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# Anti-Inflammatory Heat Shock Protein 70 Genes are Positively Associated with Human Survival

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## Abstract

A positive relationship between stress tolerance and longevity has been observed in several model systems. That the same correlation is applicable in humans and that it may be open to experimental manipulation for extending human lifespan requires studies on association of stress genes with longevity. The involvement of heat shock protein 70 (Hsp70) in cellular maintenance and repair mechanisms, including its role as an anti-inflammatory protein, makes it a suitable candidate for studying such associations. We have studied the association of three single nucleotide polymorphisms, *HSPA1A* (−110A>C), *HSPA1B* (1267A>G), and *HSPA1L* (2437T>C), present in the three *HSP70* genes, with human survival, in a cohort of individuals born in the year 1905. This population cohort is a part of the longitudinal study of Danish nonagenarians. Since DNA samples were already collected in 1998, this gave us the opportunity to perform survival analysis on these subjects. Haplotype relative risk, and genotype relative risk were calculated to measure the effects of haplotypes and genotypes on human survival in a sex-specific manner. A significant association of *HSPA1A*-AA (RR=3.864; $p$=0.016) and *HSPA1B*-AA (RR=2.764; $p$=0.039) genotypes with poor survival was observed in female subjects. Also the female carriers of haplotype G-C-T had longer survival than the non-carriers (HRR=0.550; $p$=0.015). On an average, female carriers of the G-C-T haplotype live about one year longer than non-carriers. This result corroborates our previous observations from heat shock response (HSR) study where we had shown that after heat stimulation, mononuclear cells from the carriers of genotype *HSPA1L*-TT had better HSR than cells with the *HSPA1L*-CC genotype.

### Keywords

HSP70; HSR; aging; longevity; survival; haplotype; polymorphisms



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## INTRODUCTION

At the cellular level, all organisms respond to stress by synthesizing specific stress proteins, the so-called heat shock proteins (Hsp), at the expense of other proteins. This phenomenon known as the heat shock response (HSR) protects the cells from subsequent damage and aids them to counteract the adverse effects of the stress [1–4]. Hsp are ubiquitous, cytoprotective, highly conserved chaperone proteins, which are a part of cellular safety and rescue mechanisms, preventing proteins from misfolding, permitting unfolded proteins to cross biological membranes and then allowing them to fold properly [1], or targeting the damaged proteins for degradation and removal through proteolytic pathways [5]. Furthermore, Hsp are also known to have anti-inflammatory effects by acting on NF-κB pathways [6–8].

This ability to respond rapidly to stress at the gene level determines the adaptive and, therefore, the survival capacity and longevity of the organism [9]. Genes involved in HSR have recently been studied with respect to their role in human survival, aging and longevity [10–14] Of the various stress proteins, Hsp70 is the highly inducible, most prominent and best characterised in the stress protein families [15]. In humans there are 11 isoforms of Hsp70 encoded by different genes located at dispersed loci. Three of the *HSP70* genes are mapped within the major histocompatibility complex (MHC) class III region at position 6p21.3 [16]. These are intron-less *HSPA1A (HSP70-1), HSPA1B (HSP70-2)* and *HSPA1L (HSP70-Hom)* [17], which have highly similar gene sequence but differ in their regulation. These genes are highly polymorphic [18]. Independent SNPs in these three *HSP70* genes have been found to be associated with human longevity [11,12] and certain parameters of aging, such as self rated health [13].

A widely used approach for identifying genes involved in human longevity has been the population based case-control association studies, where the frequencies of alleles and genotypes generated from a single locus candidate marker are compared between old cases and young controls [19,20]. This approach has certain drawbacks and is prone to false positive results due to sampling bias and potential secular trend in gene-environment interaction [21]. An alternative to this approach is the cohort based human survival study, where a group of individuals are genotyped for a candidate gene and then followed-up over the years to analyze their survival. This approach, though ideal, is expensive in terms of time and money, and hence rare. However, with the increase in the number of elderly, particularly in the developed world, and with the well established and extensive population based registries, it has been possible to perform cohort based survival studies in the old individuals [22].

