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Variability of the *SIRT3* gene, human silent information regulator *Sir2* homologue, and survivorship in the elderly

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Abstract

The human sirtuin 3 (*SIRT3*) gene encodes a putative mitochondrial NAD-dependent deacetylase (SIRT3) which belongs to the evolutionary conserved family of sirtuin 2 proteins. Studies in model organisms have demonstrated that *SIR2* genes control lifespan, while no data are available regarding a possible role of *SIRT3* in human longevity. By analysing the genotype-specific survival function relevant to the G477T marker of *SIRT3*, we found that in males the TT genotype increases ($p = 0.0272$), while the GT genotype decreases ($p = 0.0391$) survival in the elderly. Since *SIRT3* lies in a chromosomal region (11p15.5) where four genes potentially associated with longevity are located (*HRAS1*, Insulin-like Growth Factor 2, Proinsulin, and Tyrosine Hydroxylase) we tested for linkage-disequilibrium between G477T alleles and alleles of the above genes. The disequilibrium was not significant in any case, thus suggesting that *SIRT3* itself, or a gene strictly linked to *SIRT3*, may have a role in human longevity.

Keywords: *SIRT3* gene; Human longevity; Silent Information Regulator 2 homologue

1. Introduction

The human sirtuin 3 (*SIRT3*) gene codes for a mitochondrial protein (SIRT3) (Schwer et al., 2002; Onyango et al., 2002) which is homologous to the yeast protein Sir2p (silent information regulator 2 protein) the founding member of a large family of NAD + dependent protein deacetylases named sirtuins (Smith et al., 2000). The *SIR2* gene is a key determinant in yeast lifespan: indeed null mutations of *SIR2* shorten while an extra-copy of *SIR2* extends lifespan, probably through chromatin silencing in the ribosomal DNA repeats (Kaeberlein et al., 1999; Sinclair and Guarente, 1997). Also in *C. elegans* a *SIR2* homologue, *sir-2.1*, seems to modulate lifespan through a mechanism actually unknown, but probably associated with the insulin signalling pathway (Tissenbaum and Guarente, 2001). The role played by Sir2p as NAD + dependent protein deacetylase links cellular metabolism, transcriptional silencing and ageing (Guarente, 2000) and it has been proposed that *SIR2* genes may regulate ageing in many species, possibly by coordinating the pace of ageing to the metabolic rate (Guarente and Kenyon, 2000; Guarente, 2001).

The *SIRT3* gene (www.ncbi.nlm.nih.gov/omim: MIM 604481; contig NT_035113) lies at the telomeric terminal on 11p15.5 chromosome. Allelic association studies carried out in people older than 100 years showed that a relationship exists between longevity and polymorphism of four genes located in this region. In particular, by analysing an STR marker of the Tyrosine Hydroxylase (*TH*) gene, as well as the haplotypes defined by this marker and RFLPs of Proinsulin (*INS*) and Insulin-like Growth Factor 2 (*IGF2*) genes, significant age-related variations of the allelic pool had been observed (De Luca et al., 2001;

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Tan et al., 2002); similar age-related variations were also found for the 3′VNTR marker of the *HRAS1* gene (Bonafè et al., 2002). The *HRAS1* gene (contig NT_035113) lies at 0.665 Mb from the telomere, while the genes *IGF2*, *INS* and *TH* lie at 2.112, 2.140, and 2.145 Mb, respectively, from the telomeric terminal and are comprised within the contig NT_028310. Therefore, within a short piece of chromosome, five genes potentially involved in longevity are located.

The effect exerted on yeast and worm lifespan by sirtuin 2 genes, as well as the location of *SIRT3* in the 11p15.5 chromosomal region, prompted us to investigate whether variants of this gene were associated with longevity. Since polymorphisms of *SIRT3* were not known, we first searched for variability in the *SIRT3* region encoding for a core domain that contains short motifs of conserved amino acids (Frye, 1999, 2000). After the identification of a SNP marker in exon 3, we used this marker in searching for possible modifications of the genetic pool which may occur as the population ages and survival selection operates.

