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Polymorphic Variants of Insulin-Like Growth Factor I (IGF-I) Receptor and Phosphoinositide 3-Kinase Genes Affect IGF-I Plasma Levels and Human Longevity: Cues for an Evolutionarily Conserved Mechanism of Life Span Control

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Current literature indicates that abrogation of the IGF-I response pathway affects longevity in *Caenorhabditis elegans*, and that the down-regulation of IGF-I gene expression is associated with an extension of the life span in mice. In this paper we tested the hypothesis that polymorphic variants of IGF-I response pathway genes, namely *IGF-IR* (IGF-I receptor; G/A, codon 1013), *PI3KCB* (phosphoinositol 3-kinase; T/C, −359 bp; A/G, −303 bp), *IRS-1* (insulin receptor substrate-1; G/A, codon 972), and *FOXO1A* (T/C, +97347 bp), play a role in systemic IGF-I regulation and human longevity. The major finding of this investigation was that subjects carrying at least an A allele at *IGF-IR* have low levels of free plasma IGF-I and are more represented among long-lived people. Moreover, genotype combinations at *IGF-IR* and *PI3KCB* genes affect free IGF-I plasma levels and longevity. These findings represent the first indication that free IGF-I plasma levels and human longevity are coregulated by an overlapping set of genes, contributing to the hypothesis that the impact of the IGF-I/insulin pathway on longevity is a property that has been evolutionarily conserved throughout the animal kingdom. (*J Clin Endocrinol Metab* 88: 3299–3304, 2003)

A SUBSTANTIAL AMOUNT of data shows that mutations in genes that share similarities with the human genes involved in the insulin/IGF-I signal response pathway are responsible for an impairment of IGF-I/insulin signaling and for an extension of the *Caenorhabditis elegans* life span (1). In particular, the best characterized genes of this ancestral IGF-I/insulin response pathway are: *DAF-2*, ortholog to the human IGF-I/insulin receptor family; *AGE-1*, ortholog to human p110 catalytic subunit of phosphoinositide 3-kinase, which is involved in IGF-I/insulin signal transduction; and *DAF-16*, ortholog to the human *FOXO* family of Forkhead transcription factors, which regulates IGF-I/insulin-induced changes in gene transcription (2–5).

Further recent studies have shown that mutations in the *Drosophila melanogaster INR* gene (similar to the human insulin receptor) and in the *CHICO* gene, the ortholog to human insulin receptor substrate-1 (IRS-1), the protein associated with the IGF-I/insulin receptor, affect the life span, confirming the hypothesis that the insulin/IGF-I response pathway plays a role in the regulation of invertebrate longevity (6, 7).

Intriguingly, mutations in the *Saccaromyces cerevisiae SCH9* gene, which share similarities with insulin/IGF-I-regulated *AKT1/AKT2* kinases, affect the life span of the yeast (8). On the basis of these data it has been predicted that genes involved in the insulin/IGF-I response pathway play a role in longevity throughout evolution (9).

A major role of IGF-I in mammalian longevity has been suggested by studies on dwarf mice carrying mutations in transcription factors *PIT-1* and *PROP-1*, which are characterized by a long life span and reduced IGF-I plasma levels (10–12). Conversely, transgenic animals with elevated plasma IGF-I levels have a short life span (13). In humans there is evidence that long-lived subjects have decreased plasma IGF-I levels (14, 15) and preserved insulin action compared with aged subjects, thus indicating that an efficient insulin response has an impact on human longevity (16–18).

