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An allele of *HRAS1* 3′variable number of tandem repeats is a frailty allele: implication for an evolutionarily-conserved pathway involved in longevity

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Abstract

The human *HRAS1* belongs to an evolutionarily-conserved family of genes which enrolls among its members the yeast *RAS2*, a gene which regulates stress response and longevity in the *Saccharomyces cerevisiae*. In this paper we report that the frequency of the a3 allele of *HRAS1* 3′variable number tandem repeat (*HRAS1* 3′VNTR) decreases in centenarians in respect to young people, and we estimate that during aging a3 carriers have a relative mortality risk of 1.126 (95% CI = 1.044–1.213). We propose that the germ-line variability at the *HRAS1* locus impacts on the individual's capacity to reach the extreme limits of human life-span. Furthermore, we provide suggestive evidence that a3 *HRAS1 3′VNTR* allele and inherited variants of the mitochondrial genome (*mtDNA* haplogroups) do not affect independently human longevity, thus recalling the nucleus-mitochondrion interaction which regulates stress response and life-span in the yeast.

Keywords: *HRAS1* polymorphisms; Aging; Ras genes; Retrograde response; Stress response

1. Introduction

A great deal of data indicate that aging and longevity of invertebrates and vertebrates are strictly related to the capability to cope with endogenous and exogenous stress (Franceschi et al., 2000). In this regard, studies on model organisms such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and mice have provided evidence that mutations in genes which coordinate stress response at various levels are capable to modulate life-span (Jazwinski, 1996). A strategy to verify whether stress response genes have an impact on longevity in humans consists in the comparison of polymorphic variants of candidate genes between a group of individuals selected for successful aging and longevity, such as centenarians, and groups of individuals of younger ages (De Benedictis et al., 1998; De Benedictis et al., 1999). Accordingly, previous studies have suggested that polymorphic variants of genes involved in systemic (Tyrosine Hydroxylase, TH) and cellular (mitochondrial DNA, *mtDNA*) stress response are associated with human longevity (De Benedictis et al., 1998; De Benedictis et al., 1999; Tanaka et al., 1998). On the basis of these data, it can be proposed that genes affecting human longevity could be successfully searched for inside gene families which are involved in stress-response throughout evolution. A likely candidate is human *HRAS1*, which belongs to an evolutionarily-conserved family of GTPases that, from yeast to humans, control the response to physical stress, such as UV radiation and oxidative stress (Barbacid, 1987; Engelberg et al., 1994; Lander et al., 1995). In the yeast, a member of the family, called *RAS2,* determines life span by triggering a mitochondria-to-nucleus signaling pathway called

Abbreviations: VNTR, variable number of tandem repeats; mtDNA, mitochondrial DNA; TH, tyrosine hydroxylase; dNTP, deoxyribonucleoside triphosphate; MCχ^2, Monte-Carlo chi-square test; AR, adjusted residuals; CI, confidence interval; MH, Mantel-Haenszel test; RR, relative risk; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; df, degrees of freedom

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retrograde response which is able to cope with mitochondrial dysfunction (Sun et al., 1994; Jazwinski, 1996; Jazwinski, 1999; Kirchman et al., 1999).

HRAS1 was discovered as a proto-oncogene involved in a variety of human malignancies (Bos, 1989). Subsequently, *HRAS1* was shown to regulate a number of cell activities, such as proliferation, differentiation, senescence, apoptosis, stress response and energy metabolism (Lowy and Willumsen, 1993; Chen and Faller, 1996; Serrano et al., 1997; Lander et al., 1995). Moreover, *HRAS1* was found to be capable to replace endogenous *RAS* in the control of yeast longevity (Chen et al., 1990). Recently, a role of *HRAS1* in T-cells and endothelial response to inflammatory cytokines and neuronal long-term potentiation has been documented (Tanaka et al., 1999; Xu et al., 1998; Manabe et al., 2000). Moreover, current literature suggests that *HRAS1* may be involved in a number of age-related phenomena, such as immunosenescence, insulin resistance, atherosclerosis and neurodegeneration (Pahlavani, 1998; Draznin et al., 2000; Li et al., 1996; Gartner and Holzer, 1999). On the basis of these considerations, *HRAS1* is one of the best candidate for exploring the role of stress-responder genes in human longevity.