2. Materials and methods

2.1. Population sample

An appropriate campaign of recruitment was launched in Calabria (southern Italy) for genetic studies on ageing in 1999, excepted for centenarians whose recruitment started beginning from 1994. The recruitment campaign was focused on University students and staff for 18–60 years old people, thermal baths and aged people-Academy for 60–80 years old people. As to the centenarians, they were identified through the birth registers of the Municipalities of Calabria and then contacted. Only subjects born and living in Calabria were invited to take part to the study and after a detailed explanation of the research project, more than 90% of the contacted persons agreed to participate and gave their informed consent to studies on ageing carried out by the present research group. For each subject routine blood analyses were carried out and, in addition, subjects older than 60 years underwent a complete clinical and geriatric assessment. A total of 801 unrelated subjects (331 males and 470 females) free of clinically overt pathologies and having routine blood parameters in the normal sex- and age-specific range were enrolled in the study. The sample included 120 subjects older than 100 years (36 males and 84 females). These centenarians, classified in the categories A and B according to the criteria reported by Franceschi et al. (2000), were in a fairly good health status and did not suffer major pathologies or disabilities.

2.2. DNA analyses

After DNA extraction from blood buffy coats of 50 subjects randomly chosen, a 738 bp DNA fragment (2813–3551 nt, GeneBank accession NT_035113) which included the GAGISXXXGIPXFR conserved motif of the *SIRT3* gene (exons 2 and 3) was amplified by PCR and automatically sequenced by ABIPRISM 310 DNA sequencer. A silent transversion was identified in exon 3 at the position 477 of the coding region (G477T corresponding to Ser159Ser; AF083108). This mutation originated a *Bgl* II polymorphic restriction site. Restriction analysis was then used to analyse the G477T polymorphism in the whole sample population. Details on the experimental procedures are available on request (g.debenedictis@unical.it).

2.3. Statistical analyses

A demographic genetic approach was applied to relate the observed empirical frequencies with demographic characteristics of sub-populations formed by carriers and non-carriers of the respective genotypes (Yashin et al., 1999, 2000; Tan et al., 2002). In particular, parametric, semi-parametric, non-parametric and relative-risk models were used to discover possible differences between the survivorship of carriers and non-carriers of TT, GG and GT genotypes. Only the non-parametric model gave adequate parametrization of mortality rates in Gompertz's form, with corresponding modelled survival curves giving appropriate approximation to empirical survival function for the whole population. Therefore we tested the formal hypothesis of gene longevity effects (standard likelihood ratio test) by the non-parametric model, and calculated p-values based on upper-tail χ^2 values (MATLAB software).

Note that in this study we deal with cross-sectional data, where gene frequencies for different ages are calculated for individuals from different cohorts. We combine this information with demographic life table data to evaluate age patterns of mortality rates and survival functions for carriers of respective genes or genotypes. Before statistical calculations using cross-sectional data on genetic markers were made, we performed preliminary historic-demographic analysis of the Italian population where data came from. Such analysis was needed to make sure that migration processes did not produce much change in the initial frequencies of respective genes and genotypes in the cohorts participating in the cross-sectional study.

The method described by Weir (1996) was used to assess linkage disequilibrium D, and to check the null hypothesis $D = 0$, between the G477T marker of *SIRT3* and each of the markers previously analysed at the loci *HRAS1* (Bonafè et al., 2002) and *IGF2*, *INS*, *TH* (De Luca et al., 2001). Maximum Likelihood Estimation (MLE) was used to estimate the haplotypic frequencies (Arlequin 1.1 software).

3. Results

By searching for variability in the evolutionary conserved domain of the *SIRT3* gene (exon 2–exon 3) we

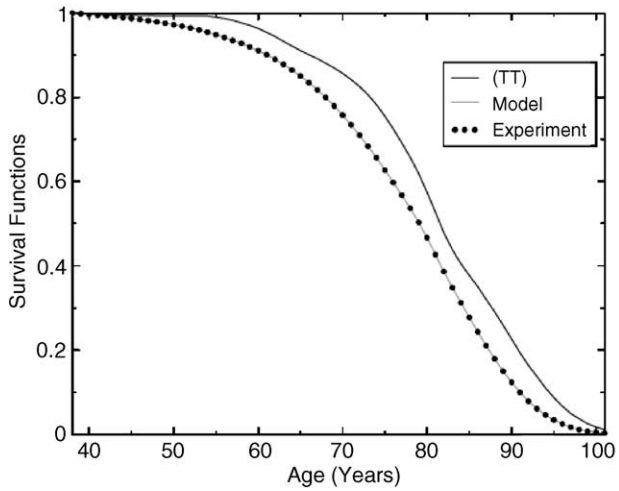


Fig. 1. Survival functions for carriers of TT genotype among Italian males. Empirical and modelled survival function for the whole population are also shown (please note that the empirical curve almost completely overlaps with the modelled one).