On the basis of the above-mentioned literature, we investigated the role of genetic variability at human loci that share similarities with the genes that regulate the insulin/IGF-I response in *C. elegans* (1–4) and *D. melanogaster*. In particular, polymorphisms at *IGF-IR* (IGF-I receptor; 34% protein sequence identity with the *C. elegans DAF-2* gene), *PIK3CB* (phosphoinositol 3-kinase; 27% protein sequence identity with the *C. elegans AGE-1* gene), *IRS-1* (30% protein sequence identity with the *D. melanogaster CHICO* gene), *FOXO1A* (49% protein sequence identity with the *C. elegans DAF-16* gene) were investigated. Their effects on IGF-I plasma levels were assessed. Moreover, the genotype distributions found in long-lived people over 85 yr of age and those found in

Abbreviations: BMI, Body mass index; HOMA, homeostatic model assessment; IGF-IR, IGF-I receptor; IR, insulin resistance; IRS-1, insulin receptor substrate-1; PI3KCB, phosphoinositol 3-kinase.

people less than 85 yr old were compared. This strategy to select long-lived people from the population was based on three findings: 1) demographic data suggesting that the selection for longevity starts at about 85 yr (19); 2) demographic models predicting that changes in the frequency of polymorphisms associated with different survival capabilities during aging are expected to occur beyond the age of 85 yr (20); and 3) previous data showing that people beyond the age of 85 yr have peculiar metabolic/anthropometric parameters [such as high insulin sensitivity, low body mass index (BMI), and low glycemia] in comparison with the rest of the aging population (18). All of these data suggest that healthy people over 85 yr of age have been highly selected by mortality forces (18, 20).

Subjects and Methods

Subjects

Four hundred ninety-six Caucasian subjects (132 men and 364 women), from 18–109 yr of age, equally distributed in northern, central, and southern Italy, volunteered for this study. On the basis of literature data (18–20), to assess the impact of genes on longevity, subjects were then subcategorized into two groups by splitting the sample at the age of 85 yr: healthy people 17–85 yr of age (n = 278; mean age, 54.8 ± 21.5; male/female ratio, 76/202) were grouped as "young people," and healthy people from 86–109 yr of age (n = 218; mean age, 98.0 ± 4.31 male/female ratio, 56/162) were grouped as "long-lived people." All subjects were contacted at home or their institutions and examined by physicians previously trained to administer a questionnaire that included cognitive and depression tests. All subjects had liver, kidney, and thyroid function tests within the normal range. According to American Diabetes Association criteria (21), no subject was diabetic or affected by impaired fasting glucose. No subject used drugs affecting insulin secretion and/or action or plasma lipid levels. In particular, long-lived subjects represented a group of carefully selected individuals free of major age-related diseases. After a clear explanation of the potential risk of the study, all subjects (as well as the relatives of the long-lived people) gave informed consent to participate in the study, which was approved by the ethical committee of our institutions.

Analytical methods

Anthropometric determinations (weight, height, and BMI) were measured as previously reported (22). Plasma glucose was determined by the glucose oxidase method (Glucose Autoanalyzer, Beckman, Fullerton, CA). Subsequently centrifugation plasma insulin (Sorin Biomedica, Milan Italy; intraassay coefficient of variation, 3.1 ± 0.3%) and free plasma IGF-I levels (Diagnostic Systems Laboratories, Webster, TX; intraassay coefficients of variation, 3.8 ± 0.4%) were determined by RIA.

Genotype typing

DNA was extracted from white blood cells by phenol/chloroform extraction, according to standard procedures (23).

The 255-bp fragment of the *IGF-IR* (chromosome 15q25-q26, GenBank GI:11068002) containing the G to A transition at nucleotide 3174 (codon 1013) in exon 16 was amplified by primers 5′-TCTTCTCCAGTGTACGTTCC-3′ (upstream) and 5′-GGAACTTTCTCTTACCAC ATG-3 (downstream) and was digested with 10 U *Mnl*I (New England Biolabs, Inc.). Allele A yielded to three restriction products of 132, 100, and 23 bp; allele G yielded to four restriction products of 132, 80, 23, and 20 bp, which were separated on 4.5% agarose gels and visualized with ethidium bromide.

The gene polymorphism due to a T to C transition located 359 bp upstream from the starting codon of *PIK3CB* (chromosome 3q22.3, GI: 5453894) was analyzed by the allele refractory method. In each of two separate PCR mixes, an allele-specific primer and an allele-nonspecific primer were used as follows: T allele, 5′-CATTGATTCAAATTCAACATAA-3′ and 5′-GAAAAACTCAGC CTAAGAAGC-3′; C allele, 5′-GTTTATTCAGATGTCAAATATC-3′ and 5′-CAAGAGTAA GTCAGCAGAAAT-3′. The PCR products were separated by agarose gel (2%) electrophoresis, followed by ethidium bromide staining.