A 28-base pair Variable Number of Tandem Repeat marker (*HRAS1 3′VNTR*) lies downstream of the coding region of the *HRAS1* locus. We used this marker to check the hypothesis that variability at *HRAS1* locus affects the individual capability to reach the extreme limits of human life-span.

2. Materials and methods

2.1. Subjects

Two groups of Italian subjects were enrolled in the study: a group of 467 people (297 males and 170 females) ranging from 20 to 65 years old, and a group of 234 centenarians (59 males and 175 females). The male/female ratio in centenarians was representative of that found in a nation-wide demographic survey on Italian centenarians (Franceschi et al., 2000). Ethnicity and geographic origin of each subject included in the study was checked as far as the grandparental generation. All subjects gave informed consent.

2.2. Analysis of allelic variation at the HRAS1 locus and of mtDNA haplogroups

Amplification of the *HRAS1* 3′VNTR was performed with the expand long template PCR system (Roche). Briefly, genomic DNA from peripheral blood lymphocytes was amplified with 300 nmoles of the following primers: 5′-GAGCTAGCAGGGCATGCCGC-3′ and 5′-AGCACGGTGTGGAAGGAGCC-3′. The reaction was carried out in 25 μl with 1 × expand long template PCR system Buffer-3, 150 ng of genomic DNA, and 500 μM dNTPs. The following thermal cycling conditions were used: 1 cycle at 93°C for 2 min, then ten cycles of denaturation for 40 s at 94°C, and annealing-elongation for 2 min and 30 s at 68°C, followed by 15 cycles of denaturation for 40 s at 94°C, annealing for 30 s at 68° and elongation for 2 min plus 20 s for each cycle at 68°C. After the 25th cycle, an extra step was performed at 68°C for 7 min, to extend the template completely. The 25 PCR cycles were carried out on a DNA thermal cycler (Delphi 1000™ Thermal Cycler, Oracle BioSystem™). A total of 15 μl of the PCR products were electrophoresed in a 2.0% agarose gel at 150 V for 5 h and visualised by ethidium bromide staining. Commercial molecular weight markers Markers VI and XIV (Roche) were employed, together with ad hoc molecular weight standards comprising common and rare *HRAS1* 3′VNTR alleles whose size was verified by automated DNA sequencing (ABI Prism 310). Data on *mtDNA* haplogroups were obtained as part of a previously published study. *MtDNA* haplogroups were named as previously reported in De Benedictis et al., 1999, with the exception of OTH haplogroup which collected the haplogroups previously named I, V, W, X, Oth in De Benedictis et al. (1999).

2.3. Statistical analysis

Hardy-Weinberg equilibrium was assessed by Monte Carlo Markov Chain (1,000,000 steps and 100,000 dememorisation steps). Comparisons of allele and genotype frequency distributions were performed by Monte Carlo chi-square test ($MC\chi^2$). Adjusted residuals (AR), which represent the difference between expected and observed frequency under the null hypothesis of independence were used for qualitative evaluations of the allele and genotype frequency distributions. Mantel-Haenszel test was applied to allow for combining information from tables stratified for a confounding variable as gender. The analyses were implemented in the SPSS package (SPSS, Inc., Chicago, IL). The genotype-specific relative risk of death (RR) of *HRAS1 3′VNTR* was assessed by the RR method, a mathematical approach which has been developed to obtain estimates of the genotype-specific RR of death from cross-sectional studies (Yashin et al., 1999). In this model, the RR of a genotype (indicated in the equations as r) is the ratio of hazard of death for carriers, $\mu(x, r)$, to that for the non-carriers or the baseline hazard, $\mu_0(x)$. Then, according to the proportional hazard assumption, $\mu(x, r) = r\mu_0(x)$. The corresponding survival function for the carriers of a genotype at age x is

