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A study into genes encoding longevity in humans

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CHAPTER 5

***SIRT1* Gene, Age-Related Diseases and Mortality. The Leiden 85-Plus Study**

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Abstract

The *Sir2* gene has been shown to regulate the lifespan of several model organisms. In mammals, the evolutionarily conserved homologue SIRT1 regulates neuroprotection, metabolism and cell survival in response to stress. Based on these data, we hypothesized that SIRT1 might influence the prevalence of age-related diseases and modify the lifespan of humans. In order to test this, we genotyped five single nucleotide polymorphisms (SNPs) in 1245 participants of the population-based Leiden 85-plus Study. *SIRT1* haplotypes were assessed and tested for association with the risks of mortality, metabolic profile, age-related diseases and cognitive functioning. These analyses revealed a trend for lower cardiovascular mortality for haplotype 2 and rs3758391 SNP carriers. In further analyses, this trend was not supported by intermediate phenotypes, albeit the rs3758391 T-allele carriers had better cognitive functioning. In conclusion, our results indicate a role for *SIRT1* in cognitive functioning, but the role in lifespan remains to be elucidated.

Introduction

Increased expression of Sir2 (Silent Information Regulator 2) either due to an extra copy of the gene or to caloric restriction, prolongs lifespan in various model organisms (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Wood et al., 2004). In mammals, there are seven Sir2 homologues, of which SIRT1 (Sirtuin 1) is the most similar to Sir2 (Frye, 1999; Frye, 2000). In response to environmental signals, SIRT1 regulates metabolism and cell survival in various types of mammalian cells (Bordone and Guarente, 2005; Cohen et al., 2004; Yang et al., 2006). To date, it is unknown whether SIRT1 influences the prevalence of age-related diseases and modifies the lifespan of humans.

SIRT1 is a NAD⁺-dependent (nicotinamide adenine dinucleotide) protein deacetylase (Imai et al., 2000) and it regulates metabolism and cell survival through influencing gene silencing, and the activity of various transcription factors and co-regulators (Bordone and Guarente, 2005; Vaquero et al., 2004). It has been shown that activation of SIRT1 increases glucose tolerance and enhances insulin response to glucose in pancreatic β -cells (Bordone et al., 2006; Motta et al., 2004; Moynihan et al., 2005). Increased SIRT1 activity also promotes hepatic gluconeogenesis and inhibits glycolysis via *PGC1- α* during fasting (Rodgers et al., 2005). Furthermore, SIRT1 has an effect on fat metabolism, via inhibition of *PPAR γ* (Picard et al., 2004). These findings suggest that increased SIRT1 activity results in a favorable metabolic profile, with decreased prevalence of diabetes and cardiovascular diseases. In addition, the role of SIRT1 in providing resistance to damage- or stress-induced apoptosis, may help to preserve organ function over time, although by doing so it may promote cancer (Hekimi and Guarente, 2003; Lim, 2006). Recent evidence also suggests a role for SIRT1 in neuroprotection and neurodegenerative disorders (Araki et al., 2004; Parker et al., 2005; Qin et al., 2006). Altogether, SIRT1 could influence lifespan through several ways. The involvement of *SIRT1* in human lifespan has been previously studied in a case-control study of elderly and young (Flachsbart et al., 2006). No differences in *SIRT1* allele and haplotype frequencies were observed between these groups. This, however, does not exclude the possibility that SIRT1 gene has an influence on human physiology and lifespan.

The aim of this study was to analyze the association between genetic variation in the *SIRT1* gene, and all-cause and cause-specific mortalities. In addition, metabolic profile, prevalence of age-related diseases, and cognitive functioning were tested in the participants of the prospective population-based Leiden 85-plus Study.

Participants and methods

Participants

The Leiden 85-plus Study is a prospective population-based study, in which all 85-year-old or older inhabitants of the city Leiden, The Netherlands, were invited to take part. The study design and data collection have been described elsewhere (der Wiel et al., 2002; Weverling-Rijnsburger et al., 1997). The study population consists of two cohorts, cohort '87 and '97, and all the study participants are of Caucasian origin. Cohort '87 includes 977 participants aged 85 or older,

enrolled between 1987 and 1989 (Weverling-Rijnsburger et al., 1997). Cohort '97 consists of 599 subjects, all members of the 1912-1914 birth cohorts, who were enrolled in the month of their 85th birthday between 1997 and 1999 (der Wiel et al., 2002). DNA was available for 682 participants from cohort '87 and for 563 participants from cohort '97. All participants were followed for mortality until August 1, 2005, with a mean follow-up period of 4.4 years. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorized according to the 10th International Classification of Diseases (ICD-10). The Medical Ethical Committee of the Leiden University Medical Center approved the study and informed consent was obtained from all participants.