In a subset of 211 individuals, direct sequencing of a 203-bp region (position −470→−267) of the *PIK3CB* promoter was performed (CEQ2000, Beckman) using primers 5′-GAA AAACTCAGCCTAAGAAGC-3′ (upstream) and 5′-AATCCATACCACCAACTAAAG-3′ (downstream) to detect a polymorphism A→G at −303 bp upstream as described by Kossila *et al.* (24). This procedure allowed confirmation of the data on the site T→C located at −359 bp and proved that no other polymorphic loci were present in that DNA region.

The region of exon I surrounding the G to A transition at codon 972 of *IRS-1* (chromosome 2q35-q36.1, GI:5031804) was amplified by primers 5′-GCAGCCTGGCAGGAGAG-3′ (upstream) and 5′-CTCACCTCCTCTGCAGC-3′ (downstream). The 220-bp PCR product was digested with *Bst*NI (New England Biolabs, Inc., Beverly, MA). The presence of the restriction site (allele A) generated two fragments of 164 and 56 bp, which were run on a 4.0% agarose gel stained with ethidium bromide.

The fragment of 296 bp spanning the T to C transition at position +97347 in intron I of the *FOXO1A* gene (GenBank GI:17476061, chromosome 13q14.1) was amplified by primers 5′-CCTGGTAAAACCGGAAGGT-3′ (upstream) and 5′-CCCTTCCTTTGGAATGACTG-3′ (downstream) and digested with *SspI* (New England Biolabs, Inc.). The presence of the T allele allowed cutting of the amplified region in two fragments of 186 and 110 bp.

Calculations and statistical analyses

The degree of insulin resistance (IR) was estimated by the homeostatic model assessment (HOMA) method (25, 26). The HOMA method has been recently validated to be a good index of insulin resistance (IR) in subjects with a broad range of insulin sensitivity (25) and has a good correlation with the insulin-mediated glucose uptake calculated by the euglycemic hyperinsulinemic glucose clamp (26). To approximate normal distributions, plasma insulin and IR (HOMA) were logarithmically transformed and used in all calculations and were back-transformed for result presentations. The difference in genotype frequency was analyzed by χ^2 test. For investigating the difference in allele frequency, Fisher's test was used. ANOVA with Scheffé's test was used for comparing IGF-I plasma levels among the genotype groups. A linear general model analysis was used to test the independent contributions of the interactions between the different genotypes as well as the effect of individual genotype on plasma IGF-I levels. A *t* test with a Bonferroni-adjusted (p_{adj}) α value was used when indicated. Statistical analyses were performed using the SPSS software package (SPSS, Inc., Chicago, IL). All metabolic parameters are presented as the mean ± SD.

Results

In the entire population, free IGF-I plasma levels displayed an age-related decrease (Fig. 1) through all ages and were negatively correlated with the degree of IR (HOMA; ρ, 0.132; $P < 0.02$) and BMI (ρ: 0.130; $P < 0.05$).

In all study groups, the genotype distributions were in the Hardy-Weinberg equilibrium ($P > 0.05$).

The impact of genetic variability at *IGF-IR*, *PIK3CB*, *IRS-1*, and *FOXO1A* loci on plasma IGF-I levels was tested by univariate analysis (Table 1). *IGF-IR* and *PIK3CB* genotypes affected IGF-I plasma levels, whereas no change in IGF-I plasma levels was observed according to genotypes of *IRS-1* and *FOXO1A* loci.

