Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity

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Summary

The insulin/IGF1 signaling pathways affect lifespan in several model organisms, including worms, flies and mice. To investigate whether common genetic variation in this pathway influences lifespan in humans, we genotyped 291 common variants in 30 genes encoding proteins in the insulin/IGF1 signaling pathway in a cohort of elderly Caucasian women selected from the Study of Osteoporotic Fractures (SOF), including 293 long-lived cases (lifespan ≥92 years (y), mean ± standard deviation (SD) = 95.3 ± 2.2y) and 603 average-lifespan controls (lifespan ≤79y, mean=75.7 ± 2.6y). Variants were selected for genotyping using a haplotype tagging approach. We found a modest excess of variants nominally associated with longevity. We then replicated nominally significant variants in two additional Caucasian cohorts containing both males and females: the Cardiovascular Health Study (CHS) and Ashkenazi Jewish Centenarians (AJC). An intronic single nucleotide polymorphism (SNP) in *AKT1*, rs3803304, was significantly associated with lifespan in a meta-analysis across the three cohorts (odds ratio (OR)=0.78 (95% confidence interval (CI)=0.68-0.89), adjusted p=0.043); two intronic SNPs in *FOXO3A* demonstrated a significant lifespan association among women only (rs1935949, OR=1.35, 95% CI=1.15-1.57, adjusted p=0.0093). Conclusion: common variants in several insulin/IGF1 pathway genes are associated with human lifespan.

Keywords

IGF1; longevity; gene; SNP; AKT1; FOXO3A

Introduction

Genes in the insulin/IGF1 signaling pathway affect lifespan in yeast, nematodes, fruit flies and mice (Kuningas *et al.*, 2008). Mutation of genes in this signaling pathway confers greater resistance to oxidative stress and phenotypic characteristics consistent with delayed or slowed aging (Tatar *et al.*, 2003).

The insulin/IGF1 pathway was first implicated in aging in *C.elegans* (Kenyon *et al.*, 1993; Larsen *et al.*, 1995; Morris *et al.*, 1996). Mutation of *daf-2*, the nematode ortholog of the IGF1 and insulin receptors, results in extended lifespan and increased stress resistance (Kenyon *et al.*, 1993; Kimura *et al.*, 1997); these effects are mediated by the nuclear transcription factor, DAF-16, which is a homolog of the mammalian forkhead (FKHRL or FOXO) transcription factors (Ogg *et al.*, 1997). Mutations in *age-1*, the homologue of mammalian PI3Kinase, which is involved in signal transduction from the insulin and IGF1 receptors (Dorman *et al.*, 1995), also increase lifespan in *C. elegans*. Similarly, in Drosophila, mutations of the insulin receptor homolog, its substrate, *chico* (Clancy *et al.*, 2001), and of the *daf-16/foxo* homolog (Hwangbo *et al.*, 2004) reduce insulin signaling and extend lifespan.

In vertebrates, growth and glucose metabolism are regulated by two parallel pathways, with separate receptors for insulin and IGF1. In mice, mutations that result in growth hormone deficiency or mutations in growth hormone receptors cause reduced size, lower insulin levels, increased stress resistance and longer lifespan (Bartke, 2005). Heterozygous deletion of the *IGF1R* gene in mice causes a modest reduction in size, and improves stress resistance and extends lifespan in females. Disruption of the *INSR* gene globally or in most tissues leads to insulin resistance and shortened lifespan (Joshi *et al.*, 1996; Okamoto & Accili, 2003); however, mice homozygous for *INSR* deletion specific to fat cells have extended lifespan in both sexes (Bluher *et al.*, 2002), indicating the presence of tissue-specific effects.

Results from model organisms suggest the hypothesis that genes in the insulin/IGF1 pathway may be involved in the control of human lifespan and variants that affect their activity may be associated with human longevity. Although a few associations of insulin/IGF1 pathway genes with longevity have been reported (Bonafe *et al.*, 2003; van Heemst *et al.*, 2005; Kuningas *et al.*, 2007; Suh *et al.*, 2008; Willcox *et al.*, 2008), most of these findings have not been replicated and the studies did not analyze genetic variation in the insulin/IGF1 pathway systematically.

We have comprehensively evaluated common variation in genes in the insulin/IGF1 signaling pathway for association with human lifespan. We performed a nested case-control association study of longevity in a cohort of elderly Caucasian women from the Study of Osteoporotic Fractures (SOF). We then replicated significant associations in two independent Caucasian cohorts of men and women.

Results

All subjects were Caucasian. The SOF cohort was all women, the CHS cohort was 47% female, and the AJC cohort was 66% female (Table 1). More than half (55.6%) of the longevity cases were still alive in SOF, about a third (33.7%) were still alive in CHS and 24% of cases were still alive in AJC.

Association of genotype with longevity in SOF

We evaluated 291 SNPs in 30 genes in the insulin/IGF1 signaling pathway (Supplemental Table A). Twenty one (7%) SNPs in 9 (30%) genes (*AKT1, FOXO1A, FOXO3A, GHR, GHRHR, IGF1R, IGFBP3, IGFBP4, PTEN*) were associated with longevity at a nominal $p<0.05$, a modest excess over the expected ~15 (Table 2). Eight SNPs had a p value < 0.01 (an excess of ~5 over the expected number) and 3 SNPs had $p<0.005$ (~1.5 more than expected). *FOXO3A* (7/16) and *GHR* (7/26) had the most SNPs associated with longevity.

For all genes except *GHR*, haplotypes containing longevity-associated SNPs exhibited a similar effect size and significance level for association with longevity, suggesting that in each case, the putative causative variant was in linkage disequilibrium (LD) with a single haplotype-tagging SNP (data not shown). For *GHR*, we observed the most significant association for a three-SNP haplotype comprising rs9292854, rs13182117 and rs4866941 ($p=0.0005$, compared to the best single-SNP association of $p=0.007$ for rs4866941; analysis not corrected for race/ethnicity), suggesting that the putative causative allele resides on this haplotype.

