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Research article

Association between low self-rated health and heterozygosity for −110A > C polymorphism in the promoter region of *HSP70-1* in aged Danish twins

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Abstract

We have studied the possible association between the −110A > C polymorphism in the promoter region of one of the heat shock protein genes *HSP70-1* with human longevity in a cohort of aged Danish twins. This cohort includes individuals aged between 70 and 91 years (mean = 75.6 years), who are categorized according to the presence or absence of various diseases and according to the various, age-related parameters for which a genetic component has already been defined. Four hundred DNA samples from the cohort were genotyped using real-time PCR. Aging phenotypes (diseases, physical and cognitive functioning) were compared with regard to genotype. Of all the aging phenotypes studied, self-rated health and relative self-rated health, which represent an individual's overall sense of physical well-being and which have been shown to be both predictors of survival at older ages and better indicators of future survival than objectively measured health status, were associated with the polymorphism. An association was found between low self-rated health and heterozygosity for −110A > C polymorphism in the promoter region of *HSP70-1* in aged Danish twins.

Introduction

In most eukaryotic cells, a slight increase in stress in the form of heat, heavy metals, nutrient deprivation, oxygen radicals or viral infection induces a heat shock (HS) response, marked by the repression of normal protein synthesis, induction of HS genes and the preferential synthesis of several HS proteins (Hsp) or stress proteins (for a review see, Verbeke et al. 2001). HS response protects cells from subsequent damage and aids them to counteract the effects of the stress. The capacity to respond rapidly to stress at the gene level determines the adaptive and, therefore, the survival capacity and longevity of the organism (Minois 2000; Rattan 2001). One way to indicate relationship between organismal aging and stress response is to demonstrate an association between polymorphisms in Hsp genes (*HSP*) and parameters of organismal aging.

Of the various stress proteins, Hsp70 is the most prominent and best characterised in the stress protein families. Hsp70 is a highly inducible and most actively synthesised protein in the cell upon HS (Tavaria et al. 1996; Verbeke et al. 2001). Hsp70 protein family members have a highly conserved sequence from *E. coli* to man (Kiang and Tsokos 1998). Cognate members of the Hsp70 family are found within all the major intercellular compartments while the inducible isoforms appear to be predominantly cytoplasmic or nuclear in distribution (Tavaria et al. 1996). Hsp70 acts either as a chaperone, or cooperates with the proteolytic pathways in the cells. In humans there are 11

different isoforms of Hsp70 encoded by different genes located at dispersed loci. Three of the *HSP70* are mapped within major histocompatibility complex (MHC) class III region on the short arm of chromosome 6 (Goate et al. 1987). These are intron-less *HSP70-1*, *HSP70-2* and *HSP70-Hom* (Milner and Campbell 1990), which have homologous gene sequence but differ in their regulation. There are 14 polymorphic sites within these three MHC-linked *HSP70*: six in *HSP70-1* and four each in *HSP70-2* and *HSP70-Hom*. Polymorphisms in these three genes have been extensively studied for their association with various autoimmune diseases such as systemic lupus erythematosus (Jarjour et al. 1996), rheumatoid arthritis (Reid et al. 1991), multiple sclerosis (Casino et al. 1994; Freedman et al. 1995; Niio et al. 2000), and insulin-dependent diabetes mellitus (Pociot et al. 1993). So far, no association between polymorphisms of the MHC-linked *HSP* and diseases has been found that would not be explained by linkage disequilibrium with other HLA markers. However, protective effects of Hsp70 against oxidative stress suggest that it might be very valuable to study *HSP* polymorphism in conditions associated with oxidant/antioxidant imbalance, for example aging.