Accordingly, the effects of *IGF-IR* and *PIK3CB* polymorphisms on IGF-I plasma levels were assessed by means of a general linear model ANOVA, including age, sex, IR (HOMA), and BMI as covariates (Table 2). The analysis revealed lower free IGF-I plasma levels in *IGF1R* A^+ subjects (AG and AA genotypes) than in A^- (GG genotype) subjects (2.95 ± 2.62 *vs.* 2.21 ± 2.34 ng/ml; F = 6.56; $P = 0.011$) as well as in *PIK3CB* T^+ individuals (TT and TC genotypes) com-

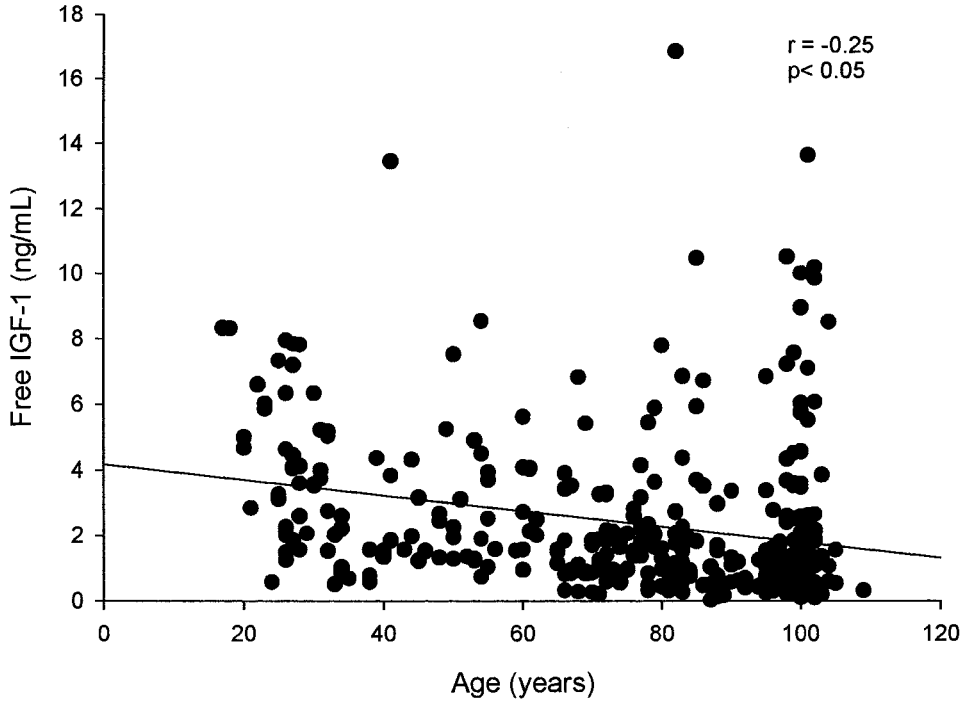

FIG. 1. Simple correlation analysis between age and IGF-I plasma levels in the entire study population (n = 496).

TABLE 1. Plasma IGF-I levels in the study population (n = 496)

	IGF-I plasma levels
IGF1R (G/A) polymorphism	
GG	2.95 ± 2.61
AG	2.34 ± 2.41
AA	1.94 ± 2.17
IRS-1 (G/A) polymorphism	
GG	2.56 ± 2.46
GA	1.95 ± 2.92
AA	0.50 ± [a]
PI3KCB (T/C) polymorphism	
TT	2.19 ± 2.05
TC	2.24 ± 2.37
CC	3.02 ± 3.10
FOXO1A (T/C) polymorphism	
TT	2.49 ± 2.56
TC	2.37 ± 2.27
CC	7.81 ± [a]

IGF1R (G/A) polymorphism: GG *vs.* AG *vs.* AA; F = 3.83, $P = 0.02$; IRS-1 (G/A) polymorphism: P = NS; PI3KCB (T/C) polymorphism: TT *vs.* TC *vs.* CC; F = 3.01, $P < 0.05$; FOXO1A (T/C) polymorphism: P = NS. NS, Not significant.

[a] Only one subject in this group.

TABLE 2. General linear model analysis with plasma IGF-I levels as dependent variable (n = 496)

	F	*P*
Age	5.52	<0.05
BMI	1.54	NS
Sex	0.08	NS
IR (HOMA)[a]	9.25	<0.05
IGF1R genotype[b]	9.92	<0.01
PIK3CB genotype[c]	7.59	<0.01
IGF1R[a] PI3KCB	3.87	<0.05

NS, Not significant. $R^2 = 0.133$.

