in centenarians ($P = 0.0002$) but not in controls ($P = 0.083$). These findings suggested that:

Table 3 PARP locus. Genotypic numbers and relative frequencies of the PARP (gt)n polymorphism recoded as diallelic

North Italy				Males			
Genotypes		*Controls (n=38)*			*Centenarians (n=24)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	17	0.447	0.081	10	0.417	0.101	1
SL	16	0.421	0.080	10	0.417	0.101	1
LL	5	0.132	0.055	4	0.166	0.076	0.725
				Females			
Genotypes		*Controls (n=39)*			*Centenarians (n=63)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	20	0.513	0.080	39	0.619	0.061	0.310
SL	12	0.308	0.074	19	0.302	0.058	1
LL	7	0.179	0.061	5	0.079	0.034	0.204
South Italy				Males			
Genotypes		*Controls (n=46)*			*Centenarians (n=19)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	27	0.587	0.073	9	0.474	0.114	0.426
SL	13	0.283	0.066	9	0.474	0.114	0.159
LL	6	0.130	0.050	1	0.052	0.051	0.663
				Females			
Genotypes		*Controls (n=41)*			*Centenarians (n=41)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
SS	23	0.561	0.077	19	0.463	0.078	0.508
SL	15	0.366	0.075	18	0.439	0.077	0.653
LL	3	0.073	0.041	4	0.098	0.046	1

S: alleles 85, 87 and 89; L: alleles 93, 95, 97 and 99. *P*: probability of falsely rejecting the hypothesis of equal genotype frequency (SS or SL or LL) in centenarians and controls $\alpha = 0.017$.

i) starting from different frequency patterns in northern and southern Italian controls, the *THO* genotypic pools attained the same pattern in northern and southern Italian centenarians by means of a hypothetical selection affecting survival;

ii) survival selection throughout *THO* locus should affect males and females differently.

Discussion

The aim of the present study was to test whether the variability of four genes controlling fundamental metabolic pathways is associated with human longevity. The approach was the genotypic analysis of individuals selected for longevity and younger people matched for sex and geographic area. Genotypes rather than alleles were considered because of a non-additive variance of the longevity trait[26] and because Hardy-Weinberg equilibrium was not fulfilled in the sample of southern Italian male centenarians ($P = 0.028$).

At present, association studies are a more reliable approach to identify the genes that affect inter-individual variability in life-expectancy, but three points should be taken into account. Firstly, the risk of false positive associations caused by confounding heterogeneity factors. To reduce such a risk, both cases and controls examined in the present study were born and resident in the specific geographic area (northern or

southern Italy) for three generations at least. Furthermore, sex-matched cases and controls were compared, on the assumption that the effect of a certain gene could vary between males and females (as expected for multifactorial traits) according to sex-specific physiological scenarios. Secondly, the age of the control group. Usually people aged 20–60 years are tested as controls with respect to centenarians, since this age range should minimise the risk of case/control overlap.[3] However, this choice introduces selective criteria for the control group which reduce its randomness. In the present study, whilst a stringent selection was made as to cases (people older than 100 years), the only selection as to controls was the availability of each person to take part in the study. Thirdly, the choice of candidate loci. The loci tested in our study are good candidates, because of their biological role in fundamental metabolic pathways that could be damaged in physiological aging[27]

Neither direct comparisons between genotypes (Table 1, Table 3, Table 4) nor three-way analyses revealed significant differences between case/control genotypic pools at *REN*, *PARP*, *SOD2* loci. Therefore HUMREN4, PARP $(gt)_{845nt}$ and SOD2$(c/t)_{401nt}$ polymorphisms are not markers of longevity. On the other hand positive results were found at the *THO* locus. The loss of LL genotypes in male centenarians with respect to controls in both southern and northern Italy is evident (Table 2), although statistical significance is borderline in northern Italian males when adjustment for multiple comparison is made. In any case, we agree with Rothman[28] that reducing the type 1 error for null

Table 4 SOD2 locus. Genotypic numbers and relative frequencies of the SOD2 (C/T) polymorphism

North Italy				Males			
Genotypes		*Controls (n = 46)*			*Centenarians (n = 25)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
CC	12	0.261	0.065	7	0.280	0.090	1
CT	23	0.500	0.074	10	0.400	0.098	0.464
TT	11	0.239	0.063	8	0.320	0.093	0.576
				Females			
Genotypes		*Controls (n = 60)*			*Centenarians (n = 80)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
CC	11	0.183	0.050	12	0.150	0.040	0.649
CT	30	0.500	0.064	41	0.512	0.056	1
TT	19	0.317	0.060	27	0.337	0.053	0.857
South Italy				Males			
Genotypes		*Controls (n = 40)*			*Centenarians (n = 19)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
CC	6	0.150	0.056	1	0.053	0.051	0.411
CT	19	0.475	0.079	12	0.631	0.111	0.282
TT	15	0.375	0.076	6	0.316	0.107	0.775
				Females			
Genotypes		*Controls (n = 40)*			*Centenarians (n = 43)*		*P*
	No.	*Rel. freq*	*St. err*	*No.*	*Rel. freq*	*St. err*	
CC	6	0.150	0.056	7	0.163	0.056	1
CT	21	0.525	0.079	24	0.558	0.076	0.827
TT	13	0.325	0.074	12	0.279	0.068	0.811

P: probability of falsely rejecting the hypothesis of equal genotype frequency (CC or CT or TT) in centenarians and controls. $\alpha = 0.017$.

associations increases the type II error for those associations that are not null. On the other hand, it is well known that the number of males who survive to very old age is much lower than that of females. If a certain genotype is unfavourable to longevity only (or primarily) in males, a gender gap in longevity should ensue. Our results suggest that the *THO* locus could be one of those which correlate with gender effect in human longevity.

The finding that the HUMTHO1 marker is associated with long life expectancy and that such an effect is sex-related gives rise to two questions. Could a tandem-repeat polymorphic region affect life expectancy? Why does such an effect occur in males but not in females? As to the first question, HUMTHO1 lies in intron 1. It was proposed[7] that secondary structures of the first intron and between first and second introns could affect THO alternative splicings. We might speculate that the variability of HUMTHO1 may play a role in the efficient production of spliced mRNAs. Indeed recent data has shown that the biological role of VNTR alleles of different size may be different.[29,30] On the other hand, since HUMTHO1 and INS-VNTR markers are in linkage-disequilibrium and INS-VNTR allele frequencies show a sex-specific age-related trend,[31] it may also be that sex-specific age-related variations of the genotypic pool embrace the entire DNA region comprising both *THO* and *INS* loci. In our samples at present, we are analysing further markers occurring in the (11p15.5) DNA region in order to clarify this point. As to the second question, we must consider that the effect of a gene on a multifactorial trait depends on the physiological background in which the gene is expressed. Therefore, if the age-related physiological scenario changes in males and females differently, the effect of a certain gene on survival could vary between the sexes.

In conclusion, the data here reported shows that the genotypic pools at *THO* locus differ between male centenarians and male controls, the LL homozygous genotypes being very rare in centenarians. Furthermore, the specificity of the positive association found at the *THO* locus is validated by the negative findings at *REN, PARP* and *SOD2* loci.

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