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RESEARCH ARTICLE

The frequency of Klotho KL-VS polymorphism in a large Italian population, from young subjects to centenarians, suggests the presence of specific time windows for its effect

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Abstract In mice a defect of Klotho gene expression results in multiple aging-like phenotypes including short lifespan, osteoporosis and atherosclerosis, while its over-expression suppresses aging and extends lifespan. Contrasting data have been reported as far as the importance of the functional variant of Klotho termed "KL-VS" on human longevity, depending on the average age of the old subjects that were compared with young controls. We therefore performed a study on a large Italian population sample including people from very young to very old age (centenarians). A total of 1,089 (669 women and 420 men) unrelated individuals from 19 to 109 years, born and residing in northern and central Italy, were subdivided into three age classes defined on the basis of the survival curve constructed using Italian demographic mortality data, and genotyped for the KL-VS allele. We found a significant increase of the heterozygous Klotho genotype in the class of elderly people compared to young controls. On the contrary, no difference was present between centenarians and young controls. Such a non monotonic trajectory is evident only when a large, comprehensive age range is investigated, and is compatible with the hypothesis that this KL-VS heterozygous genotype is favorable for survival in old people, its beneficial effect decreasing thereafter, and becoming no more evident at the extreme ages. Such unusual age-related changes in the Klotho KL-VS genotype frequency is compatible with the hypothesis that alleles and genotypes involved in aging and longevity may exert their biological effect at specific time windows.

Keywords Klotho KL-VS polymorphism · Human longevity · Centenarians · Allele timing · Genetic-demographic approach

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Introduction

Klotho (MIM 604824), originally identified by insertional mutation in a transgenic mouse, is an aging

suppressor gene. Its defective expression in mice results in aging-like phenotypes including short life span, hypoactivity, infertility, osteoporosis and atherosclerosis (Kuro-o et al. 1997). On the other hand, its over-expression extends mice lifespan by about 20–30% (Kurosu et al. 2005). Mouse Klotho gene encodes a 130 kDa protein with a single transmembrane domain and an extracellular domain that contains two repeats (KL1 and KL2), both of which have up to 40% homology to β-glycosidase family-1. Klotho is expressed predominantly in the choroid plexus of the brain and the distal convoluted tubule of the kidney (Kuro-o et al. 1997; Tohyama et al. 2004). An alternatively spliced transcript that lacks the transmembrane domain also exists (Shiraki-Iida et al. 1998). Klotho can be found as either transmembrane or secreted protein, this latter form being predominant in humans (Matsumura et al. 1998). Secreted Klotho is detectable in the blood and cerebrospinal fluid of mice and humans, which suggests that Klotho could function as a hormone (Kurosu et al. 2005; Imura et al. 2004). The secreted Klotho protein can regulate multiple growth factor signaling pathways, including insulin/IGF-1 and Wnt, and the activity of multiple ion channels. Klotho protein also protects cells and tissues from oxidative stress, yet the precise mechanism underlying this antioxidant activity remains to be determined (Kuro-o 2008). It has been hypothesized that Klotho-mediated resistance to oxidative stress may explain, at least in part, the anti-aging effects of this protein (Bartke 2006; Kuro-o et al. 1997; Yamamoto et al. 2005).