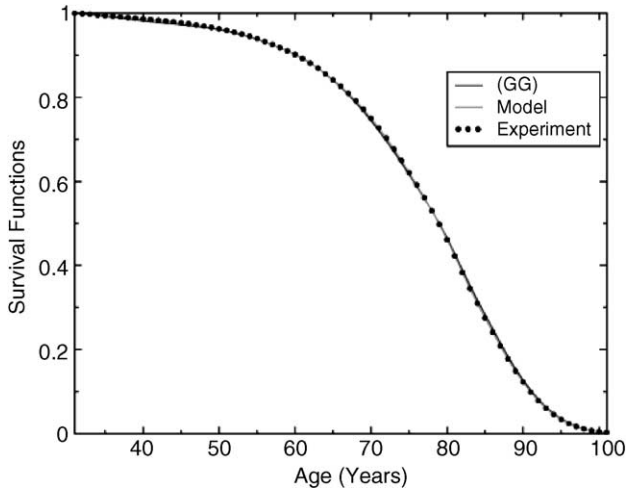


Fig. 3. Survival functions for carriers of GG genotype among Italian males. Empirical and modelled survival function for the whole population are also shown (please note that the three curves almost completely overlap).

identified a silent G/T transversion at the position 477 of the coding region (G477T corresponding to Ser159Ser; AF083108). We used this marker to investigate possible modifications of the *SIRT3* gene pool in people of increasing age, including the oldest old. We found that, in males, the TT genotype increased ($p = 0.0272$) while the GT genotype decreased ($p = 0.0391$) the value of the survival function; the GG genotype did not affect significantly the survival function ($p = 0.871$) (Figs. 1–3; note that the modelled functions start from the minimal age present in the subpopulation of carriers of the respective genotype, and then scales along the age axes are different). As previously observed for other loci (see Franceschi et al., 2000; De Luca et al., 2001 and references therein) the association between *SIRT3* variability and survival was restricted to males, because no genotype specific variation of the survivorship was observed in females ($p = 0.674$ for TT; $p = 0.845$ for GT; $p = 0.777$ for GG).

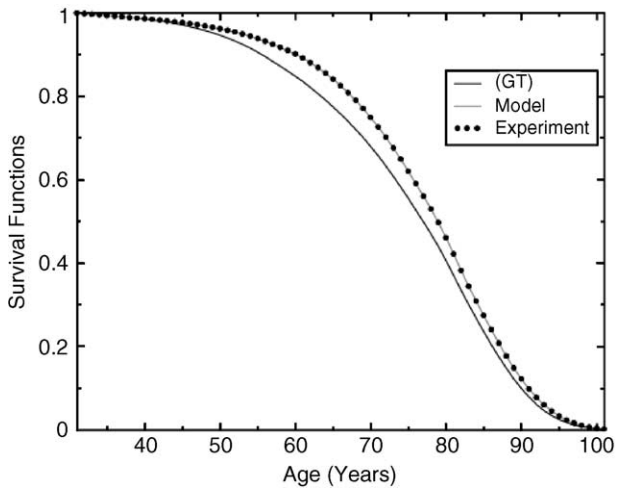


Fig. 2. Survival functions for carriers of GT genotype among Italian males. Empirical and modelled survival function for the whole population are also shown (please note that the empirical curve almost completely overlaps with the modelled one).