Another drawback in the conventional genes-for-longevity studies is that, single locus marker are tested for their association with human longevity. Since lifespan is a complex trait where, along with the environment, a network of genes and genetic variables play a critical role, it is prudent to study genetic variations in a group (haplotype), rather than single polymorphisms for mapping longevity determinant genes. In this context recently a lot of attention has been focused on the effects of haplotypes on human longevity [23,24].

Taking these two crucial factors into consideration, we have performed a longitudinal study of human survival on a group of individuals born in the year 1905 in Denmark (1905 cohort) [25]. These subjects were genotyped for three single nucleotide polymorphisms (SNPs) present in the three heat shock protein 70 genes (*HSP70*), and the effects of genotypes and haplotypes on human survival were studied.

The three SNPs which we studied are, −110A>C (marker rs1008438), present in the promoter region of *HSPA1A*; the synonymous 1267A>G (marker rs1061581), present in the coding region of *HSPA1B*; and the non-synonymous 2437T>C (Marker rs2227956), present

in the coding region of *HSPA1L*. The latter SNP leads to a change in amino acid at position 493 from a non-polar hydrophobic acid methionine (Met) to a polar neutral threonine (Thr) and may have molecular functional significance with respect to the stability and activity of the chaperoning protein Hsp70.

## MATERIALS AND METHODS

168 DNA samples (males = 40, mean age = 92.8 ± 0.4 yr; females = 128, mean age = 92.8 ± 0.4 yr), collected in 1998 from individuals born in year 1905, were used for typing the three SNPs in the three genes. These samples were collected in Denmark as part of the first ever nationwide survey of nonagenarians [26]. Various physical and cognitive parameters were also measured on these individuals.

Genotyping was done using real-time (RT) PCR on the LightCycler system (Roche Applied Sciences), which helps to monitor the amplification of PCR product in real time, using fluorescent labelled oligonucleotide probes and primers specific for each SNP. The protocol for the RT-PCR is described elsewhere [13], and the program can be made available on request.

The effects of genotypes and haplotypes on human survival was estimated by using the relative risk method developed by Tan *et al.* [21]. With this method sex-specific haplotype relative risk (HRR) and genotype relative risk (RR) on human survival was estimated for every haplotype and genotype derived from the three SNPs. For every carrier of genotype/haplotype a relative risk of more than or less than 1 was considered to be harmful or beneficial for human survival respectively. All statistical analyses were conducted by GAUSS programming package (http://www.aptech.com) [21].

## RESULTS

Gene and genotype frequencies of the three SNPs are given in Table 1. Fig. (1) and Table 2 show that the survival duration of the female carriers of genotypes *HSPA1A*-AA (RR=3.864; $p$=0.016) and *HSPA1A*-AC (RR=2.577; $p$=0.056) was shorter than the carriers of *HSPA1A*-CC.

Also the females carriers of genotype *HSPA1B*-AA (RR=2.764; $p$=0.039) had shorter survival duration than carriers of *HSPA1B*-GG [Fig. (1)]. These results on the association of genotypes with female survival were maintained when Kaplan-Meier's test of survival was performed [Fig. (2)]. No significant results were obtained in analyzing the male subjects.

Estimating the sex-specific HRR on human survival showed that the female carriers of haplotype G-C-T had longer survival duration than the non-carriers (HRR=0.550; $p$=0.015) (Table 3), while all other haplotypes failed to reach the significance level. Fig. (3) (left), shows that the estimated frequency of the G-C-T haplotype goes up with increasing age indicating the beneficial effect of the haplotype. On an average, female carriers of the G-C-T haplotype live about one year longer than non-carriers [Fig. (3), right]. Similar to the genotype-based analysis, no haplotype displays a significant effect on male survival.

## DISCUSSION

The conventional method for performing the genetics of human longevity, in the population based association studies, is the cross-sectional design. In this method the gene, genotype or haplotype frequencies are compared between a group of old 'cases' and young 'controls'. This methodology, though inexpensive and straightforward, suffers from a drawback that the observed differences in the frequencies in the two groups can be due to secular changes in

population demographic and genetic parameters of the two groups and not necessarily age. An alternate to this approach is the longitudinal cohort design for studying the effects of genes on human survival in a cohort of long lived individuals. In this, a group of individuals are genotyped for polymorphisms in the candidate genes, and then followed up for studying their survival. This methodology mitigates the drawbacks thus encountered by the case-control design. In the present study we have applied the longitudinal cohort design for studying relative risk of haplotypes and genotypes on human survival in a cohort of individuals born in Denmark in the year 1905 [21].