[a] Back log transformed.

[b] Calculated as A+ *vs.* A−.

[c] Calculated as T+ *vs.* T−.

pared with T^- (CC genotypes) individuals (2.22 ± 2.22 *vs.* 3.02 ± 3.11; F = 6.017; $P = 0.015$). Inasmuch as the interaction (IGF-IR × PIK3CB) was significant, a univariate two-factor general linear model ANOVA was performed, which revealed that *IGF-IR*/*PIK3CB* A^-/T^- individuals had the highest IGF-I levels in the population; this was independent of age and gender (A^-/T^- *vs.* all other groups, 4.25 ± 4.03 *vs.* 2.17 ± 2.31; $t = 3.63$; $p_{adj} = 0.005$). All other comparisons among *IGF-IR*/*PIK3CB* genotype combinations were not significant (Fig. 2).

The entire sample was then divided into two subgroups characterized by the same gender proportion, designated young people (median age, 60 yr) and long-lived people (median age, 99 yr; 74.3% *vs.* 72.7% for females; 25.7% *vs.* 27.3% for males; $P > 0.05$ for both sexes).

As expected (17, 18), long-lived people had lower BMI (23.1 ± 3.7 *vs.* 25.2 ± 2.8 kg/m^2; $P < 0.001$), lower fasting plasma glucose (5.02 ± 1.48 *vs.* 5.63 ± 1.37 mmol/ml; $P < 0.001$), lower plasma insulin (7.36 ± 4.36 *vs.* 8.99 ± 3.56 mU/liter; $P < 0.001$), lower IR (HOMA) degree (1.70 ± 1.08 *vs.* 2.33 ± 1.38; $P < 0.001$), and lower plasma free IGF-I plasma levels (1.89 ± 2.42 *vs.* 2.75 ± 2.38; $P < 0.005$) than young people. These data confirm that subjects more than 85 yr of age have peculiar metabolic and anthropometric characteristics

The genotype frequency distributions at *IGF-IR*, *PIK3CB*, *IRS-1*, and *FOXO1A* loci in young people and long-lived people were studied in the two groups. Carriers of the A allele (A^+ subjects) at the *IGF-IR* locus were more represented among long-lived people than in young people (Table 3). No significant differences were found when *PIK3CB*, *IRS-1*, and *FOXO1A* gene polymorphisms were examined. Differences in genotype combinations were also tested by combining *IGF-IR* A^+ and A^- carrier status with R^+/R^-