Metabolic profile in cohort '97

HbA1c (hemoglobin A1c), triglycerides, C-reactive protein (CRP) and high-density lipoprotein (HDL)-cholesterol concentrations were determined in serum using fully automated analyzers (Hitachi 747 and 911; Hitachi Ltd, Tokyo, Japan). Low-density lipoprotein (LDL)-cholesterol was estimated with the Friedewald equation (Friedewald et al., 1972).

Cardiovascular pathologies and diabetes in cohort '97

The prevalence and number of cardiovascular pathologies and diabetes were obtained from the participants' general practitioner or nursing home physician. For cardiovascular pathologies, also electrocardiograms were recorded (Macfarlane and Latif, 1996). Cardiovascular pathologies were classified as myocardial infarction, myocardial ischemia, stroke, arterial surgery and intermittent claudication (van Exel et al., 2002). Subjects were classified as having diabetes when they met at least one of the following criteria: 1) history of diabetes obtained from the general practitioner or the subject's treating physician; 2) use of sulfonylurea, biguanide, or insulin, based on information obtained from the subject's pharmacist; or 3) non-fasting glucose of ≥ 11.1 mmol/l.

Cognitive function and depressive symptoms in cohort '97

Overall cognitive functioning was measured with Mini-Mental State Examination (MMSE) (Folstein et al., 1975). From specific domains of cognitive functioning attention was assessed with Stroop Test (Klein et al., 1997), processing speed with Letter Digit Coding Test (LDT) (Houx et al., 2002) and memory with 12-Word Learning Test, which assesses immediate- (WLTI) and delayed recall (WLTD) (Brand and Jolles, 1985). The prevalence of depressive symptoms was assessed with 15-item Geriatric Depression Scale (GDS-15) (de Craen et al., 2003). The tests assessing specific domains of cognitive functioning could not be administered to 92 participants because of severe cognitive impairment (MMSE score < 19 points). All participants were visited annually for re-measurement of cognitive functioning and depressive symptoms during a mean follow-up period of 4.2 years. In addition to the specific tests, a composite cognitive score was calculated by converting the scores of the individual tests (Stroop Test, LDT, WLTI and WLTD) into a z-score ((individual level – mean level)/SD), and computing the average.

SNP selection and genotyping

The single nucleotide polymorphisms (SNPs) were selected from the public database of National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>) to cover the *SIRT1* gene region (GeneID: 23411) in equally spaced intervals. The minor allele frequencies (MAF) of the polymorphisms had to be > 5 %. The selected SNPs were rs3758391 (promoter), rs3740051 (promoter), rs2236319 (intron), rs2273773 (exon) and rs3818291 (intron). All these SNPs were genotyped using the MassArray platform, according to the protocols of the manufacturer (Sequenom Inc., San Diego, CA, USA).

Statistical analysis

The program Haploview (Barrett et al., 2005) was used to estimate the SNPs' allele frequencies, test the genotypes for Hardy-Weinberg equilibrium, and estimate pair-wise linkage disequilibrium (LD) between the polymorphisms. Haplotypes and haplotype frequencies were calculated using SNPHAP software (<http://www-gene.cimr.cam.ac.uk/clayton/software>). The posterior probabilities of pairs of haplotypes per subject, as estimated by the SNPHAP, were used as weights in all analyses. The haplotype analyses approach used in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere (Wallenstein et al., 1998). CRP, triglyceride and HbA1c levels were not normally distributed and were ln-transformed. All-cause and cause-specific mortality risks with 95 % confidence intervals (CI) were calculated with Cox proportional hazard model, using left censoring to correct for the delayed entry into the risk set according to age. Associations between haplotypes and metabolic profile were analyzed using general linear model. Differences in the prevalence of cardiovascular pathologies and diabetes between the haplotypes were tested using binary logistic regression. The associations between cognitive functioning, depressive symptoms and haplotypes were tested with linear mixed model. All analyses were sex adjusted, except the analyses of cognitive functioning and depressive symptom, which were additionally adjusted for education. The analyses were performed with STATA version 9.0 (StataCorp LP, Texas, USA) and SAS version 8.2 (SAS Institute Inc., Cary, NC, USA) statistical software.