Human Klotho gene maps on chromosome 13q12, and its product is highly conserved (86% amino acid identity with the mouse Klotho protein) (Matsumura et al. 1998). A common variant, termed "KL-VS", composed of six single nucleotide polymorphisms (SNPs) in perfect linkage disequilibrium, spans exon 2 and its flanking sequence and is present in $\sim$15% of Caucasians (Arking et al. 2002). It has been suggested that the KL-VS allele influences the trafficking and catalytic activity of Klotho protein (Arking et al. 2002). In studies on humans, Klotho gene has been associated with some features of the aging process such as osteopenia/osteoporosis and atherosclerosis (Arking et al. 2002, 2003, 2005; Kawano et al. 2002; Low et al. 2005; Mullin et al. 2005; Yamada et al. 2005). Association studies of Klotho gene variants with human longevity yielded contrasting results, likely depending on the different age of the groups of old subjects analysed and their controls. In particular, a survival advantage was observed for the heterozygous but not homozygous genotype in a population of Bohemian Czech elderly subjects (Arking et al. 2002, 2005). However, these results were not confirmed in Baltimore Caucasian and Baltimore African-American subjects (Arking et al. 2002, 2005). In another study performed in two independent populations (Ashkenazi-Jewish and Czech) the advantage for the heterozygous genotype was observed in subjects aged $\geq$79 years (Arking et al. 2005). Recently, Puca et al. tested the frequency of KL-VS variant in Caucasian long-lived ($\geq$93 years) and young control subjects (<35 years). This study reported no association between the functional variant of the Klotho gene and exceptional longevity (Novelli et al. 2008).

In order to clarify the importance of Klotho KL-VS genotype in human longevity, we performed a study on a large Italian population sample, including people from very young to very old age (centenarians).

Materials and methods

To determine whether KL-VS allele status influences human longevity, we analyzed a sample of Italian subjects divided into gender-specific age classes according to demographic information obtained from the survival function $S(x)$ for the Italian population of the XX century (Passarino et al. 2006; Cardelli et al. 2008). In brief, the limit between the youngest class and the intermediate class has been chosen as the age at which the curvature of the survival function $S(x)$ is negative and has the largest absolute value (formally, the age corresponding to the minimum value of the second derivative $S''(x)$ of the survival function). Equivalently, the limit between the intermediate class and the oldest class was chosen as the age at which the curvature of the graph is positive and has the largest absolute value (formally, the age at which the second derivative $S''(x)$ of the survival function attains the maximum value). The age classes defined according to this criterion are gender-specific and account for the different survival of men and women in the Italian population. For men [women], the first class consists of individuals aged <66 [<73] years, the second class of individuals aged 66–88 [73–91] years, the third class of individuals aged >88 [>91] years.

A total of 1,089 (669 women and 420 men) unrelated individuals (age range: 19–109 years), born and residing in northern and central Italy, were recruited to participate in this study. Information about health status (based on their clinical history and on blood tests) was obtained for all participants, and no major pathological conditions were present (diabetes, cancer, dementia, cardiovascular diseases). The sample included 238 centenarian subjects (191 women and 47 men), who were classified as healthy (categories A and B according to the criteria reported in Franceschi et al. 2000).

DNA was extracted from peripheral blood mononuclear cells (PBMCs) using phenol/chloroform, according to standard procedures. Since all six SNPs occur in perfect linkage disequilibrium, a single variant, 1155G>A, can be used to tag the haplotype. The presence of **G** (*wild-type* allele) and/or **A** (KL-VS allele) at position 1155 in the exon 2 of the Klotho gene was determined by a PCR amplification using the following primers: Fw 5′-AggCTCATgCCAAAg TCTgg-3′ and Rv 5′-gTTCCATgATgAACTTTTT gAgg-3′; PCR conditions: 40″ at 95°C, 40″ at 61°C and 2 min at 68°C, for 29 cycles. The PCR product was digested by Acs I enzyme (*Roche Diagnostics*) for 5 h at 50°C and visualized after electrophoresis on 2% MS gel (*Roche Diagnostics*).

Genotype frequencies of Klotho variant were tested for Hardy–Weinberg equilibrium in all subjects using Arlequin (V 3.11) software in order to check whether our experimental sample was representative of the Caucasian population.

Analyses were performed using the SPSS statistical package (V 13.0). Linear regression analysis was then applied to genotype frequency data. Statistical significance was set at $P \leq 0.05$.