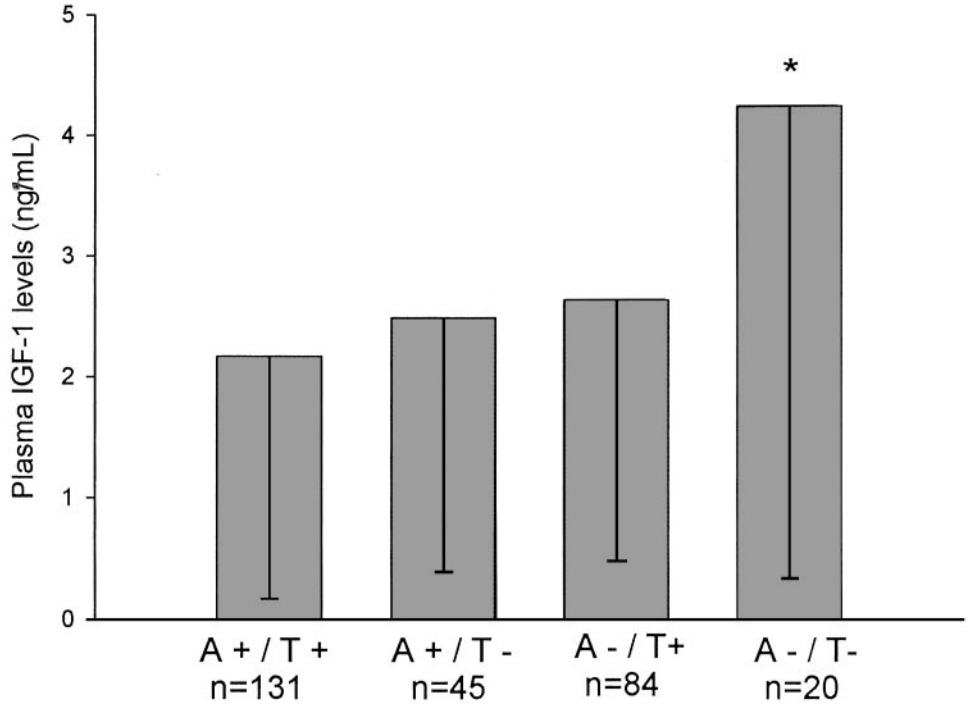

FIG. 2. Free IGF-I plasma levels among different genotypes independently of age and gender. *, $P < 0.05$ (variation in group means).

carrier status at the *IRS-1* locus, C^+/C^- status at the *PI3KCB* locus, and C^+/C^- carrier status at the *FOXO1A* locus. *IGF-IR/PI3KCB*-A^+/T^+ carriers increased from 43.5% to 54.5%, in long-lived people ($\chi^2 = 4.382$; df = 1; $P = 0.036$), and A^-/T^- individuals dropped from 9.3% to 5.5%; this latter change was not significant ($\chi^2 = 1.763$; df = 1; $P = 0.184$). No significant changes were found as far as the remaining genotype combinations were concerned (data not shown).

Discussion

The major findings of this investigation are the following: 1) IGF-I plasma levels, which decrease significantly with age, are affected by the polymorphisms at *IGF-IR* and *PI3CKB* genes, both alone and in combination. In particular, individuals bearing at least one allele A at the *IGF-IR* locus (*IGF-IR* A^+) have lower plasma IGF-I levels than the rest of the population. 2) *IGF-IR* A^+ subjects are found in increased proportion in long-lived individuals. 3) Genotype combinations of an A allele at the *IGF-IR* locus and a T allele at the *PI3CKB* locus (A^+/T^+ subjects) affect IGF-I plasma levels (having A^-/T^- individuals the highest free IGF-I plasma levels), as well as longevity, and the proportion of A^+/T^+ subjects significantly increased among long-lived individuals.

Collectively, these data suggest that genetic variants of genes involved in the intracellular response to IGF-I (*IGF-IR* and *PI3CKB*) jointly affect two phenotypes: free IGF-I plasma levels and longevity. The fact that the two traits are coregulated by overlapping sets of genes is in line with previous findings on the genetics of longevity in animal models. Indeed, in both invertebrates (*C. elegans*) and mammals (*Mus musculus*), the down-regulation of the IGF-I pathway or IGF-I plasma levels is associated with an extension of the life span (9–12, 27), whereas animals with elevated plasma IGF-I levels display a shortened life span (13). Indeed, in humans there is evidence that centenarians, the best example of successful aging, have lower plasma IGF-I levels (14, 15). Interestingly, caloric restriction, the most reproducible intervention to extend animal life span (28), substantially reduces plasma free IGF-I levels (29, 30).

In accordance with previous studies (16–18, 22), remarkably low IR as well as BMI were found in our sample of long-lived people, confirming that these individuals show metabolic characteristics different from those in the rest of aging population. To date no genetics have been found that can be associated with these parameters (18); it cannot be excluded that BMI and IR (HOMA) as well as plasma IGF-I (whose variance was explained by only 13% by the variables used in this report) could be influenced by the peculiar nutritional regimen of these long-lived people, which could, in fact, share some similarities with moderate caloric restriction. In this regard, the preserved insulin pathway efficiency observed in long-lived subjects might be indirectly due to an enhanced tissue response to IGF-I secondary to an increase in IGF-I receptor number/activity (30). Our previous study (14) demonstrated that centenarians had a plasma IGF-I/IGFBP-3 molar ratio greater than that of aged subjects. This finding appears to contradict the present study, because the greater molar ratio was due to the lower IGFBP-3 present in long-lived subjects rather than to higher IGF-I plasma levels (no significant difference in total plasma IGF-I levels was found among long-lived and aged subjects), thus suggesting that in centenarians the bioavailability of IGF-I is enhanced despite its lower levels. The significance of plasma IGF-I remains to be established. It could be hypothesized that the decrease in plasma IGF-I observed in long-lived subjects might minimize the generalized mitogenic stimulus to tissues and contribute to the reduction in age-related pathologies due to the effect of IGF-I on cellular replication. Such