Table 1. Characteristics of the study subjects

	Leiden 85-plus Study	
	Cohort '87	Cohort '97
Number	682	563
Age (median, IQR)	89 (88-92)	85 (-)
Female (n, %)	491 (72 %)	375 (67 %)
Polymorphisms*		
rs3758391 (C/T)	0.33	0.36
rs3740051 (A/G)	0.07	0.07
rs2236319 (A/G)	0.07	0.07
rs2273773 (T/C)	0.07	0.08
rs3818291 (G/A)	0.13	0.13

IQR – interquartile range; *minor allele frequency

Results

All 1245 participants of the Leiden 85-plus Study were genotyped for the five *SIRT1* polymorphisms (Table 1). The genotype frequencies of the SNPs were in Hardy-Weinberg equilibrium, and the allele frequencies were similar between the two elderly cohorts (Table 1). The polymorphisms were in strong linkage disequilibrium ($D' 0.97-1.00$), and gave rise to five different haplotypes of which four were common (frequencies $> 5\%$) and cumulatively accounted for 99.9 % of the haplotypes (Figure 1).

All-cause and cause-specific mortality risks were assessed after a mean follow-up period of 4.4 years. During that time 1001 (80 %) of the 1245 participants had died. From these 406 (41%) had died due to cardiovascular disease, 162 (16%) due to cancer, and 431 (43%) due to other causes. The cause of death was unknown for two participants. The mortality risk analyses revealed a trend for lower cardiovascular mortality (HR 0.82, 95 % CI: 0.66 to 1.01, $p=0.062$) for haplotype 2 carriers, compared to the reference haplotype (Figure 2). A similar trend was observed in the two Leiden 85-plus Study cohorts separately (data not shown). For the other haplotypes, no differences in all-cause or cause-specific mortality risks were observed (Figure 2).

In order to study further the role of *SIRT1*, we analyzed the associations between *SIRT1* haplotypes and metabolic profile, prevalence of cardiovascular diseases and diabetes. The data on these endpoints were available for 563 participants of the cohort '97. At baseline, no associations between the *SIRT1* haplotypes and various metabolic profile parameters were observed, except for LDL-cholesterol and haplotype 3. These haplotype carriers had 0.18 mmol/l lower (95 % CI: -0.34 to -0.02, $p=0.030$) LDL levels compared to the reference haplotype. In contrast, none of the *SIRT1* haplotypes were associated with the prevalence of cardiovascular pathologies or diabetes. For haplotype 2 carriers, non-significant trends for lower prevalence of arterial surgery (OR 0.82, 95 % CI: 0.41-1.65, $p=0.574$) and intermittent claudication (OR 0.76, 95 % CI: 0.36-1.60, $p=0.471$) were observed (Table 2).

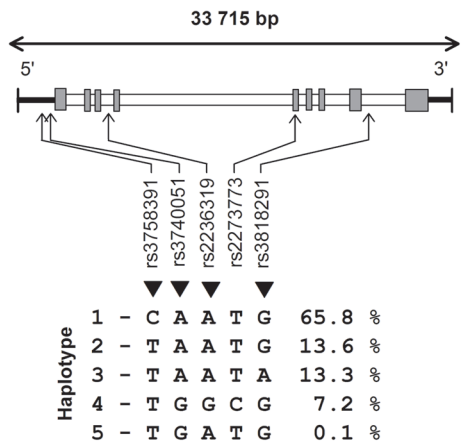


Figure 1. Structure and haplotypes of the *SIRT1* gene. The *SIRT1* gene is located in chromosome 10 and the coding region spans 33 715 bp. It contains nine exons, which are represented by gray boxes in the figure. The approximate positions of the SNPs are indicated with arrows. All SNPs were in strong LD and resulted in five haplotypes, of which four had frequencies $> 5\%$.