The survival function of the studied subjects was determined by applying a genetic-demographic approach that allows the estimation of the survival function for candidate alleles and genotypes in cross-sectional samples of unrelated individuals (Yashin et al. 1999). A detailed description of the analytical procedure is reported elsewhere (Dato et al. 2007). Briefly, in order to take into account the changes in mortality experienced by all the cohorts considered in the study, a synthetic survival function of the general population is constructed. Then, the marginal survival functions $S_a(x)$ and $S_b(x)$ of carriers and non carriers of a certain genotype is determined according to the relationship $S(x) = PS_a(x) + (1 - P)S_b(x)$ where $S(x)$ is the survival function of the whole population and P $(1 - P)$ is the initial frequency of carriers (non carriers) in the population, that is before the survival selection begins to operate consistently (Vaupel and Yashin 1985). In the present case we analyzed the survival function of people carrying the KL-VS/wt heterozygous or KL-VS homozygous genotype in comparison with that of people carrying the wt homozygous genotype. The Matlab 6.1 package was used for genetic-demographic analyses.

Results

To compare genotype distributions of KL-VS variant, we subdivide our sample into three gender-specific age classes as described in materials and methods (Table 1). These classes have been previously introduced on the basis of a synthetic survival curve constructed using historic mortality data taken from the Italian population from year 1890 onward (Passarino et al. 2006; Cardelli et al. 2008). In each age class and in each gender, the observed genotype frequencies were in agreement with those expected at Hardy–Weinberg equilibrium ($P > 0.05$).

Table 2 displays the distribution of genotype frequencies among the three above mentioned age classes. Univariate analysis indicates that in our sample the genotype frequencies are significantly different ($P = 0.03$). In particular the analysis shows a relevant increase of KL-VS heterozygous state in elderly subjects (age class 2) compared to young controls (age class 1). This result is in accordance with data previously published in Caucasian and Ashkenazi-Jewish populations (Arking et al. 2002, 2005). On the contrary, no difference is present between long-

Table 1 Distribution of the people who participated to the study, categorized according to gender and age

	Age class 1	Age class 2	Age class 3
Men (years)	<66	66–88	>88
Women (years)	<73	73–91	>91
Age (mean ± SD) (years)	53.0 ± 13.0	78.4 ± 6.1	99.6 ± 3.1
N	463	300	326
(M, W)	(191, 272)	(147, 153)	(82, 244)

Table 2 Genotype frequencies of KL-VS variant<colspec colnum="2" colname="c2" align="left/>

Whole subjects (1089)	Age class 1 ($n = 463$)	Age class 2 ($n = 300$)	Age class 3 ($n = 326$)
Genotypes			
wt/wt *n* (%)	348 (75.2%)	203 (67.7%)	236 (72.4%)
KL-VS/wt *n* (%)	103 (22.2%)	94 (31.3%)	80 (24.4%)
KL-VS/KL-VS *n* (%)	12 (2.6%)	3 (1.0%)	10 (3.1%)
P value		**0.008**	0.428
Odds ratio (CI)		**1.564 (1.126–2.174)**	1.145 (0.819–1.601)
Men (420)	Age class 1 ($n = 191$)	Age class 2 ($n = 147$)	Age class 3 ($n = 82$)
Genotypes			
wt/wt *n* (%)	142 (74.3%)	99 (67.3%)	59 (72.0%)
KL-VS/wt *n* (%)	46 (24.1%)	46 (31.3%)	20 (24.4%)
KL-VS/KL-VS *n* (%)	3 (1.6%)	2 (1.4%)	3 (3.7%)
P value		0.336	0.553
Odds ratio (CI)		1.434 (0.885–2.324)	1.046 (0.571–1.919)
Women (669)	Age class 1 ($n = 272$)	Age class 2 ($n = 153$)	Age class 3 ($n = 244$)
Genotypes			
wt/wt *n* (%)	206 (75.7%)	104 (68.0%)	177 (72.5%)
KL-VS/wt *n* (%)	57 (21.0%)	48 (31.4%)	60 (24.6%)
KL-VS/KL-VS *n* (%)	9 (3.3%)	1 (0.7%)	7 (2.9%)
P value		**0.018**	0.605
Odds ratio (CI)		**1.668 (1.063–2.617)**	1.225 (0.809–1.854)