In a recent cross-sectional study on samples from Calabria, a south Italian region, Altomare et al. (2003) have reported that the presence of an allele carrying adenine (allele A), in the $-110A > C$ polymorphism present in the 5′ flanking region, 3 base pairs upstream of the heat shock element (HSE) of the promoter region of *HSP70-1* gene, was unfavourable for longevity in women. In another association study between polymorphism ($2437T > C$) in the coding region of *HSP70-Hom* with human aging, done on the Irish population (Ross et al. 2003), the frequency of T allele has been shown to increase in the old population (80–90 years) as compared to the young controls (19–45 years). However, as will be discussed later, there are confounding factors which occur while performing such cross-sectional studies, where allele frequencies in a group of young controls are compared against the allele frequencies in the oldest-old. Taking this into account, we report the results of our study performed on DNA samples collected from Danish dizygotic (DZ) twin cohort of 400 individuals aged 70–91 years (mean = 75.6), from the Longitudinal Study of Aging Danish Twins (LSADT), and who have been scored for various age associated physical and cognitive parameters.

Material and methods

DNA samples

The possible association between $-110A > C$ polymorphism in *HSP70-1* gene and successful/unsuccessful aging was studied on DNA samples collected from a cohort of 200 DZ twin pairs aged 70–91 years, from LSADT study. These samples form a part of the Danish Twin Registry (Skytthe et al. 2002), which is the first nationwide twin registry in the world. The LSADT is an ongoing longitudinal study of Danish twins (Christensen et al. 1999) who are more than 70 years old (about 4000 individuals). With an interval of two years these individuals have been offered an examination at their home, which includes both a traditional health-related interview and a number of objective and cognitive tests, and a collection of blood sample. Till date five rounds have been performed in the years 1995, 1997, 1999, 2001, and 2003. The DZ twins from LSADT-1999 wave are the basis of this investigation. These twins are between the ages 70 and 91 years, and have been categorised according to the absence or presence of various age-related diseases viz. lung disease, osteoporosis, arthritis, osteoarthrosis, cardiovascular diseases (ischaemic heart disease (AMI/angina), heart failure and stroke), hypertension, hyperthyroidism, cancer and diabetes, and for various age-related parameters including sex- and age-adjusted scores of physical and cognitive tests (Frederiksen et al. 2002b). Various parameters which have been used as measures of aging include scorings of the physical function such as 'hand grip strength', 'activity of daily living (ADL) strength' and 'ADL endurance' (Nybo et al. 2001); scorings of the mental state of the individual viz. 'cognititive composite score' and 'mini mental state examination' (MMSE) (Frederiksen et al. 2002b). Furthermore, 'self-rated health' (scored both in absolute terms, measured in the scale of 1–5, with the possible responses, 'very good', 'good', 'fair', 'poor', 'very poor'; and in relation to others of the same age, measured in the scale of 1–3, with the possible re-

sponses, 'better', 'the same' and 'worse') was scored by asking the individuals to assess their own health.

A substantial genetic component has been found for all the above traits, although the extent of the genetic component differs (Frederiksen and Christensen, 2003). For example, a quarter of the variation in self-rated health score has been explained by genetic factors for the groups 60–69 years and 70 years and older (Christensen et al. 1999). Also, 'hand grip strength' has been shown to be a suitable phenotype for identifying genetic variants of importance to mid- and late-life physical functioning and has a heritability of 52% (Frederiksen et al. 2002a). Once heritability is defined to all these age-related traits the next step lies in associating these traits with the specific genes. Based on these scorings we have analysed the association of parameters correlated with human longevity with −110A > C polymorphism in *HSP70-1* gene.

Genotyping

Genotyping was done using the real-time PCR on the LightCycler system (Roche Applied Sciences), which helps to monitor the amplification of PCR product simultaneously, using fluorescent labelled sequence specific oligonucleotide probes. Probes were designed such that the oligonucleotides were complementary to the allele carrying the nucleotide cytosine (C). Hence on performing the melting peak analysis, the high or low melting peak corresponded to the presence of cytosine or adenine respectively (Figure 1). LightCycler reaction was performed on 3 μl DNA in the reaction mix of 10 μl containing the following reagents: 10× Titanium Taq PCR buffer (1 μl), ADV Taq 50× (0.25 μl), 10 mM dNTPs (0.13 μl), Primer HSP70-1-forw (5 pmol/μl; 1.0 μl), Primer HSP70-1-rev (5 pmol/μl; 1.0 μl), Anchor probe (5 pmol/μl; 0.2 μl), Sensor probe (5 pmol/μl; 0.2 μl), DMSO (0.5 μl) and double distilled water (2.72 μl). The sequence of the primers and probes used is given in Table 1. The program used for the LightCycler run can be available from the authors on request.