Replication of association of AKT1 and FOXO3A SNPs with longevity

Since the case-control associations we observed in SOF were not significant after correction for multiple hypothesis testing, we replicated the nominally associated SNPs in two independent Caucasian cohorts, the Cardiovascular Health Study (CHS) and Ashkenazi Jewish Centenarians (AJC). We selected all 21 SNPs individually associated with longevity in SOF ($p<0.05$) as well as 4 SNPs significantly associated with longevity ($p<0.001$) in 2-SNP interaction analyses for replication genotyping (Table 3). We observed the most consistent results across all 3 cohorts for 1 SNP in *AKT1* (rs3803304), which was nominally associated with longevity in each of the 3 cohorts, with a consistent effect size (OR) of 0.77-0.79. In a meta-analysis of results from all three cohorts, this association was statistically significant ($p=0.0004$; permutation-based correction for multiple testing $p = 0.043$).

Two SNPs in *FOXO3A* were nominally associated with longevity in SOF and in AJC, but not in CHS, although the magnitude and direction of the effect were consistent in CHS. The

p values in the meta-analysis were 0.0014 for rs1935949 and 0.0009 for rs4946935; these approached statistical significance in the meta-analysis after correction for multiple testing.

We observed a borderline effect of sex on the *FOXO3A* rs1935949 SNP association with longevity, suggesting a stronger effect of female sex (OR=0.72, 95% CI=0.52-0.99, p=0.049), as also seen when comparing the odds ratio for association with longevity between the mixed-sex (OR=1.24, 95%CI=1.09-1.41) and female-only subsets (OR=1.35, 95% CI=1.15-1.57). When restricted to female subjects, the association of *FOXO3A* rs1935949 and rs4946935 with longevity in the meta-analysis achieved statistical significance (adjusted P values were 0.0093 and 0.019 respectively).

Next, we evaluated the association with longevity for the replicated SNPs in a genotypic model (Table 4). For *AKT1* rs3803304, the association with longevity appeared driven by the minor allele homozygous genotype (CC). Subjects homozygous for the minor allele were underrepresented among long-lived cases (OR=0.41-0.5, p=0.00016 in the meta-analysis). A single allele did not confer a significant effect (p=0.12), suggesting a recessive effect, with homozygosity for the minor allele decreasing the probability of longevity. For the two *FOXO3A* SNPs, the effect appeared to be consistent with a multiplicative model; the probability of surviving to exceptional longevity increased with each additional copy of the minor allele. Results were similar when evaluated in women only (rs1935949: CT, OR=1.33 (1.08-1.63), p=0.067; TT, OR=1.82 (1.25-2.65), p=0.0017).

Follow-up of significant findings in *AKT1* and *FOXO3A*

Complete coverage (at $r^2 > 0.8$) of common variation in both *AKT1* and *FOXO3A* based on HapMap Caucasians (CEU) has been achieved.

The longevity-associated *AKT1* rs3803304 SNP is not in LD with any other HapMap SNPs. Therefore to follow-up we selected other variants in the surrounding area from dbSNP. We genotyped 8 additional *AKT1* SNPs in SOF, including 5 coding SNPs, (1 rare, 4 unvalidated), 2 synonymous SNPs, and 1 intronic SNP located 22bp from an intron-exon junction. Three of these 8 SNPs were polymorphic in SOF and none were associated with longevity (Supplementary Table A). Since no coding SNPs in *AKT1* were associated with longevity, we searched for potential regulatory sites in the region. *AKT1* rs3803304 lies in an intron, 70bp away from a conserved exon-intron boundary and in an area of high predicted regulatory potential (0.3 score on the evolutionary and sequence pattern extraction through reduced representations (ESPERR) prediction metric (King *et al.*, 2005). Data from the Affymetrix Transcriptome Phase 3 gene tiling arrays (Kapranov *et al.*, 2007) show a long (>200bp) RNA signal at this intron/exon border encompassing rs3803304 (Figure 2A); a CpG island is also found here. It is possible that rs3803304 disrupts an RNA regulatory mechanism.

Initial SNP discovery in *FOXO3A* was by sequencing – no other common exonic SNPs were found. The two SNPs most strongly associated with longevity, *FOXO3A* rs1935949 and rs4946935, lie in intron 3, 1.7 kilo-base-pairs (kb) from the intron-exon boundary of the 3'UTR, but not in an area of evolutionary conservation (Figure 2B and C).

Interaction analyses

We included 4 SNPs in the replication genotyping that were not individually associated with longevity in SOF, but had a significant association with longevity in interaction analyses with another SNP. Two *IGFBP5* SNPs (rs3770472 and rs11575134) had significant interactions with *GHR* SNP rs2940935 in SOF (both P < 0.00004); these interactions appeared to replicate in the AJC cohort (p=0.012 for rs3770472 and 0.026 for rs11575134), but not in the CHS cohort. SNP rs1009728 was included in the replication genotyping

because of a significant interaction with a SNP in *PIK3R1*, but this latter SNP could not be genotyped in the replication cohorts.

Association of lifespan-associated SNPs with cause-specific mortality

We evaluated associations of the longevity-associated SNPs with reported cause of death in SOF and CHS. *AKT1* rs3803304 was associated with deaths due to cardiovascular disease and deaths from non-cardiovascular, non-cancer causes. The *FOXO3A* SNPs rs4946935 was associated with cardiovascular causes of death in SOF and with non-cardiovascular, non-cancer other causes of death in CHS. The *FOXO3A* SNP rs1935949 was associated with cardiovascular and cancer causes of death in SOF, and with other causes of death in CHS.

Discussion

We have comprehensively evaluated common variants in 30 genes in the insulin/IGF1 signaling pathway for their associations with human lifespan in a cohort of elderly Caucasian women. Replication of nominally significant findings in two independent Caucasian cohorts revealed a SNP in *AKT1* which was significantly associated with lifespan, and 2 SNPs in *FOXO3A* which were significantly associated with lifespan in women. Overall, the longevity-associated SNPs were associated with the three major categories (cardiovascular, cancer, other) of cause of death, suggesting that their association with lifespan is due to a general effect rather than to a specific disease process.