A group of nonagenarians, born in year 1905, and from whom DNA was collected in the year 1998, when they were 93 years old, were genotyped for polymorphisms in the three *HSP70* genes. Thus we could study if, over the years, the carriers of one genotype/haplotype survived better or worse than the non-carriers. Nonagenarians form an ideal group on which such follow-up studies can be performed. A large degree of heterogeneity with respect to physical and cognitive functioning can be expected among them. Nonagenarians are also better representative of the older population than a group of rare and selected centenarians. A follow up study of this age group can shed light on the aging processes among extremely old individuals. It has also been found in the Danish 1905 cohort that the selection pressure that an individual has to face in order to survive from ages 92 to 100 is comparable to the selection pressure faced in order to survive from ages 0 to 92 (http://www.mortality.org). So even if we cannot use a younger cohort for studying the survival, the use of an older group such as nonagenarians can be a good representative of the general population [21].

In our study we found that the female carriers of genotype *HSPA1A*-AA survived for a shorter duration than the non-carriers. Our result is consistent with the observations of Altomare *et al.* from a case-control study done on Italian subjects where they found that the frequency of allele A was unfavorable for female longevity [11]. However, the current survival study, being performed on a cohort, is more powerful to show this association. *HSPA1A* (−110A>C) SNP is present in the promoter region of the gene, 3′ upstream of the heat shock element (HSE). Favatier *et al.* [27] had observed that this SNP does not affect the binding of heat shock factor with the HSE and hence may not affect the synthesis of Hsp. In another recent study it was shown that the reporter constructs containing the −110A allele cloned into the luciferase reporter plasmid drove marginally higher transcriptional activity of *HSPA1A* as compared with the −110C allele in both control and heat shocked cells [28], proposing that this polymorphism may be a functional one. Since we have observed a significant association of this SNP with female survival, the mechanisms involved in this association need to be further explored.

In the current study we also observe that female carriers of genotype *HSPA1B*-AA survive for a shorter duration as compared to the non-carriers. This synonymous SNP is present at position 1267, in the coding region of *HSPA1B*. We had recently observed that a high level of linkage disequilibrium (LD) exists between *HSPA1A*-A and *HSPA1B*-A ($D'$=1) [14]. This is consistent with our present observation where we see that carriers of both the alleles, *HSPA1A*-A and *HSPA1B*-A, are shorter survivors. Because *HSPA1B* (1267A>G) is not a functional variation, it remains to be seen how the negative association of this allele with human survival is manifested at the biofunctional level.

Because longevity is a complex trait, statistical approaches that take into account a group of genetic variants (haplotypes) are crucial in mapping genes that modulate human survival. In our analyses on the association of haplotypes on human survival, we observed that the female carriers of haplotype G-C-T were longer survivors as compared to the non-carriers. Our current results are consistent with our earlier observations of haplotype survival analysis, in the cross-sectional study design (manuscript in preparation). Moreover in the

cross-sectional study design we had also seen that allele *HSPA1L*-T was favourable, whereas allele *HSPA1L*-C was unfavourable for female longevity. From the current results on the 1905 cohort individuals, we see that allele *HSPA1L*-T is defined in the haplotype G-C-T which is shown to have longer survival duration for females. We also observed a trend towards shorter survival duration of the carriers of *HSPA1L*-C as compared to the non-carriers (Table 2).

The polymorphism *HSPA1L* (2437T>C) is present in the coding region of the gene and leads to an amino acid change at position 493 from a non-polar hydrophobic methionine (Met) to a polar neutral threonine (Thr). This may have biofunctional relevance, since amino acid 493 corresponds to amino acid position 489 in *E. coli* DnaK, which in the crystal structure of the DnaK peptide binding domain is localised on the beta sheet beneath the peptide binding groove [29]. An amino acid change at this position could be associated with the peptide-binding specificity of Hsp70. A change to a polar neutral Thr (allele C) may affect the chaperone activity and hence the functional efficiency of *HSPA1L* by lowering the strength of the hydrophobic interactions between chaperones and the target protein [12]. Also in a recent study we were able to show that after heat stimulation mononuclear cells from the carriers of genotype *HSPA1L*-TT had better HSR than *HSPA1L*-CC [30]. And this difference was more prevalent among females. This fits quite well into our current observations of the effects of haplotypes, carrying these alleles, on human survival.