Statistical analysis

We grouped the participants according to their *HSP70-1* genotype and compared these groups with respect to various aging phenotypes. Continuous phenotypes (physical ability scores, grip strength, depression and cognitive scores) were compared across the genotypes using the one way ANOVA test. Discrete variables (diseases – see Table 3) were compared across the genotypes using Pearson's χ^2 test. Bonferroni correction on repeated testing was applied.

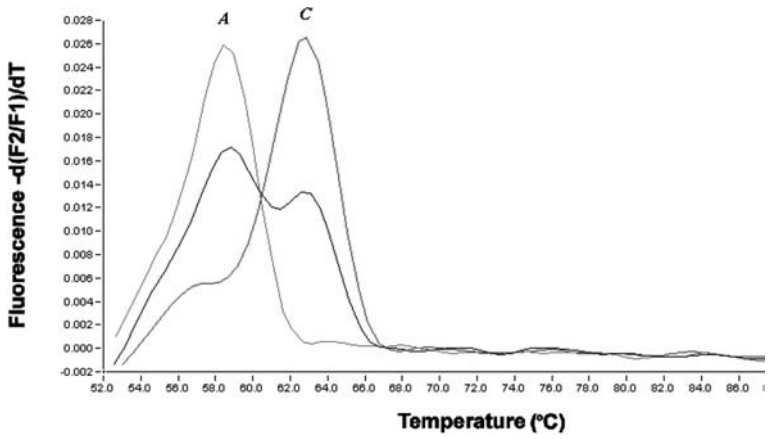

Figure 1. Melting peak analysis of three samples on the LightCycler: The fluorescent probe complementary to the Allele carrying adnine (A) melts at 58 °C and that carrying (C) melts at 63 °C, respectively. The samples homozygous for allele A or C show one peak corresponding to 58 or 63 °C, respectively. Whereas the sample heterozygous for A and C shows two peaks corresponding to both the temperatures.

Table 1. List of the primers and oligonucleotide probes used for genotyping using LightCycler system.

Primers	
HSP70-1-forw	5′-AGAAGACTCTGGAGAGTTC-3′
HSP70-1-rev	5′-AAAGGTAGTGGACTGTCGC-3′
Probes	
HSP70-1-se1	5′-AATATTCCAGGGGTTTCGCCTC-X
HSP70-1-an1	5′-LCRED640-CGTCCTGCCCCCCAGCCTT-p

X – Fluorescent dye LCRED640; p – Phosphorylated.

Results

The genotype frequencies of the individuals homozygous for allele A (AA), heterozygous for alleles A and C (AC) and homozygous for allele C (CC) were 35% ($n = 142$), 42.7% ($n = 189$) and 17.2% ($n = 69$), respectively (Table 2). The allele frequencies for allele A and allele C were 59.1% and 40.9%, respectively (Table 2) and the genotype frequency was found to be in Hardy–Weinberg equilibrium (*P*-value 0.59).

The relationship of aging phenotypes with *HSP70-1* genotype is shown in Tables 3–6. Of all the age-related parameters studied, only self-rated health and relative self-rated health (compared to others of the same age), showed an association (*P*-values of 0.0046 and 0.018, respectively) with polymorphism at −110 (Tables 5 and 6). As evident from Tables 5 and 6, it is the heterozygous individuals who are more inclined to rate their heath as 'poor', 'very poor' or 'worse'.

Table 2. Genotypic and gene frequencies (g.f.) at −110 in the promoter region *HSP70-1* gene in elderly Danish twins.

Genotype	$n = 400$ *n* (%)
AA	142 (35.5)
AC	189 (47.2)
CC	69 (17.2)
Alleles	*n* (g.f. %)
A	473 (59.1)
C	327 (40.9)

Discussion

Aging and longevity are highly complex traits influenced by genes, environment and chance (Rattan 2003). Heritability of human longevity has been estimated to be 0.26 for males and 0.23 for females from Danish twin studies (Herskind et al. 1996). However, the molecular basis of inherited components in aging is far from clear. For study-

Table 3. Genotype distribution in cases (with disease) and controls (without disease) and *P*-values calculated for various diseases using Pearson's χ^2 test.