TABLE 3. Allele and genotype distribution of different gene polymorphisms involved in insulin signaling pathway

	Young	Long lived	
IGF1R A/G polymorphism	(n = 248)	(n = 162)	
G/G	0.399 (n = 99)	0.296 (n = 48)	$\chi^2 = 6.04$; $P < 0.04$
A/G	0.407 (n = 101)	0.426 (n = 69)	
A/A	0.194 (n = 148)	0.278 (n = 45)	
Allele frequency			
g	0.603	0.509	
a	0.397	0.491	
A+ subjects	0.601 (n = 149)	0.704 (n = 114)	Fisher's test: $P = 0.03$
IRS-1 G/A polymorphism	(n = 197)	(n = 153)	
G/G	0.858 (n = 169)	0.862 (n = 132)	
G/A	0.137 (n = 27)	0.131 (n = 20)	
A/A	0.005 (n = 1)	0.007 (n = 1)	
Allele frequency			
g	0.926	0.928	
a	0.074	0.072	
A+ subjects	0.142 (n = 28)	0.137 (n = 21)	
PI3KCB T/C polymorphism	(n = 245)	(n = 179)	
T/T	0.351 (n = 86)	0.335 (n = 60)	
T/C	0.384 (n = 94)	0.447 (n = 80)	
C/C	0.265 (n = 65)	0.218 (n = 39)	
Allele frequency			
t	0.543	0.559	
c	0.457	0.441	
C+ subjects	0.649 (n = 159)	0.665 (n = 119)	
FOXO1A T/C polymorphism	(n = 198)	(n = 153)	
T/T	0.934 (n = 185)	0.948 (n = 145)	
T/C	0.061 (n = 12)	0.052 (n = 8)	
CC	0.005 (n = 1)	–	
Allele frequency			
t	0.965	0.974	
c	0.035	0.026	
C+ subjects	0.066 (n = 13)	0.052 (n = 8)	

When not reported, χ^2 and Fisher's tests were not significant ($P > 0.05$).

a hormone is also linked to the development of several diseases, such as cancer (31, 32), but the local expression (bioavailability) of IGF-I may be an important factor contributing to the maintenance of normal tissue function.

To our knowledge the results reported here are the first findings supporting the hypothesis that genetic variability in the genes responsible for IGF-I regulation plays a role in human longevity. The ortholog of human *IGF-IR*, *DAF-2*, is one of the best characterized longevity genes in *C. elegans* (1–3). It has been proposed that *DAF-2* mutations lead to the up-regulation of genes involved in heat stress, UV, and antioxidant responses, *i.e.* pathways that are expected to affect the life span in all species (33). Furthermore, it has been recently shown that *IGF-IR* inactivation prologs the life span in mice and increases their resistance to oxidative stress (34). However, the functional effects of the structural variation in *IGF-IR* due to the polymorphism studied here are not known.

The association design is a potential limitation of our study, and a longitudinal study might provide more robust data. Unfortunately, no longitudinal studies in humans have been performed to obtain more robust conclusions, especially in long-lived subjects (>85 yr of age), in which a long follow-up period would be extremely difficult to achieve. Furthermore, it should be pointed out that the rationale for assessing the potential role of the IGF-I signaling pathway on longevity derives from a large number of studies performed in laboratory models of longevity and in which a cause-effect mechanism has already been proven.

In conclusion, our study shows that genetic variability at the insulin/IGF-I signaling response pathway plays a role in human longevity, indicating that the impact of these genes on species longevity is an evolutionarily conserved property. We hope that our data will be the starting point for future longitudinal studies, including the insulin response pathway genes as a possible trigger for longevity.

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