The sample we were analysing in the present study had already been typed, at least in part, for markers of other genes spanning for 1.5 Mb about in the same region and associated with longevity (*HRAS1*, *IGF2*, *INS*, *TH*). Since the association between *SIRT3* and survivorship could be due to allelic associations, we tested for linkage disequilibrium (LD) between the G477T marker of *SIRT3* and markers of the above genes. In particular, after verification of the Hardy Weinberg equilibrium for the *SIRT3* polymorphism (see Supplementary material), LD was checked between G477T polymorphism and *HRAS1*-3′VNTR; *IGF2-Ava* II-RFLP; *INS-Fok* I-RFLP, and *HUMTHO.1*-STR. Results are shown in Tables 1–4, respectively, where it can be seen that the hypothesis $D = 0$ cannot be rejected in any case.

Table 1
SIRT3-$(G/T)_{3223}$*/HRAS1-3′VNTR* linkage disequilibrium (*D*) analysis in a sub-sample of 145 subjects

Haplotypes ($n = 290$)	Frequency (× 100)	Standard errors (× 100)
G-a1	43.50	3.36
G-a2	8.67	1.79
G-a3	7.93	1.74
G-a4	4.23	1.40
T-a1	16.84	2.47
T-a2	0.99	0.86
T-a3	1.38	0.99
T-a4	4.04	1.38
Others	12.42	1.90

Haplotypic frequencies are only reported for *HRAS1-3′VNTR* common alleles (a1, a2, a3, a4) while haplotypes containing rare alleles are grouped as Others (Bonafè et al., 2002). Haplotypic frequencies are estimated by maximum likelihood method (Arlequin 1.1 software). The hypothesis $D = 0$ is not rejected ($p = 0.124 \pm 0.001$).

Table 2
SIRT3-$(G/T)_{3223}$/*IGF2-Ava* II-RFLP linkage disequilibrium (*D*) analysis in a sub-sample of 130 subjects

Haplotypes ($n = 260$)	Frequency (× 100)	Standard errors (× 100)
G-P′	25.54	2.99
G-A′	47.16	3.28
T-P′	7.92	2.10
T-A′	19.38	2.87

IGF2-AvaII-RFLP alleles are named according to the presence/absence (P′/A′) of the AvaII restriction site (De Luca et al., 2001). Haplotypic frequencies are estimated by maximum likelihood method (Arlequin 1.1 software). The hypothesis $D = 0$ is not rejected ($p = 0.499 \pm 0.002$).

Table 3
SIRT3-$(G/T)_{3223}$/*INS-Fok* I-RFLP linkage disequilibrium (*D*) analysis in a sub-sample of 130 subjects

Haplotypes ($n = 260$)	Frequency (× 100)	Standard errors (× 100)
G-P	10.57	2.17
G-A	62.12	3.26
T-P	4.43	1.68
T-A	22.88	2.83

INS-FokI-RFLP alleles are named according to the presence/absence (P/A) of the FokI restriction site (De Luca et al., 2001). Haplotypic frequencies are estimated by maximum likelihood method (Arlequin 1.1 software). The hypothesis $D = 0$ is not rejected ($p = 0.810 \pm 0.001$).

Table 4
SIRT3-$(G/T)_{3223}$/*HUMTHO.1-STR* linkage disequilibrium (*D*) analysis in a sub-sample of 718 subjects

Haplotypes ($n = 1436$)	Frequency (× 100)	Standard errors (× 100)
G-6	20.84	1.19
G-7	10.01	0.92
G-8	9.06	0.86
G-9	13.52	1.03
G-10*	16.35	1.08
G-10	0.62	0.25
T-6	9.80	0.96
T-7	4.89	0.73
T-8	3.26	0.61
T-9	6.26	0.82
T-10*	4.96	0.78
T-10	0.43	0.22

HUMTHO.1-STR alleles are named according to the number of STR repeats; allele 10* is that containing the imperfect repeat (De Luca et al., 2001; Tan et al., 2002). Haplotypic frequencies are estimated by maximum likelihood method (Arlequin 1.1 software). The hypothesis $D = 0$ is not rejected ($p = 0.482 \pm 0.002$).