Diseases	Genotypes						
	N (cases)			*N* (controls)			*P* (χ^2)
	AA	AC	CC	AA	AC	CC	
Hyperthyroidism	8	19	8	134	170	61	0.243
Hypertension	38	49	16	104	140	53	0.854
Heart failure	10	23	5	132	166	64	0.226
AMI/angina	16	20	9	126	169	60	0.858
Stroke	9	3	3	136	186	66	0.292
Cancer (except skin cancer)	7	17	6	135	172	63	0.349
Pulm disorder	10	27	7	132	162	62	0.110
Osteoporosis	10	12	2	132	177	67	0.474
Arthritis	5	9	4	137	179	65	0.683
Osteoarthrosis	41	61	27	101	128	42	0.327
Diabetes	8	14	3	134	175	66	0.622

Table 4. Mean and standard deviation (SD) of performance scores for each genotype, and *P*-value calculated using one way ANOVA.

Parameter	Mean ± SD (*N*) AA	Mean ± SD (*N*) AC	Mean ± SD (*N*) CC	*P* (ANOVA)
Grip strength	−0.19 ± 1.02 (131)	0.05 ± 0.88 (183)	0.09 ± 0.97 (65)	0.298
ADL strength	−0.16 ± 0.86 (142)	−0.22 ± 0.74 (187)	−0.25 ± 0.71 (69)	0.331
ADL endurance	−0.16 ± 0.95 (138)	−0.17 ± 0.89 (185)	−0.19 ± 0.88 (67)	0.670
MMSE	0.06 ± 0.88 (141)	0.03 ± 1.01 (189)	0.12 ± 0.72 (69)	0.180
Cognitive composite	−0.03 ± 1.06 (138)	0.19 ± 0.98 (188)	0.19 ± 1.05 (67)	0.450
Depression	−0.42 ± 4.61 (139)	−0.41 ± 4.61 (188)	−1.32 ± 2.69 (69)	0.474

Table 5. Genotypic distribution of the self-rated health response.

Genotype	Very good	Good	Fair	Poor/very poor	Total
AA	55 (39%)	55 (39%)	27 (19%)	4 (3%)	141
AC	47 (25%)	87 (46%)	36 (19%)	18 (10%)	188
CC	25 (36%)	27 (39%)	17 (24%)	0	69
Total	127	169	80	22	398

$P = 0.0046$; Pearson's $\chi^2 = 18.749$.

Table 6. Genotypic distribution of the relative self-rated health response.

Genotype	Self-rated health compared to others of the same age			Total
	Better	The same	Worse	
AA	86 (61%)	49 (35%)	6 (4%)	141
AC	95 (50%)	69 (37%)	24 (13%)	188
CC	41 (59%)	26 (38%)	2 (3%)	69
Total	222	144	32	398

$P = 0.018$; Pearson's $\chi^2 = 11.919$.

ing the genetic basis of human longevity, the gene-longevity association studies, which look for association between polymorphisms at candidate loci and lifespan, have been a popular and useful approach. Although a number of genes have been studied for their association with human longevity, the overall picture still remains unclear (Caruso et al. 2001). This is due to the various confounding factors which affect the longevity association studies, such as heterogeneity, which may affect the Hardy–Weinberg equilibrium of the population on which such studies are to be carried out. Special problems occur when doing cross-sectional studies, where allele frequencies a group of young controls are compared against the allele frequencies in the oldest-old. In this design a difference in allele frequency might not relate directly to age but to confounding factors, as the mortality for genotypes may depend on the time of birth of the cohort because of the changes in the incidence and prevalence of the number of diseases (De Benedictis et al. 2001). Another weakness of cross-sectional studies is what epidemiologists call 'population stratification', that is that the ethnic compositions of various cohorts are not identical and therefore can create spurious correlations.