Several previous studies have demonstrated associations between variants in other genes in the insulin/IGF1 signaling pathway and human aging phenotypes. Most of these studies investigated only a small number of SNPs or genes, and their results have not been replicated. Our current study analyzed 291 SNPs in 30 genes providing the first comprehensive screen of the insulin/IGF1 signaling pathway in humans.

Association of genetic variation in *AKT1* with human lifespan has not been previously reported; however several studies have reported associations with *FOXO3A* SNPs or haplotypes. A recent case-control association study in elderly Japanese men found an association with 3 SNPs in *FOXO3A* and longevity (Willcox *et al.*, 2008); this study did not report replication of findings in independent cohorts. While the tag SNPs genotyped were different in our study, 2 of the 3 associated SNPs share strong LD (r2>0.8 in HapMap Caucasians) with the *FOXO3A* SNPs associated with longevity in our study. The effect size is larger in the Japanese cohort, but, as in our study, homozygosity for the minor allele is associated with longevity. The most strongly associated SNP from the Japanese cohort lies in the first intron, and has lower LD in Caucasians (r2=0.48) than in Japanese (r2=0.71) with the best SNPs in our study, which are located in the last intron; this difference in LD patterns may account for the different localization of the top signal in our two studies.

The population-based Leiden 85-plus study (van Heemst *et al.*, 2005) also found a *FOXO3A* haplotype, but no single SNPs, associated with an increase in mortality (Kuningas *et al.*, 2007) in Caucasians. The *FOXO3A* haplotype identified contains some of the SNPs we found nominally associated with longevity in SOF, but resides in a different part of the gene from the two *FOXO3A* SNPs we found associated with longevity in our 3-cohort meta-analysis. Taken together, these results suggest that 5 independent cohorts may be detecting an association between *FOXO3A* variants and longevity. A combined analysis in which all cohorts genotype the same SNPs will be useful to confirm whether these findings are true replications of the same effect, and to identify the region of the gene with the strongest signal in order to prioritize fine mapping efforts.

Other insulin/IGF1-pathway genes implicated in human longevity include *FOXO1A* and *IGF1R*. A genome-wide association scan in the Framingham Study showed association between age at death with two *FOXO1A* SNPs (Lunetta *et al.*, 2007), which are in complete LD with *FOXO1A* rs1334241, which we found to be nominally associated with longevity in SOF. However, this finding did not replicate in CHS or AJC. In the AJC cohort, rare nonsynonymous mutations of *IGF1R* were significantly more common in female centenarians (Suh *et al.*, 2008). Female centenarians carrying those mutations had lower IGF1R levels and decreased IGF signaling compared with other female centenarians (Suh *et al.*, 2008). In a study of long lived Italians, those carrying a common variant allele at the *IGF1R* locus had lower plasma IGF1 levels than those without the allele; this allele was over-represented in long-lived Caucasians (Bonafe *et al.*, 2003). We genotyped this variant but did not replicate the association with lifespan in the SOF cohort.

The association of lifespan with *FOXO3A* variants in several studies and the novel association we identified with a SNP in *AKT1* strongly suggest that the same components of the insulin/IGF1 signaling pathway that affect lifespan in *C. elegans, Drosophila* and mice are also involved in determining lifespan in humans. FOXO3A is one of the three mammalian homologues of the *C. elegans* transcription factor DAF-16, which is required for the lifespan effects of the insulin/IGF1 signaling pathway. AKT1 is one of the tyrosine kinases in the signaling cascade that links IGF1 binding to its receptor to transcriptional activation and downstream effects on cell metabolism and oxidative stress response; AKT1 phosphorylates FOXO3A and prevents it from entering the nucleus thus inhibiting transcriptional activation (Brunet *et al.*, 1999; Zheng *et al.*, 2000). Thus the two genes most strongly associated with longevity in our results are also functionally related.

How the variants we identified affect lifespan remains uncertain. All three longevity-associated SNPs that replicated across 3 independent cohorts are intronic SNPs of no known function and they do not reside in motifs conserved through evolution. In the case of *FOXO3A*, the two longevity-associated SNPs are in linkage disequilibrium with other variants across an area of approximately 67kb. Since the linkage disequilibrium is so strong in this area, it may not be possible to discern which is causative from association studies in Caucasians alone; association studies in other populations, locus re-sequencing and functional studies are needed.

The *AKT1* SNP associated with lifespan is not in high LD with any other HapMap variants; evaluation of 8 unlinked variants in the nearby area, including 5 amino-acid coding polymorphisms, did not reveal any further associations with lifespan. Since *AKT1* rs3803304 resides 70 base-pairs (bp) away from an intron-exon boundary, and in an area of high predicted regulatory potential (King *et al.*, 2005), which potentially encodes a non-coding regulatory RNA (Kapranov *et al.*, 2007), it may be a candidate SNP for influencing RNA splicing or other regulatory mechanisms. *In vitro* functional studies will be needed to explore such potential mechanisms.

Our study has several potential limitations. First, although we adjusted for self-reported ethnicity in the SOF study, population stratification may have affected our results. In addition, we did not have self-reported ethnicity information in CHS. Second, subtle effects on longevity might have been missed because of our relatively small sample size (896 participants in SOF); we had adequate (80%) power to detect an odds ratio of ~1.7. As with other complex traits (Easton *et al.*, 2007; Frayling, 2007; Harley *et al.*, 2008), much larger sample sizes will be required to identify modest risk modifiers. Third, like most studies of longevity in humans, our study may be influenced by birth cohort effects, since controls were generally born later (e.g., in the 1910's-20's) than the cases (in the 1890's-1900's). Finally, we only tested common SNPs and small insertion-deletions. We did not genotype

copy number variants or large insertion-deletions and, with the exception of a few amino-acid coding variants, we only evaluated the effects of common variants (minor allele frequency (MAF)>0.05). The relative dearth of common SNPs associated with longevity in the genes we analyzed does not exclude the existence of other genetic effects in the pathway. To obtain a complete picture of the association of genetic variation in the insulin/IGF1 pathway with phenotypes, discovery of rare variants by resequencing of the same set of candidate genes will be necessary.