Figure 2. *SIRT1* haplotypes, all-cause and cause-specific mortality risks. Mortality risks were calculated in the combined cohort of the Leiden 85-plus Study (n=1245). All-cause and cause-specific mortality risks are presented as hazard ratios (HR) with 95 % confidence intervals (CI). The most common haplotype 1 was used as a reference group.

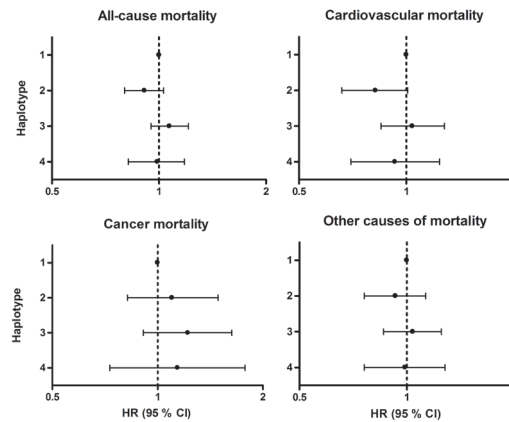


Table 2. *SIRT1* haplotypes, prevalence of cardiovascular diseases and diabetes at baseline in the Leiden 85-plus Study cohort '97 (n=563)

	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4
	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)
CVD total (n=365)*	1 (ref)	1.17 (0.83-1.64)	1.11 (0.77-1.60)	0.84 (0.53-1.33)
Myocardial infarction (n=137)	1 (ref)	0.99 (0.69-1.44)	0.90 (0.60-1.36)	0.98 (0.58-1.67)
Myocardial ischemia (n=286)	1 (ref)	1.02 (0.74-1.40)	1.08 (0.77-1.51)	0.91 (0.58-1.44)
Stroke (n=57)	1 (ref)	1.07 (0.61-1.88)	1.14 (0.67-1.95)	0.90 (0.37-2.23)
Arterial surgery (n=37)	1 (ref)	0.82 (0.41-1.65)	0.98 (0.48-1.97)	0.75 (0.27-2.08)
Intermittent claudication (n=36)	1 (ref)	0.76 (0.36-1.60)	0.92 (0.43-2.00)	0.78 (0.29-2.09)
Diabetes (n=92)	1 (ref)	1.37 (0.89-2.11)	1.46 (0.93-2.28)	0.79 (0.40-1.55)

CVD – cardiovascular disease; OR – odds ratio; CI – confidence interval; * Participants with one or more cardiovascular pathology

In the cohort '97, cognitive functioning and the prevalence of depressive symptoms were assessed at baseline, age 85 years, and re-examined annually during a mean follow-up period of 4.2 years. Compared to the reference haplotype, there were no differences in cognitive functioning or in prevalence of depressive symptoms between the *SIRT1* haplotypes (data not shown).

In addition to haplotype analyses, univariate analyses with the individual polymorphisms were performed. From the five polymorphisms, associations with only one (rs3758391), which also resides in the haplotype 2, were observed. Heterozygous (HR 0.77, 95 % CI: 0.62 to 0.96, $p=0.018$) but not homozygous (HR 1.01, 95 % CI: 0.73 to 1.39, $p=0.965$) carriers of rs3758391 T-allele, had lower cardiovascular mortality risks. These differences were not attributable to changes in metabolic profile or in prevalence of age-related diseases (data not shown). In contrast, homozygous but not heterozygous carriers of the rs3758391 T-allele performed better on all tests measuring cognitive functioning (Table 3). These differences were most pronounced for immediate memory (2.26 points, 95 % CI: 0.62 to 3.89, $p=0.007$) and for delayed memory (1.06 points, 95 % CI: 0.29 to 1.84, $p=0.007$) (Table 3).