$$s(x, r) = e^{-\int_0^x \mu(t, r)\,dt} = e^{-\int_0^x r\mu_0(t)\,dt}$$

$$= e^{-r\int_0^x \mu_0(t)\,dt} = e^{-rH_0(x)} = s_0(x)^r.$$

where $r_i = e^{\beta U}$ (take $U = 0$ for non-carriers of the genotype and $U = 1$ for carriers of the genotype), $s_0(x)$ is the survival distribution corresponding to the baseline hazard function

and $H_0(x)$ is the cumulative hazard at age x. r can take any value greater than zero, an allele with r larger than 1 (frailty allele) increases the hazard of death, while an allele with r smaller than 1 (robust allele or longevity allele) reduces it.

3. Results

3.1. Allele and genotype frequency distributions in young people and centenarians

A total of 24 different *HRAS1 3′VNTR* alleles, ranging from 0.91 to 2.55 Kbp were found. Both in young people, and centenarians the genotype distributions were in agreement with Hardy-Weinberg equilibrium (HWE) expectations ($P > 0.05$ for all groups). For cross-tabulations and data analysis, common alleles corresponding to size of 0.97, 1.45, 2.00, 2.50 Kbs were named as a1, a2, a3, a4, respectively. The remaining 20 alleles, whose individual frequency was below 2%, were collectively coded as rare (aR) alleles (Krontiris et al., 1993). The allele distribution of *HRAS1 3′VNTR* in young people and centenarians is (reported in Table 1. A decrease of a3 allele frequency was evident: 0.113 (95% CI 0.092–0.134) in young people versus 0.062 (95% CI 0.040–0.084) in centenarians ($\chi^2 = 9.959$, df = 4, $P = 0.041$). The Mantel-Haenszel (MH) test revealed that the decrease a3 allele was associated with age after stratification for sex ($\chi^2 = 6.577$, df = 1, $P = 0.010$); odds ratio (OR) = 0.551, 95% CI 0.349–0.870, $P = 0.011$.

Accordingly, the MH test revealed that the proportion of a3 allele carrier genotypes decreased from young people (0.203, 95% CI 0.240–0.166) to centenarians (0.124, 95% CI 0.102–0.167) after stratification for sex ($\chi^2 = 4.566$, df = 1, $P = 0.033$; OR = 0.592, 95% CI 0.366–0.958, $P = 0.033$). Even though these data suggested that a3 allele confers disadvantage for longevity, it has been recently pointed out that OR calculations and the comparison of gene frequencies between young people and centenarians are not satisfactory strategies to estimate the impact of genetic factors on longevity (Yashin et al., 1999; Yashin et al., 2000). Consequently, the genotype-specific RR of death of *HRAS1 3′VNTR* was assessed by the RR method (Yashin et al., 1999; Yashin et al., 2000). The analysis revealed that a3 carriers have a RR of 1.126 (95% CI = 1.044–1.213, Log-likelihood = 9.57716, df = 1, $P = 0.002$) to die in respect to the rest of the population, indicating that a3 allele is a frailty allele. No significant results were obtained when the approach was employed to assess the impact of *HRAS1 3′VNTR* aR alleles, which have been associated with an increased individual susceptibility to various cancer (Krontiris et al., 1993).

3.2. mtDNA haplogroups and HRAS1 3′VNTR interaction

Considering the functional relationship between *HRAS1* related-genes and mitochondria and their involvement in pathways related to longevity in classical experimental models, and the involvement of *mtDNA* haplogroups in human longevity (De Benedictis et al., 1999) we considered interesting to assess for possible interaction between *HRAS1* 3′VNTR genotypes and *mtDNA* haplogroups taking advantage of a pre-existing *mtDNA* haplogroup genotypization on 158 centenarians and 208 young individuals presented in this investigation. Subjects were subdivided in a3 carriers and a3 non carriers. The distribution of *mtDNA* haplogroups was different between the two groups in centenarians ($MC\chi^2 = 11.13$, df = 5; $P = 0.045$) (Table 2). In particular, the haplogroups K and U were over-represented in a3 allele carrier centenarians. Since the K and U haplogroups share several variants, and are molecularly related (K is one of the U sub-clades, Macaulay et al., 1999) we could also test the difference after pooling K and U haploytypes ($MC\chi^2 = 8.272$, df = 1, $P = 0.004$). No interaction between *HRAS1* 3′VNTR a3 allele and *mtDNA* haplogroups was found when the same approach was used to analyze the data from young individuals ($MC\chi^2 = 3.271$, df = 5; $P = 0.67$) (Table 3).