Despite these limitations, replication among three independent Caucasian samples, including a founder population (AJC), strengthens our findings. It is unlikely that population stratification could lead to false-positive results that would be consistent across a European American/Caucasian American population and the Ashkenazi population, since the patterns of stratification would be different. Any birth cohort effects should be relatively modest, since the differences in birth-year were small, and since both groups were born before there were substantial changes in early-life mortality rates due to advances in medical care.

In summary, we screened the insulin/IGF1 signaling pathway for association with human longevity and identified common variants in 2 genes, *AKT1* and *FOXO3A*, that are consistently associated with longer lifespan in 3 independent Caucasian cohorts. Our results extend the remarkably consistent effect of this pathway on lifespan in model organisms to human populations. Although the effects we observed are subtle, they implicate the insulin/IGF1 signaling pathway in human aging, confirming that studies in model organisms can help us to gain insights into the biology of human aging.

Experimental Procedures

Samples

Data from 3 studies were analyzed. Two are large cohort studies of older adults: SOF and CHS. The third study is a case-control study (AJC).

Study of Osteoporotic Fractures (SOF)—A total of 9704 ambulatory community-dwelling Caucasian women age 65 and older were recruited from September 1986 through October 1988 from 4 areas in the United States: Baltimore, MD: Monongahela Valley, PA; Minneapolis, MN; and Portland, OR (Cummings *et al.*, 1990). The study protocol was approved by the appropriate institutional review committees and written informed consent was obtained from all participants. Self-reported ethnicity was recorded as Northern, Southern, or Central European, or mixed/other. The cohort has been followed for over 20 years with age and cause of death recorded for participants who have died. 896 SOF participants were selected for the present study. These included 293 long-lived cases (lifespan ≥92y, mean ± SD = 95.3 ± 2.2y) of whom approximately 56% were still living at the last study visit. We selected 603 controls who were the youngest to die in this cohort (lifespan ≤79y, mean=75.7 ± 2.6y); however, the age of death in the controls is not substantially different compared to the general population, since all women had to be relatively healthy to enter the cohort (Cummings *et al.*, 1990). Cause of death was adjudicated by an experienced internist based on review of death certificates and hospital records.

Cardiovascular Health Study (CHS)—The original CHS cohort includes 5201 Caucasian and African-American participants recruited from 1989 to 1990 in 4 communities in the United States (Fried *et al.*, 1991; Tell *et al.*, 1993) and 684 additional African-American participants recruited from 1992 to 1993. All participants were 65 years of age or older. At enrollment, participants were not institutionalized, wheelchair-bound at home, or receiving treatment for cancer. Informed consent was obtained from all participants. Deaths

were ascertained through surveillance and at semiannual contacts. For all deaths, the CHS collected information from medical records, physician interviews, autopsy reports, coroner reports, and next of kin interviews. Data were reviewed by the CHS Events Committee and cause of death classified according to pre-specified criteria (Ives *et al.*, 1995). From the 4383 Caucasian CHS participants, we defined a case-control replication sample of 1076 for the longevity analysis using the same age cutoffs as in the SOF study. This subset included 279 long-lived cases (lifespan ≥92y, mean = 94.5 ± 2.1 years (y)) and 797 average-lifespan controls (lifespan ≤79y, mean=76.1 ± 3.1 y).

Ashkenazi Jewish Centenarians (AJC)—Subjects were of self-reported Ashkenazi Jewish ancestry (Barzilai *et al.*, 2003; Atzmon *et al.*, 2006; Suh *et al.*, 2008). Informed written consent was obtained in accordance with the policy of the Committee on Clinical Investigations of the Albert Einstein College of Medicine. The 383 cases (74.8% female) were required to be living independently at 95 years of age as a reflection of good health (mean age 97.7y, range 95–108y). The 363 controls (mean age 79.5 years, age range 43-94y, 57% female), all living at time of recruitment, were of self-reported Ashkenazi Jewish descent without a family history of unusual longevity (parents' age <85y). Participants' ages were defined by birth certificates or passports. 4% of controls and 76% of centenarians have died since recruitment.

Gene and Variant Selection

Variants were selected for genotyping in 30 genes encoding the key proteins in the human insulin/IGF1 signaling pathway, including ligands (IGF1, insulin, GH1, GHRH), binding proteins (IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP6), receptors (IGF1R, INSR, INSRR, GHR, GHRHR), signal transduction proteins (AKT1, AKT2, AKT3, IRS1, IRS2, IRS4, PDPK1, PIK3CA, PIK3CB, PIK3R1, PIK3R2, PTEN, SHC1) and transcription factors (AFX, FOXO1A, FOXO3A). GH1 was included initially, but all 3 SNPs available in dbSNP failed genotyping attempts. Initially, variants in *FOXO1A* and *FOXO3A* were identified by sequencing exons, intron-exon boundaries and the promoter in 80 Caucasians and selecting haplotype-tagging variants. Subsequently, we utilized the Human Haplotype Consortium data from CEU Caucasians (2005; Frazer *et al.*, 2007) (www.hapmap.org) to select haplotype-tagging SNPs. Briefly, tag SNPs were picked for the gene ± approximately 10 kb genomic flanking sequence using the Tagger algorithm (de Bakker *et al.*, 2005) implemented in the Haploview program (Barrett *et al.*, 2005) (www.broad.mit.edu/mpg/haploview/download.php). The entire genomic sequence was tagged for all genes except *IGF1R* and *INSR*, where most of the large intron 1 was excluded. We set the r^2 cutoff at 0.8 and minor allele frequency cutoff at 0.05 (except for amino acid coding variants, which were all included). Pair-wise tag selection was used for all genes except *IGFBP2, IGFBP5, IRS2* and *PIK3R1*, where 2-marker haplotypes were used to reduce the number of tag SNPs. Amino-acid coding variants were prioritized and previously published candidate variants were included (Bonafe *et al.*, 2003). Eight additional SNPs in *AKT1* were selected for follow up. We selected 339 SNPs overall; 293 SNPs were successfully genotyped.