$P = 0.008$, Odds ratio (CI) 1.564 (1.126–2.174, $P = 0.008$) and $P = 0.018$, Odds ratio (CI) 1.668 (1.063–2.617, $P = 0.026$)

lived individuals (age class 3) and young controls (age class 1). Moreover, we observed that, when ranking the subjects according to the gender, the association was particularly evident in women, where it reached statistical significance, but not in men (see Table 2).

The genetic-demographic analysis was carried out on the whole sample categorized according to the KL-VS polymorphism. Figure 1 shows the maximum likelihood estimates of the survival functions of heterozygous subjects (blue curve) and of wt homozygous subjects (red curve), compared to the general population (green curve). We noted that the survival function of heterozygous subjects crosses twice the survival function of the wt homozygous subgroup, roughly around the ages of 70 and 90 years. This trend suggests that the heterozygous genotype has a different effect on survival at different ages. In particular it indicates that heterozygous subjects have higher chance to survive beyond 70 years of age than the general population; on the other hand, among old subjects the chance of heterozygous subjects to survive beyond 90 years seems to be lower than the rest of the population. Incidentally, it may be worth mentioning that the algorithm for the construction of the curves leads to an initial slope of the curve of heterozygous subjects, which is not due to an excessive mortality at very young age, but to the great excess of wt homozygous subjects before 70 years of age. To verify the statistical significance of the pattern shown in Fig. 1 we carried out pairwise heterogeneity tests between age classes as defined on the basis of the crosses between survival functions. Only the cross at 70 years of age reflects a statistically significant change of the population survival in the heterozygous subgroup with respect to the wt homozygous subgroup ($P = 0.023$). When stratified by gender (Fig. 1, inset A and B), the same trend was noted, being more evident for women (inset B) than for men (inset A).

Discussion

The studies regarding the role of Klotho gene variants in human longevity performed by the groups of Dietz