4. Discussion

In this paper we found a decrease of *HRAS1 3′VNTR* a3

Table 1
3′VNTR alleles in young individuals and centenarians[a]

Allele size (Kb)	Name	Young individuals ($n = 467$)			Centenarians ($n = 234$)		
		Number	Frequency	AR	Number	Frequency	AR
0.97	a1	554	0.593	−0.8	288	0.615	0.8
1.45	a2	89	0.095	−0.4	48	0.103	0.4
2.00	a3	106	0.113	3.1	29	0.062	−3.1
2.50	a4	83	0.089	−1.0	49	0.105	1.0
Rare	aR	102	0.109	−0.3	54	0.115	0.3
Total		934			468		

[a] *HRAS1* 3′VNTR alleles in young individuals and centenarians. Four common alleles (a1, a2, a3, a4) were found. A total of 20 rare alleles (each with a frequency lower than 2%) were grouped as aR. Difference in the overall allele frequency distribution was found when young individuals and centenarians were compared ($MC\chi^2 = 9.959$, df = 4, $P = 0.041$). Inspection of adjusted residuals (AR), which quantify the magnitude of the difference between observed and expected allele frequencies, revealed a decreased frequency of a3 allele in centenarians (AR = ±3.1).

Table 2
Interaction between *HRAS1 3′VNTR* genotypes and mtDNA haplogroups in centenarians[a]

mtDNA haplogroups	Centenarians ($n = 158$) *HRAS1* 3′VNTR					
	a3 non-carriers			a3 carriers		
	Number	Frequency	AR	Number	Frequency	AR
H	54	0.380	0.5	5	0.313	−0.5
J	17	0.120	0.7	1	0.063	−0.7
K	7	0.049	−3.0	4	0.250	3.0
T	16	0.113	0.6	1	0.063	−0.6
U	14	0.099	−1.1	3	0.188	1.1
OTH	34	0.239	1.0	2	0.125	−1.0
Total	142			16		

[a] Interaction between *HRAS1* 3′VNTR genotypes and *mtDNA* haplogroups in 158 centenarians ($MC\chi^2 = 11.13$, df = 5; $P = 0.045$).

allele frequency in centenarians in respect to young people. Moreover, we employed an analytical approach which estimates the impact of specific genotypes on mortality during aging by combining data from cross-sectional studies and data from demographic life-tables (Yashin et al., 1999; Yashin et al., 2000). A significant RR could be attributed to a3 carrier status (RR = 1.126), suggesting that people who carry an a3 allele at *HRAS1 3′VNTR* are likely to experience higher mortality during aging in respect to the rest of the population.

So far, associations between *HRAS1 3′VNTR* and common diseases mostly regard common cancer, such as breast cancer (Krontiris et al., 1993). However, we found that cancer-predisposing alleles of *HRAS1 3′VNTR* (aR alleles) are represented in centenarians at the same frequency of young people. This finding is reminiscent of the results we previously obtained on Codon 72 variants of p53 (Bonafè et al., 1999. One possible explanation of these results is that cancer death does not impact enough on population mortality, and thus genetic factors involved in cancer susceptibility cannot be detected in studies involving young people and centenarians (Bonafè et al., 1999.