To analyze the genomic regions surrounding *FOXO3*A and *AKT1*, we used the UCSC Genome Browser (www.genome.ucsc.edu) including data from several analysis tracks (ESPRR regulatory potential (King *et al.*, 2005), Phastcons conservation, CpG islands, Affymetrix Transcriptome Phase 3 (Kapranov *et al.*, 2007)) on the March 2006 assembly of the human genome.

Genotyping

SNPs were genotyped in the SOF and CHS cohorts using two genotyping platforms: template directed primer extension with fluorescence polarization detection (FP-TDI,

AcycloPrime II detection kit, Perkin Elmer, Boston, MA) (Hsu & Kwok, 2003) and SNPstream 48plex (Beckman Coulter, Fullerton, CA). Oligonucleotide primers were designed using www.autoprimer.com, primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and as previously described (Hsu & Kwok, 2003). Assay conditions are available upon request. Genotyping in AJC was performed at the Albert Einstein College of Medicine using the Sequenom platform and pyrosequencing.

Samples were genotyped by investigators blinded to phenotype. All data was scored by at least 2 investigators. DNA samples were genotyped on 384-well plates with negative and positive controls (duplicates) included in each plate. There were 19 duplicates among the SOF and 267 among the CHS samples. Plates with fewer than 90% successful genotypes or with duplicate discrepancies were repeated. Plates where duplicate discrepancies persisted after re-genotyping were excluded. Overall genotyping accuracy was >99.5%.

SNP genotype distributions in SOF controls were tested for consistency with Hardy Weinberg equilibrium using a γ^2 test (Bonferroni-corrected significance threshold p=1.7E^{-04}). 2/293 SNPs: *IRS4* rs1801162 and *IGFBP2* rs9341156, were not in Hardy-Weinberg equilibrium and were removed from further analysis.

Statistical Analyses

Association of SNP genotype with longevity—For all 291 SNPs analyzed, minor allele homozygotes were coded 2, heterozygotes were coded 1, and major allele homozygotes were coded 0. Logistic regression was used to evaluate the association between genotype and longevity case/control status. We assumed an additive model as our baseline model; significant associations were also tested with a genotypic model. In SOF, the logistic regression model was adjusted for self-reported ethnicity. In CHS and AJC, models were adjusted for sex; we also analyzed data separately for women in these two cohorts. Association of significant genotypes with the three main categories of cause of death (cardiovascular disease, cancer, or other) was evaluated in the 2 cohorts (SOF and CHS) that had this information. OR and 95% CI are reported. We also performed haplotype case-control association analyses using 2- and 3-SNP sliding window and haplotype analysis across entire LD-blocks using the Haploview program (Barrett *et al.*, 2005).