In a recent study it was shown that the females from the 1905 cohort had lower self reported disability levels, and were fitted with respect to different parameters of Activities of Daily Living (ADL) as compared to the females from another centenarian cohort [31]. However this difference was not observed among males. It remains to be seen if those females who have better ADL carry the genotypes/haplotypes which have predisposition towards longer duration of survival.

These results from the association of genotypes/haplotypes of *HSP70* genes on human survival reiterate the view that the genes involved in stress response are suitable candidates for studying the genetics of longevity.

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## ABBREVIATIONS

| | |
|---|---|
| **ADL** | Activities of Daily Living |
| **GRR** | Genotype Relative Risk |
| **HRR** | Haplotype Relative Risk |
| **Hsp** | Heath shock proteins |
| **HSP70-1** | HSPA1A |
| **HSP70-2** | HSPA1B |
| **HSP70-Hom** | HSPA1L |
| **HSR** | Heat Shock Response |
| **MHC** | Major Histocompatibility Complex |

**SNP** Single Nucleotide Polymorphism

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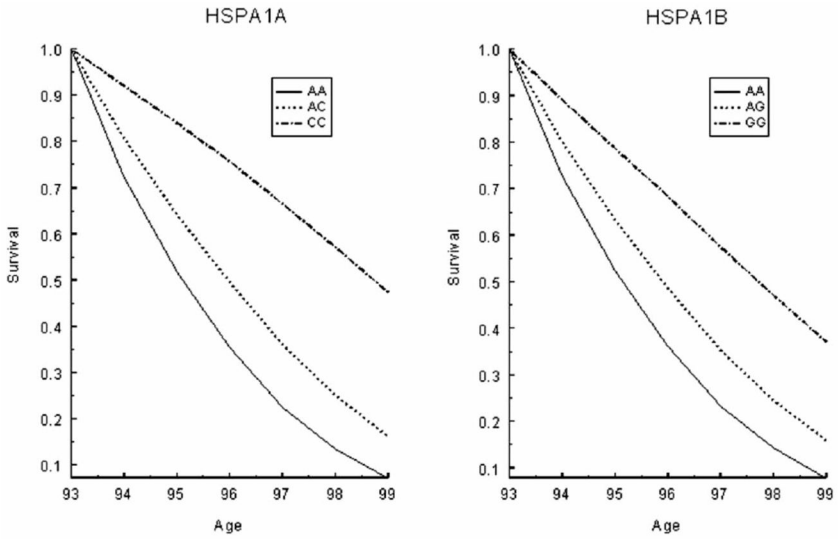


**Fig. 1.**
Survival of genotypes in ***HSPA1A*** (left) and ***HSPA1B*** (right) among females.

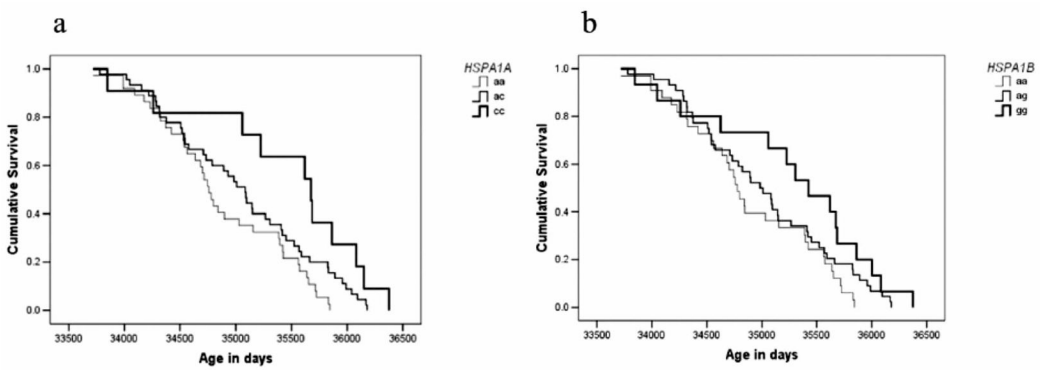


**Fig. 2.**

Association of female survival with genotypes of ***HSPA1A*** and ***HSPA1B*** respectively, using Kaplan-Meier's survival test.