Table 3. *SIRT1* rs3758391 SNP, cognitive functioning and depressive symptoms during mean follow-up period of 4.2 years in the Leiden 85-plus Study cohort '97 (n=563).

	rs3758391			p-trend
	CC Mean (SE)	CT Difference (SE)	TT Difference (SE)	
Composite cognitive score	-0.23 (0.06)	0.02 (0.08)	0.28 (0.11)*	0.031*
Global cognitive function (points)	22.7 (0.43)	0.24 (0.59)	0.63 (0.83)	0.443
Attention (seconds)	87.0 (2.12)	1.67 (2.90)	-2.28 (4.10)	0.823
Processing speed (digits)	15.7 (0.46)	-0.11 (0.63)	0.97 (0.88)	0.414
Immediate memory (pictures)	20.1 (0.43)	-0.10 (0.59)	2.26 (0.83)*	0.035*
Delayed memory (pictures)	6.86 (0.20)	0.02 (0.28)	1.06 (0.39)*	0.029*
Depressive symptoms (points)	2.97 (0.18)	0.18 (0.25)	-0.17 (0.36)	0.912

SE – standard error; * p-value < 0.05

Discussion

In this study, we tested the role of *SIRT1* in age-related diseases, cognitive functioning and mortality in humans. The analyses of *SIRT1* haplotypes revealed a trend for decreased cardiovascular mortality for haplotype 2 carriers, and for the rs3758391 SNP carriers, which resides in the haplotype 2. None of these, however, were associated with metabolic profile or cardiovascular pathologies. In contrast, carriers of the rs3758391 polymorphism performed better on tests measuring cognitive functioning.

A specific role of *SIRT1* in cell survival and in development of cancer has been proposed (Alcendor et al., 2004; Giannakou and Partridge, 2004; Luo et al., 2001). In this study, we found no associations between *SIRT1* haplotypes and cancer mortality, but we observed a trend for lower cardiovascular mortality for haplotype 2 carriers. This trend was observed in the combined, but also in the separate cohorts. Altogether, these observations are in accordance with the results from cell culture studies, where a protective effect of *SIRT1* on cardiac myocytes has been demonstrated (Alcendor et al., 2004; Pillai et al., 2005). In addition, *SIRT1* appears to be important for the development of heart, since *Sirt1* knock-out mice presented cardiac abnormalities (Cheng et al., 2003; McBurney et al., 2003). Based on these data, the lower cardiovascular mortality in the haplotype 2 carriers in our study population is in line with the expected functions of *SIRT1*. This implies that these *SIRT1* haplotype carriers might suffer less from cardiovascular diseases. In order to test that, we analyzed the prevalence of various cardiovascular pathologies dependent on *SIRT1* haplotypes. However, no associations were found, and also the parameters of metabolic profile, which underlie atherosclerosis, did not differ. For the latter, a beneficial profile was expected for the *SIRT1* haplotype 2 carriers. The lack of a consistent risk profile suggests that the association between the lower cardiovascular mortality and the *SIRT1* haplotype 2 could have arisen either due to other mechanisms or due to chance. It might also be that the beneficial effects of *SIRT1* only appear in acute disease states, thereby decreasing the severity of outcomes from crisis events. This mode of action is consistent with the effects of *SIRT1* on apoptosis.

Besides mortality and various intermediate phenotypes, we tested the role of SIRT1 in cognitive functioning and in prevalence of depressive symptoms. The involvement of SIRT1 in neurophysiological functioning has been discovered recently. Several studies have linked the SIRT1 protein and its biological activator, resveratrol, to axonal protection and survival of neurons (Araki et al., 2004; Parker et al., 2005; Qin et al., 2006). Axonal degeneration often precedes the death of neuronal cell bodies in neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Raff et al., 2002). As a result, impairments in cognitive functioning occur. In this study, we found no associations between *SIRT1* haplotypes and cognitive functioning and depressive symptoms. In contrast, a promoter polymorphism (rs3758391), which is the only variant allele in the haplotype 2, was associated with better cognitive functioning. From the specific domains of cognitive functioning, memory was the best preserved. These data, together with the evidence from recent literature, support a possible role of SIRT1 in the brain.

Our results are partly in accordance with a recent case-control study, where also no associations between *SIRT1* polymorphisms/haplotypes and lifespan were found (Flachsbart et al., 2006). In both studies, five polymorphisms from the *SIRT1* gene were analyzed, although only two were the same between the studies. However, besides analyzing individual polymorphisms we calculated haplotypes and tested their association with various phenotypes. The *SIRT1* gene is embedded in a region of strong LD (Supplementary Figure 1) and a haplotype-based approach enables to capture the majority of the genetic variation in the *SIRT1* gene. Since no associations with intermediate phenotypes were observed, we reason that the association between the rs3758391 SNP and cognitive functioning has arisen due to polymorphisms in LD with those analyzed in this study. These SNPs could reside in neighboring genes (DNAJC12 in 5' and HERC4 in 3') or in the regulatory regions of the *SIRT1* gene. We speculate that functional variability in the SIRT1 gene itself is constrained because it plays diverse but essential roles in human physiology.