HRAS1 3′VNTR alleles have been suggested to differ in their enhancement of transcriptional regulatory activity of reporter genes, and to contain binding sites for NF-κB (Green and Krontiris, 1993), a transcription factor which plays a pivotal role in stress response and inflammation (Baeuerle and Henkel, 1994. Thus, the hypothesis that the a3 allele could confer peculiar properties to inflammatory and stress response could be proposed. In fact, *HRAS1* is a pivotal regulator of stress response at cell level (Jazwinski, 1999; Lander et al., 1995)

The involvement of stress-response genes in longevity is predicted on theoretical grounds, and it is reminiscent of what occurs in a number of species (Jazwinski, 1996); Consequently, the existence of evolutionarily-conserved strategies to attain resistance to stress, with longevity as a consequence, can be argued. One of such biochemical pathways could have relationship with retrograde response in *S. cerevisiae*, in which *RAS2* an homologue of the human *HRAS1,* modulates an intracellular signalling pathway from the mitochondrion to the nucleus, which compensates for mitochondrial dysfunction and extends longevity (Kirchman et al., 1999). In this regard, a role for *mtDNA* germ-line variability in human longevity has been suggested in recent investigations (Tanaka et al., 1998; De Benedictis et al., 1999). In particular, sets of evolutionarily-conserved *mtDNA* polymorphisms, i.e. *mtDNA* haplogroups, have been found to be differently represented in centenarians, compared to young people (De Benedictis et al., 1999;

Table 3
Interaction between *HRAS1 3′VNTR* genotypes and mtDNA haplogroups in young people[a]

mtDNA haplogroups	Young individuals ($n = 208$) *HRAS1* 3′VNTR					
	a3 non-carriers			a3 carriers		
	Number	Frequency	AR	Number	Frequency	AR
H	71	0.420	0.1	16	0.410	−0.1
J	11	0.065	0.1	2	0.051	−0.1
K	15	0.089	−0.1	2	0.051	0.1
T	15	0.089	−0.5	7	0.179	0.5
U	26	0.154	−1.0	6	0.154	1.0
OTH	31	0.183	−1.2	6	0.154	1.2
Total	169			39		

[a] Interaction between *HRAS1* 3′VNTR genotypes and *mtDNA* haplogroups in 208 young people ($MC\chi^2 = 3.271$, df = 5; $P = 0.67$).

Tanaka et al., 2000). It seemed therefore interesting to search for a possible interaction between *HRAS1* and mitochondria, by investigating the combinations of *HRAS1 3′VNTR* and *mtDNA* haplogroups in young people and centenarians. Indeed, we found that *HRAS1 3′VNTR* and *mtDNA* haplogroups are randomly associated in young people, but not in centenarians in which K and U haplogroups are over-represent among a3 carriers in respect to a3 non carrier people. These results suggest that K and U haplogroups could exert beneficial effects for longevity which may be able to compensate the detrimental effects of a3 allele. In this regard, a putative protective role of K and U haplogroups in respect to the effects of *APOE4* allele on the susceptibility to Alzheimer's disease has been recently proposed (Carrieri et al., 2001). Intriguingly, K and U are closely molecularly related, being K part of the U sub-clade (Macaulay et al., 1999).

Interestingly, *mtDNA* haplogroups have been recently found to affect inter-individual variability in oxidative phosphorylation capacity (Ruiz-Pesini et al., 2000). Moreover, recent literature has elucidated that stress response at cell levels involve an exchange of p21/HRAS1 from mitochondrial compartment to cytoplasm (Rebollo et al., 1999). Hence, it can be proposed that the combination of different germ-line variability at both loci could be responsible for differences in stress response at cellular levels, which could have long-term consequences on the performances in a number of metabolic functions. The mitochondria-nucleus interaction involving a gene of *RAS* family recalls *S. cerevisae* retrograde response (Kirchman et al., 1999). This biochemical pathway could be an evolutionarily-conserved strategy which affects life span within a species by compensating the age-related stress due to mitochondrial dysfunction. If this interpretation is correct, future research on human longevity could benefit from studies on mechanisms regulating stress response and longevity in lower invertebrates, as in the case of *S. cerevisae* (Jazwinski, 1999).

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