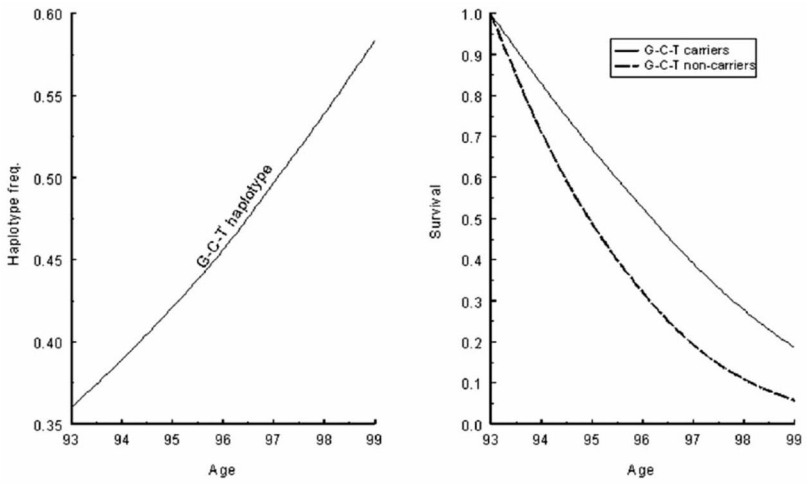


**Fig. 3.**
Frequency (left) and survival (right) of G-C-T haplotype carriers in females over the follow-up ages.

**Table 1**

Gene/Genotypic Frequency Distribution of the Three SNPs in 1905 Cohort Individuals

| *HSPA1A* (−110A>C) | AA | | AC | | CC | | A | | C | |
|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | M | F | M | F | M | F | M | F |
| | 25 (10) | 41.9 (49) | 65 (26) | 47 (55) | 10 (4) | 11.1 (13) | 57.5 (46) | 65.3 (153) | 42.5 (34) | 34.6 (81) |
| *HSPA1B* (1267A>G) | AA | | AG | | GG | | A | | G | |
| | M | F | M | F | M | F | M | F | M | F |
| | 25 (10) | 38 (46) | 62.5 (25) | 47.9 (58) | 12.5 (5) | 14 (17) | 56.2 (45) | 61.9 (150) | 43.7 (35) | 38.1 (92) |
| *HSPA1L* (2437T>C) | TT | | TC | | CC | | T | | C | |
| | M | F | M | F | M | F | M | F | M | F |
| | 62 (23) | 57.7 (67) | 35.1 (13) | 37.9 (44) | 2.7 (1) | 4.3 (5) | 79.7 (59) | 76.7 (178) | 20.2 (15) | 23.2 (54) |

M= male; F= female. Data are presented in percentage with number of individuals given in parentheses.

## Table 2

Estimated Relative Risks of the Genotypes at the Three Loci in Females

| Genotype | Frequency | Risk | *p*-value |
|---|---|---|---|
| *HSPA1A* | | | |
| AA | 0.398 | 3.864 | 0.016 |
| AC | 0.484 | 2.577 | 0.056 |
| CC | 0.118 | 1 | - |
| *HSPA1B* | | | |
| AA | 0.359 | 2.764 | 0.039 |
| AG | 0.478 | 1.932 | 0.188 |
| GG | 0.163 | 1 | - |
| *HSPA1L*[*] | | | |
| C allele carriers | 0.359 | 1.913 | 0.328 |

[*]Genotype analysis was not possible due to low frequency of C allele.

## Table 3

Estimated Relative Risks of the Frequent Haplotypes* over the Three Loci in Females

| Haplotype | Frequency | Risk | *p*-value |
|---|---|---|---|
| AAT | 0.379 | 1.481 | 0.115 |
| AGT | 0.027 | 2.266 | 0.180 |
| GCT | 0.358 | 0.550 | 0.015 |
| AAC | 0.230 | 1.148 | 0.589 |

*The first position in the haplotypes corresponds to the marker in gene *HSPA1B*, second in *HSPA1A*, and third in *HSPA1L*.