4. Discussion

By applying a demographic-genetic approach (Yashin et al., 1999) a significant variation of the survivorship, related to the genotypes defined by the G477T polymorphism of the *SIRT3* gene, was found in the elderly. Although the possibility that such association is due to chance cannot be ruled out, the statistical significance of the finding seems to be sound. On the other hand the possibility that the association between *SIRT3* variability and survival were due to population stratification is unlikely. Indeed, the population structure of Calabria was proved to be homogeneous and with no stratification by genotyping a moderate number of unlinked genetic markers in the same population (Rose et al., 1996; and unpublished results). What is more, the *SIRT3* gene is a strong candidate in longevity taking into account the findings in model organisms. Thus, both the choice of the population sample and the choice of the candidate gene fit with the criteria suggested for genetic association studies of complex traits (Tabor et al., 2002).

On the other hand, since the G477T transversion does not change the amino acid in the conserved sirtuin domain, the observed association could be due to LD either with a functional variant of the *SIRT3* gene itself or of other genes occurring in this neighbourhood. As an LD map relevant to the 11p15.5 chromosomal region is not yet available, it is not known if such a region is organised in *hot* or *cold* LD blocks (Ardlie et al., 2002). In any case, the negative findings obtained in LD analysis (Tables 1–4) show that the relationship between the G477T marker of *SIRT3* and survivorship is not due to the gene/longevity associations previously found in the same 11p15.5 region, thus reinforcing the hypothesis of D-linkage between the G477T variant and an unknown functional variant of *SIRT3* itself. In particular, the puzzling effects of different genotypes on the survival curves (Figs. 1–3) suggest a linkage disequilibrium of G477T with a putative multiallelic polymorphism displaying complex allelic interactions as previously observed for the STR alleles of the TH gene (Tan et al., 2002). Intriguingly, molecular analyses we are carrying out in the surrounding region revealed a functional multiallelic polymorphism located in the 5th intron of the *SIRT3* gene; at present, a linkage disequilibrium map of the 11p15.5 region is under construction (manuscript in preparation).

Anyway, whatever the functional variant may be, an association between a marker of *SIRT3* and survivorship has been found. The possibility that the *Sir2p* human homologue has a role in ageing is in line with the idea that, although ageing is not a programmed process as mechanisms of development are, the modulators of the rate of ageing are conserved over large evolutionary distances (Partridge and Gems, 2002). On the other hand a link among rate of ageing, gene-expression modulation, and metabolic rate is suggested by an elegant study of genome-wide transcript profiles in ageing *D. melanogaster* which showed age-dependent changes in transcription level nearly in 23% of the genome (Pletcher et al., 2002). These findings indicate that Sir2p activity may be an evolutionary conserved check point of this complex interplay (Chang and Ming, 2002) and therefore agree with the idea that *SIRT3* variability may modulate human ageing. What is more, the localisation of the SIRT3 protein to mitochondria,

as well as its NAD + dependent activity in these organelles, is in line with models putting the mitochondria as central actors in the network which connects metabolic rate, gene expression modulation, and lifespan (Salvioli et al., 2001; Rose et al., 2002).

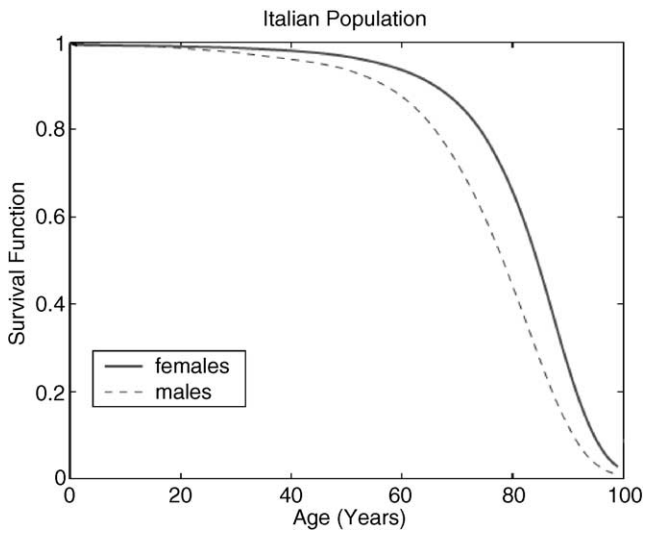
The location in a restrict chromosomal region, 11p15.5, of five genes potentially involved in human ageing (*HRAS1*, *SIRT3*, *IGF2*, *INS*, *TH*) is very intriguing and suggests an evolutionary conserved control of shared regulatory elements (Hurst et al., 2002). Some preliminary results we obtained by gene expression studies indicate that this may be the case at least for some of the above 11p15.5 genes (Bellizzi et al., 2002).