Replication and meta-analysis—The logistic regression results from all 3 cohorts (or from 2 cohorts only in some cases) were combined using inverse variance weighting. To evaluate statistical significance in the replication we corrected for all of the SNPs we replicated and used only the data from the 2 replication cohorts (AJC+CHS) to assess significance. We determined statistical significance by permutation. Case/control status was permuted randomly for all participants for the 25 SNPs genotyped in the replication stage. In each permutation, we recorded the maximum absolute value of the Z scores. We performed 2000 permutations. We then compared the Z scores from the observed data with the distribution of the maximum Z scores from the permutations. The percentile of the observed absolute z score among the permuted z scores was calculated as the adjusted P value for determining statistical significance corrected for multiple testing. Statistical significance in the replication was defined as an adjusted p<0.05 in the AJC+CHS replication cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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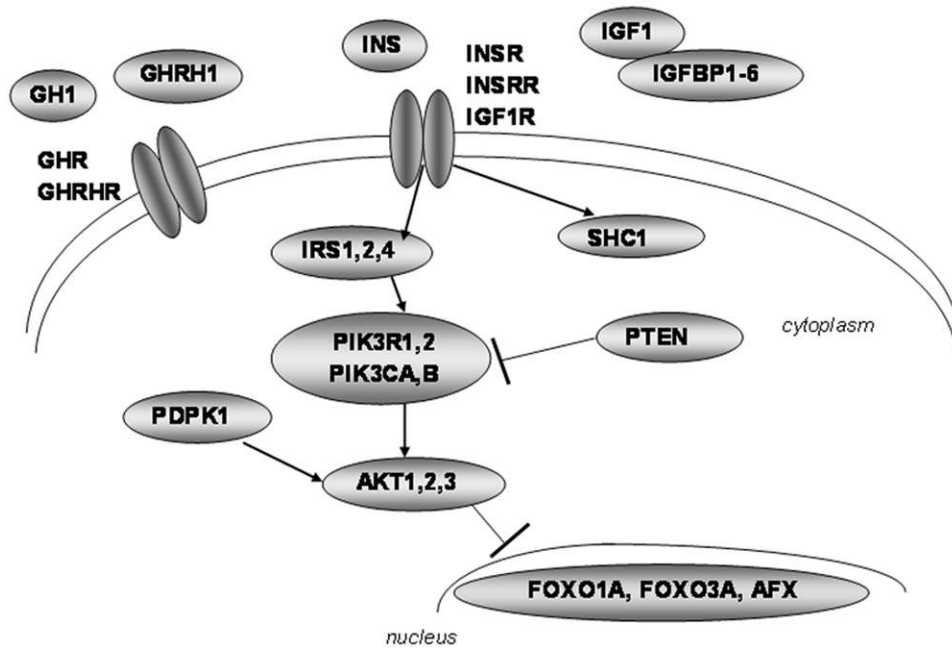
Figure 1. Graphic of insulin/IGF1 signaling pathway showing proteins encoded by scanned genes
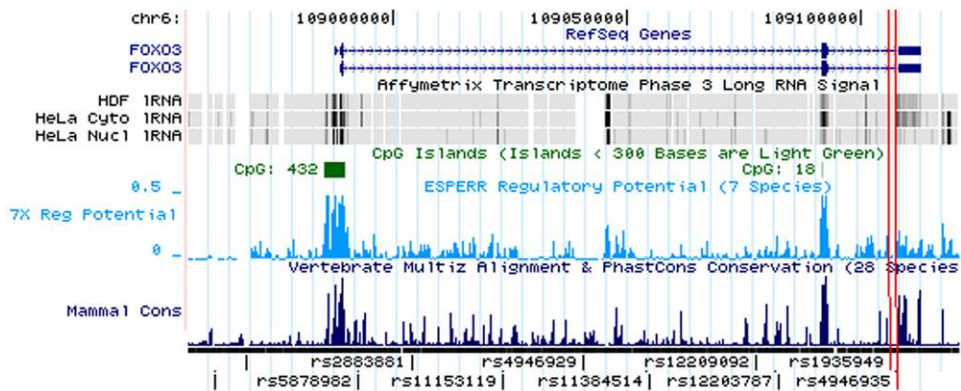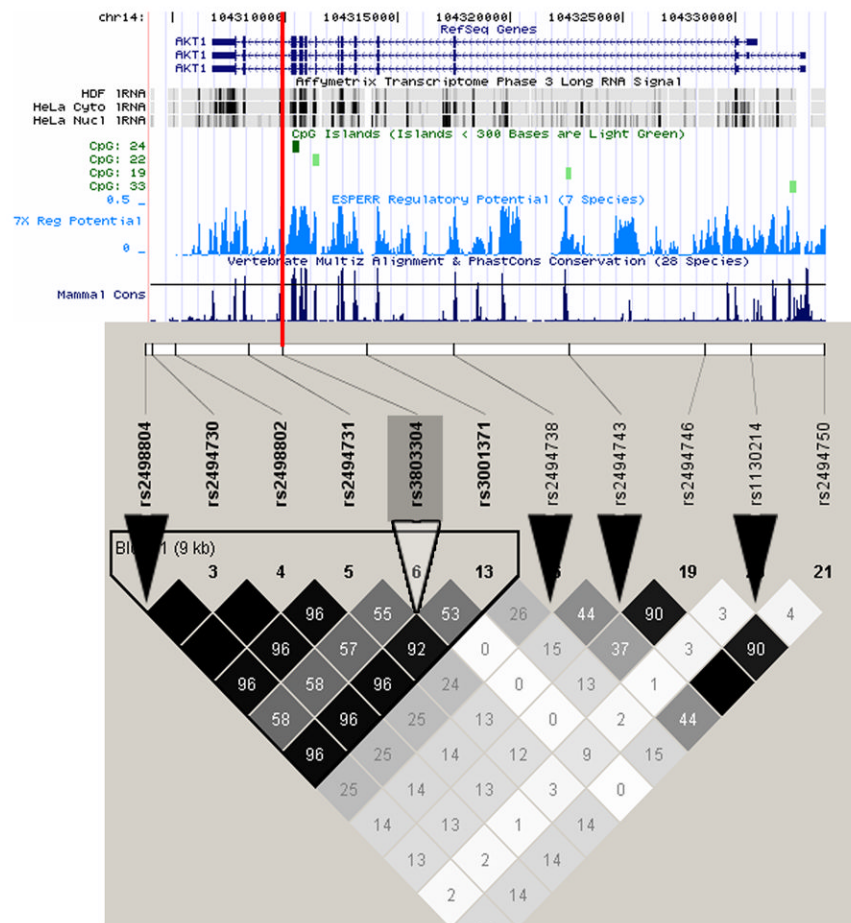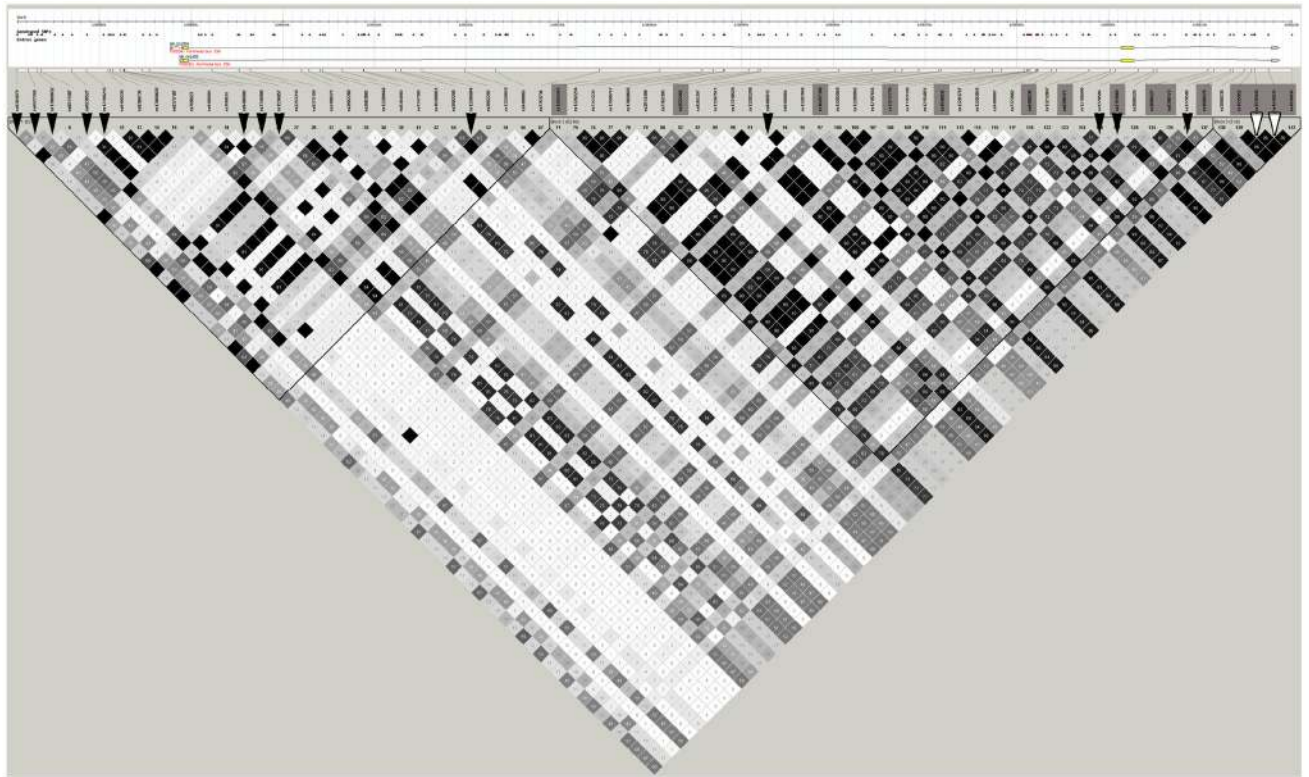
Figure 2. Genomic context and linkage disequilibrium for (A) *AKT1* **and (B, C)** *FOXO3A*
UCSC genome browser tracks show mRNA isoforms for both genes, CpG islands, ESPERR regulatory potential and conservation across 28 mammalian species (indicator line at 70%). Linkage disequilibrium plots below show r2 in HapMap Caucasians. All HapMap variants are tagged at r2>0.8. SNPs genotyped in SOF are marked by black triangles. Longevity-associated SNPs are marked by white triangles and red lines and other SNPs in LD with them are boxed.