The association of *HSP70-1* with human longevity has been reported for the first time in a recent case/control study on samples from Calabria, a south Italian region (Altomare et al. 2003). The study showed that the presence of allele A, in the −110 position of the promoter region of *HSP70-1*,

was unfavourable to longevity in women. In order to avoid various confounding factors discussed earlier, we in our study, have introduced a model where we took a group of 400 individuals, aged 70–91 years, from LSADT, who have been scored for parameters which are characteristic of successful/unsuccessful aging. These parameters have been studied for their association with polymorphism at −110 of *HSP70-1*. Of all the parameters studied, self-rated health (scored both in absolute terms and in relation to others of the same age) shows a striking association (*P*-value 0.0046 and 0.018, respectively). This self-rated health, an ordinal trait with multifactorial etiology, represents an individual's overall sense of physical well being and has been shown to be both a predictor of survival at older ages (McCallum et al. 1994), and a better indicator of future survival than objectively measured health status (Mossey and Shapiro 1982). In our series we observed lower health score in the heterozygous individuals. This seems somewhat surprising, first because Altomare et al. found lower survival in the females homozygous for allele A (AA genotype), and second because it is difficult to envision a molecular mechanism causing phenotypic domination in heterozygotes. We also did a gender-specific analysis for the association of 'self-rated health' with −110A > C polymorphism, but did not find any sex specificity for this association (data not shown).

A genetic component for the variation in the self-rated health can be demonstrated by genetic factors for those who are more than 70 years old (Christensen et al. 1999). In a follow-up, it was investigated if low self-rated heath score could be due to depression (McGue and Christensen 2003), by including the depression score for the individuals, assessed using an adaptation of the depression section of the Cambridge Mental Disorders of Elderly Examination (CAMDEX), but we could not find any association to it (*P*-value 0.47), thus excluding this possibility. Although the two *P*-values obtained for self-rated health are quite low, the possibility of type 1 error due to multiple testing cannot be disregarded. Doing a Bonferroni correction, and taking into account that some of the parameters are dependant, revealed that a *P*-value should be below 0.0048 before type 1 error can be disregarded on the 5% level of significance level. Our finding of a possible association between a polymorphism in position −110 in the 5′UTR of the *HSP70-1* gene and self-rated health (*P*-value 0.0046) nevertheless requires corroboration with further studies.

Standard statistical methods require independent observations. In this study we use twins and it is known that twins correlate for many phenotypes including a number of health outcomes such as self-rated health (Christensen et al. 1999, 2000). This leads to an underestimation of the standard error but has no impact on the mean and hence does not bias the results (Holm 1983). However, this is primarily a problem while using monozygotic twin pairs and for highly heritable traits such as 'height'; but not for moderately heritable traits and dizygotic twins, where the problem is negligible.

One way to substantiate the finding is by extending the investigation to include other markers in the region. There are 14 polymorphic sites within the three MHC-linked *HSP70*, and the present study does not rule out a more significant association of human longevity with one of these polymorphisms. Moreover, this polymorphism at position −110, and other polymorphic sites within *HSP70-1, HSP70-2* and *HSP70-Hom* are shown to be in linkage disequilibrium with the other markers in the MHC region, which have been studied for their association with human longevity. For example, in Caucasian populations, Proust et al. (1982) and Rea and Middleton (1994) have reported a positive association of haplotype A1B8Cw7DR3 with longevity in male nonagenarians. Similarly, haplotypes bearing DR3 were increased in male nonagenarians (Ivanova et al. 1998). Effects of genetic variation at the HLA-DR locus were found on survival beyond age 90 years, whenever sex was taken into account. Also, allelic combinations from three *HSP70* genes have been defined in the context of MHC ancestral haplotype in the Caucasian population (Kok et al. 1999). In this study the polymorphism at *HSP70-Hom*, *HSP70-2* and *HSP70-1* gene has been shown to be associated with HLA-DR3 antigen which confirms the result from the previous study (Casino et al. 1993). The present study can therefore also be extended by defining haplotypes and using them instead of single nucleotide polymorphisms SNPs.

Because many markers within MHC have been studied for their association with human longevity,

and because the genetic markers within the MHC region are shown to be in high linkage disequilibrium with each other, we do not rule out the further possiblity of finding a strong association between the MHC-III linked *HSP70* polymorphism and human longevity.

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