In mammals, the *Sir2* gene has several homologues (Michishita et al., 2005) and perhaps one or more of the other SIRT family members (SIRT1-7) have bigger influences on lifespan. From this point of view, *SIRT3* gene which encodes a mitochondrial protein, has been implicated to play a role in human longevity (Bellizzi et al., 2005; Rose et al., 2003). In addition, recent evidence suggests a similar role for SIRT6, since *Sirt6*-deficient mice displayed genomic instability and premature aging-like phenotype (Mostoslavsky et al., 2006). Therefore, the analyses of other SIRT family members might shed more light into the regulation of human lifespan.

The strengths of the study include the possibility to estimate several phenotypes in one cohort, and the prospective analyses with high number of deaths during follow-up. A limitation of the study is the lack of data on the activity or levels of SIRT1, which would reflect the functionality of the polymorphisms analyzed. In addition, considering the number of tests performed, adjustment for multiple testing would eliminate all the statistically significant associations observed. Consequently, the results of this study are not exhaustive.

In conclusion, the results of this study provide evidence for a role of SIRT1 in cognitive functioning, but due to the lack of associations with intermediate phenotypes, the influence of genetic variation in the *SIRT1* gene on lifespan remains to be elucidated.

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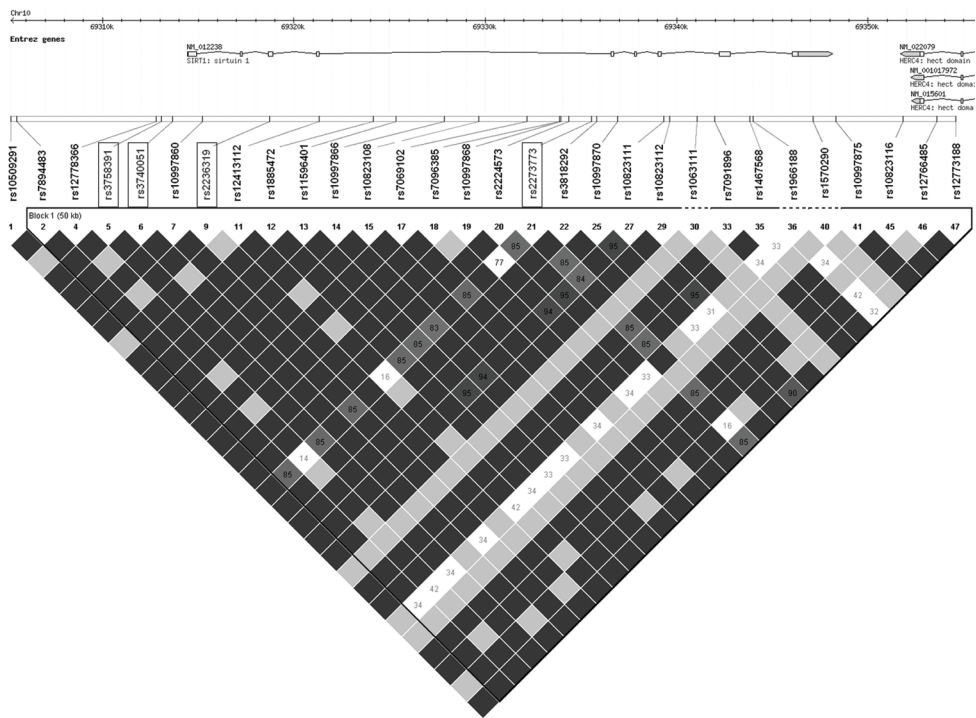
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Supplementary materials



Supplementary Figure 1. Overview of the physical and genetic structure of the *SIRT1* gene region as generated by Haploview from the Caucasian HapMap data (release nr. 21a). The polymorphisms genotyped by the HapMap are shown in the top panel. The SNPs marked with boxes were analyzed in this study. The lower panel gives an overview of the linkage disequilibrium structure of the locus (D')