Table 1

Characteristics of the three study cohorts.

Characteristic	SOF		CHS		AJC	
	cases	**controls**	**cases**	**controls**	**cases**	**controls**
N	293	603	279	797	383	363
Age (years)	95.3 ± 2.2	75.7 ± 2.6	94.5 ± 2.1	76.1 ± 3.1	99.8 (95-108)	85.5 (43-94)
Sex (% male)	0	0	47.0	52.6	25.2	42.7
% Alive	55.6	0	33.7	0	24	96
% Atherosclerosis death	17.7	30.2	16.8	26.1	NA	NA
% Cancer death	4.1	40.8	12.9	37.5	NA	NA
% Other death	22.5	29.0	36.6	39.1	NA	NA

SOF, Study of Osteoporotic Fractures, CHS, Cardiovascular Health Study, AJC, Ashkenazi Jewish Centenarians. Age is the age at death for deceased subjects, and age at last followup for subjects still alive. NA, not available

Table 2

Association of variants in insulin/IGF1 pathway genes with longevity.

Gene	# SNPs	# p<0.05	# p<0.01	# p<0.005
AFX	7	0	0	0
AKT1	8	**1**	0	0
AKT2	5	0	0	0
AKT3	13	0	0	0
FOXO1A	7	**1**	0	0
FOXO3A	16	**7**	**4**	**1**
GHR	26	**7**	**3**	**1**
GHRH	1	0	0	0
GHRHR	13	**1**	0	0
IGF1	6	0	0	0
IGF1R	34	**1**	**1**	**1**
IGFBP1	11	0	0	0
IGFBP2	8	0	0	0
IGFBP3	5	**1**	0	0
IGFBP4	7	**1**	0	0
IGFBP5	11	0	0	0
IGFBP6	4	0	0	0
INS	2	0	0	0
INSR	32	0	0	0
INSRR	4	0	0	0
IRS1	6	0	0	0
IRS2	13	0	0	0
IRS4	1	0	0	0
PDPK1	3	0	0	0
PIK3CA	5	0	0	0
PIK3CB	3	0	0	0
PIK3R1	28	0	0	0
PIK3R2	2	0	0	0
PTEN	9	**1**	0	0
SHC1	1	0	0	0
All genes, observed	**291**	**21**	**8**	**3**
All genes, expected	**-**	**15**	**2.9**	**1.5**

Number of SNPs in each gene associated with longevity at a nominally significant level is shown. Results shown are for an additive model corrected for self-reported ethnicity. Total number of SNPs observed at 3 different significance thresholds is compared with the number expected.

Table 3

Meta-analysis of association with longevity for SNPs genotyped in SOF, CHS, and AJC.