In conclusion, by a demographic genetic approach a male-specific relationship between a marker of *SIRT3* and survivorship has been discovered. This finding shows that the variability of a gene which affects lifespan in experimental organisms is associated with longevity in humans. Furthermore, the *SIRT3* chromosomal location close to other genes associated with longevity opens intriguing questions about the significance of this finding in the light of the current evolutionary theories on ageing and longevity (Partridge and Gems, 2002).

5. Supplementary material

The following supplementary material is available on the web site of the Department of Cell Biology of the University of Calabria: http://biologia.unical.it/genetica.

1. A graph reporting the survival curve of the general Italian population (year 2000)



2. A map of the chromosome 11p15.5 region, showing the—in scale—position of the genes *HRAS1*, *SIRT3*, *IGF2*, *INS* and *TH*

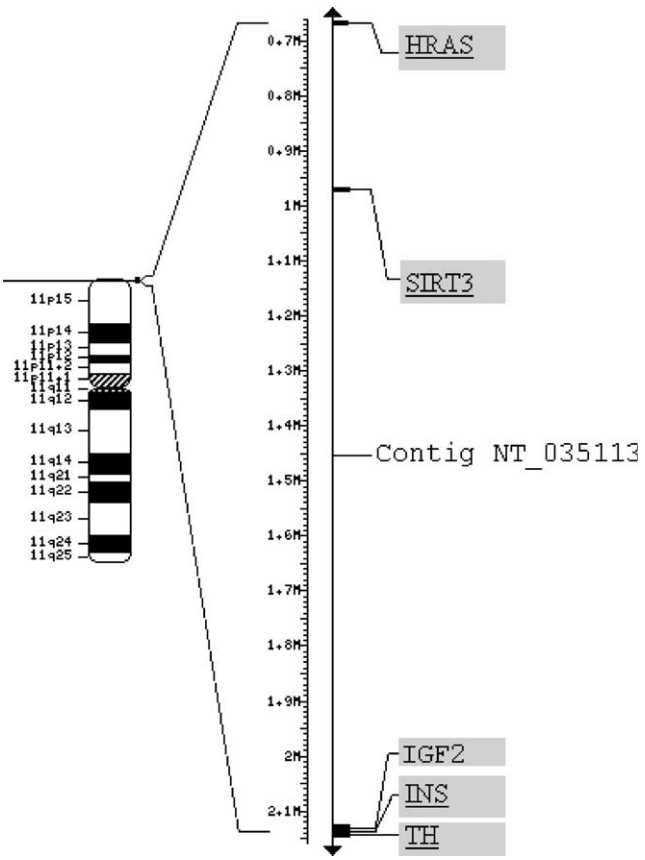


Table S1
SIRT3 (G477T) polymorphism

Genotype	Absolute frequency	Relative frequency ± SE	HWE expected values
Genotypic distribution in the male population ($\chi^2 = 2.22$ *with 1 d.f.*; $p = 0.155$)			
GG	152	0.51 ± 0.029	157.1
GT	128	0.43 ± 0.029	117.8
TT	17	0.06 ± 0.013	22.1
Total	297	1	297.0
Genotypic distribution in the female population ($\chi^2 = 3.28$ *with 1 d.f.*; $p = 0.068$)			
GG	197	0.45 ± 0.024	205.1
GT	202	0.47 ± 0.024	185.8
TT	34	0.08 ± 0.013	42.1
Total	433	1	433.0
Alleles	Counts	Relative frequenvy ± SE	
Allelic distribution in the male population			
G	432	0.73 ± 0.018	
T	162	0.27 ± 0.018	
Total	594	1	
Allelic distribution in the female population			
G	596	0.69 ± 0.016	
T	270	0.31 ± 0.016	
	866	1	

χ^2 and p values refer to expected values at Hardy Weinberg Equilibrium (HWE).

3. The table of allele and genotypic frequencies in the whole sample (Table S1).

Acknowledgements

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