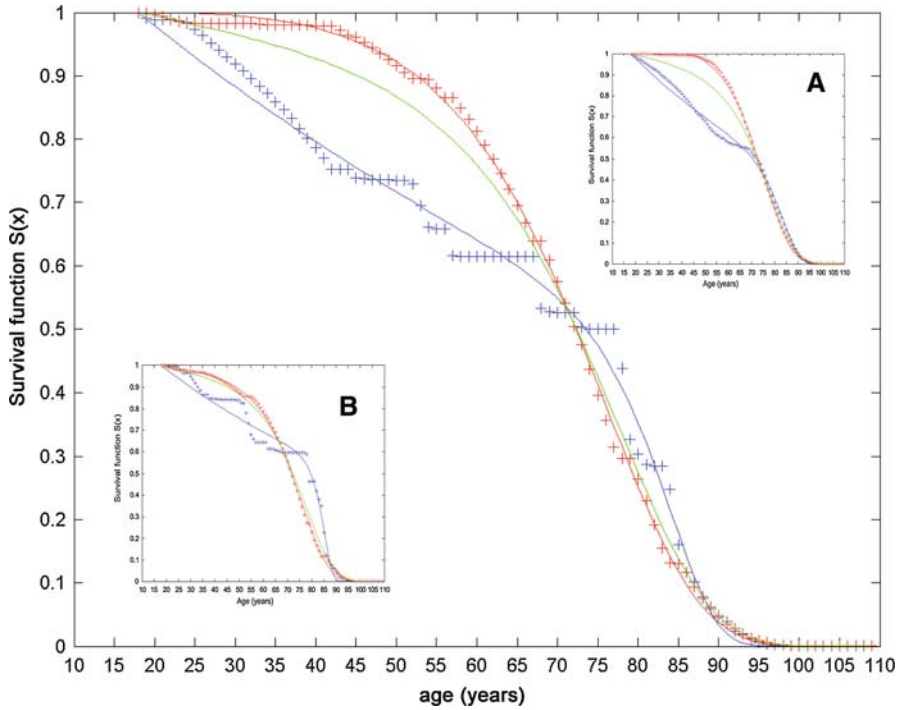

Fig. 1 Maximum likelihood estimates of the survival functions of KL-VS/wt heterozygous subjects ($S_a(x)$, *blue curve*) and wt homozygous subjects ($S_b(x)$, *red curve*). The synthetic survival function of the Italian population ($S(x)$, *green curve*) is also reported as a reference. *Inset A* the same for male subjects; *Inset B* the same for female subjects. (Color figure online)

and Puca arrived to contrasting results. The group of Dietz basically reported a survival advantage for the KL-VS/wt heterozygous genotype in elderly people, while the group of Puca reported no difference between young and long-living subjects. In this study we analysed the distribution of Klotho KL-VS genotypes in a large group of Italian people of different age, from young to centenarians. We predicted in a previous work that changes in cohort mortality may influence the results of a genetic analysis (Yashin et al. 1999), and consequently we subdivided the study group into three age classes on the basis of demographic data, as indicated by Passarino et al. (2006), that considered gender specific life expectancies. This approach allowed us to compare genotype frequency without introducing arbitrary age classes, as in previous studies on TNF-alpha polymorphisms and human longevity (Cardelli et al. 2008). From previous studies it appears that many genotypes are associated with human longevity in a gender-specific fashion, leading to the hypothesis that men and women follow different strategies to attain longevity (Bonafè et al. 2001; De Luca et al. 2001; Barbieri et al. 2004; Cederholm et al. 2007). In this case we did not observe any significant difference between the two genders, and the frequency distribution of the genotypes was remarkably similar in the two genders, suggesting that statistical significance is likely a matter of number of subjects in the two groups (women in population aging studies usually outnumber men) rather than a real biological difference. In any case we detected a significant increase of the heterozygous Klotho genotype in the class of elderly people, but not in the class of long-living people. On the whole these results show that the heterozygous genotype exerts a beneficial effect on survival when population mortality begins to accelerate (Ukraintseva and Yashin 2001), indeed it is found to be more frequent in the group of people aged from 66 to 91 years, while, on the contrary, in the group of long-living people its frequency is nearly identical to that of young people. Thus the KL-VS/wt heterozygous genotype modulates survival according to an age-specific complex pattern, probably shaped by physiological age-related variations (Passarino et al. 2006).

A possible explanation for these figures is that the heterozygous genotype is favorable at old age but less favorable, if not detrimental later on at very old age. Such a non-monotonic trend may be due to an age-specific effect of the genotypes, similar to that originally described for alleles in the "antagonistic pleiotropy" theory (Williams 1957), a hot topics that some of us recently critically revisited (Leroi et al.

2005). This theory proposes that individuals with alleles that are beneficial for reproductive fitness could be selected for by evolution even if the same alleles may have negative effects at later ages. We surmise that the same considerations may apply to genotypes and indeed it is not unusual to find non-monotonic age-related trajectories of genotype frequencies, in which centenarians display frequencies quite similar to those of young people (De Benedictis and Franceschi 2006). In conclusion, all the available data and those reported in the present study indicate that Klotho genetic variability has likely a role on human survival that is more complex than previously thought, since it appears that the same genotype has different effects (positive or negative for survival) according to the age of the subjects. On the basis of these data, we suggest that Klotho genetic variants may influence human longevity in a peculiar, unexpected way. The Klotho polymorphism here examined may be one example of a larger category of genes and gene variants exerting their effects at specific time windows.

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