All (males + females)			SOF (n=896)			CHS (n=1076)			AJC (n=746)			Meta-analysis		
SNP	Reason	Gene	p	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p	OR	95% CI
rs3803304	**longevity**	**AKT1**	**0.049**	**0.79**	**0.62-0.99**	**0.033**	**0.77**	**0.61-0.98**	**0.041**	**0.77**	**0.59-0.98**	**0.00040**	**0.78**	**0.68-0.89**
rs1334241	longevity	FOXO1A	0.032	1.30	1.02-1.65	0.17	0.84	0.65-1.08	0.48	0.90	0.67-1.20	0.87	1.01	0.87-1.17
rs1044891	longevity	FOXO3A	0.0083	1.48	1.10-1.97	0.53	0.91	0.66-1.22	NA	NA	NA	0.14	1.17	0.95-1.45
rs1935949	**longevity**	**FOXO3A**	**0.043**	**1.25**	**1.00-1.56**	**0.23**	**1.14**	**0.92-1.41**	**0.016**	**1.36**	**1.05-1.74**	**0.0014**	**1.24**	**1.09-1.41**
rs2153960	longevity	FOXO3A	0.015	1.31	1.05-1.63	0.19	1.15	0.93-1.41	0.81	1.03	0.81-1.30	0.019	1.16	1.02-1.32
rs3778588	longevity	FOXO3A	0.0047	1.51	1.13-1.99	0.84	0.97	0.73-1.29	0.19	1.24	0.89-1.72	0.023	1.22	1.03-1.45
rs4946935	**longevity**	**FOXO3A**	**0.041**	**1.26**	**1.00-1.57**	**0.11**	**1.19**	**0.96-1.47**	**0.029**	**1.33**	**1.03-1.72**	**0.00087**	**1.25**	**1.10-1.43**
rs9486902	longevity	FOXO3A	0.0050	1.47	1.12-1.92	0.93	0.99	0.74-1.31	0.99	1.00	0.66-1.50	0.073	1.18	0.98-1.40
rs9486913	longevity	FOXO3A	0.0051	1.49	1.12-1.96	0.60	0.93	0.69-1.23	0.32	1.19	0.84-1.66	0.053	1.18	0.99-1.40
rs12153009	longevity	GHR	0.017	0.76	0.60-0.95	0.55	0.94	0.75-1.16	0.47	0.91	0.71-1.16	0.033	0.87	0.76-0.99
rs2940923	longevity	GHR	0.0094	1.31	1.06-1.61	0.44	1.08	0.88-1.31	0.023	1.28	1.03-1.59	0.0013	1.21	1.09-1.37
rs2940935	interaction	GHR	0.61	1.07	0.82-1.38	0.011	1.37	1.07-1.73	0.046	1.30	1.00-1.67	0.0031	1.24	1.08-1.44
rs3764451	longevity	GHR	0.0093	0.68	0.50-0.90	0.86	1.03	0.77-1.35	0.61	0.92	0.66-1.26	0.094	0.86	0.73-1.03
rs4292454	longevity	GHR	0.043	1.25	1.00-1.54	0.49	0.93	0.76-1.13	NA	NA	NA	0.39	1.07	0.92-1.23
rs4866941	longevity	GHR	0.0027	1.42	1.12-1.78	0.27	0.88	0.70-1.10	NA	NA	NA	0.19	1.11	0.95-1.31
rs6883523	longevity	GHR	0.042	1.30	1.00-1.67	0.48	0.91	0.70-1.17	0.64	0.92	0.63-1.32	0.52	1.05	0.90-1.24
rs6887528	longevity	GHR	0.012	1.72	1.12-2.62	0.38	0.83	0.54-1.26	NA	NA	NA	0.25	1.19	0.88-1.61
rs2228078	longevity	GHRHR	0.048	2.68	1.00-7.14	0.96	1.02	0.46-2.21	NA	NA	NA	0.21	1.48	0.81-2.72
rs2272037	longevity	IGF1R	0.0048	1.39	1.10-1.74	0.55	1.06	0.86-1.29	NA	NA	NA	0.021	1.19	1.03-1.39
rs6670	longevity	IGFBP3	0.047	1.28	1.00-1.63	0.36	0.89	0.70-1.13	0.63	0.94	0.74-1.19	0.75	1.02	0.89-1.18
rs1009728	interaction	IGFBP4	0.43	1.09	0.87-1.36	0.78	1.03	0.82-1.28	0.35	1.10	0.89-1.35	0.24	1.08	0.95-1.22
rs7214466	longevity	IGFBP4	0.012	0.75	0.59-0.93	0.68	1.04	0.84-1.28	0.37	0.91	0.73-1.12	0.094	0.90	0.79-1.02
rs11575134	interaction	IGFBP5	0.71	1.05	0.80-1.37	0.40	0.90	0.70-1.15	0.043	1.28	1.00-1.63	0.36	1.07	0.93-1.24
rs3770472	interaction	IGFBP5	0.91	1.01	0.78-1.31	0.23	0.87	0.68-1.09	0.042	1.29	1.00-1.63	0.60	1.04	0.90-1.20
rs1022427	longevity	PTEN	0.033	1.65	1.04-2.61	0.99	1.00	0.63-1.58	NA	NA	NA	0.13	1.28	0.93-1.78

We show OR, 95% CI and p values for an additive model for each study and for the meta-analysis. SOF analyses are adjusted for self-reported ethnicity, CHS and AJC analyses are adjusted for sex. Nominal p-values are shown for all analyses. For replication, only *AKT1* rs3803304 meets the permutation-based threshold for statistical significance in the entire cohort; *FOXO3A* rs1935949 and rs4946935 meet the threshold for women only (data not shown). Reason = reason for replication. NA = not available.

Table 4

Meta-analysis of association with longevity for top SNPs genotyped in SOF, CHS, and AJC.

SNP	Geno type	SOF (n=896)				CHS (n=1076)				AJC (n=746)				Meta-analysis		
		N	P	OR	95% CI	N	P	OR	95% CI	N	P	OR	95% CI	OR	95% CI	P
***AKT1* rs3803304 G/C**	GG	465	ref	-	-	607	-	-	-	455	-	-	-	-	-	-
	GC	309	0.53	0.90	0.66-1.23	345	0.25	0.84	0.62-1.13	249	0.36	0.86	0.63-1.18	0.87	0.73-1.04	0.12
	CC	70	0.025	0.50	0.27-0.91	67	0.040	0.50	0.25-0.97	34	0.023	0.43	0.20-0.89	0.48	0.32-0.70	0.00016
***FOXO3A* rs1935949 C/T**	CC	420	ref	-	-	531	-	-	-	429	-	-	-	-	-	-
	CT	366	0.10	1.29	0.95-1.75	394	0.15	1.24	0.92-1.67	274	0.10	1.30	0.95-1.77	1.27	1.07-1.52	0.0064
	TT	81	0.10	1.52	0.92-2.52	87	0.56	1.16	0.70-1.94	39	0.039	2.08	1.04-4.16	1.46	1.07-2.01	0.019
***FOXO3A* rs4946935 G/A**	GG	415	ref	-	-	521	-	-	-	437	-	-	-	-	-	-
	GA	358	0.16	1.25	0.92-1.69	403	0.050	1.34	1.00-1.80	211	0.090	1.34	0.96-1.87	1.31	1.09-1.56	0.0035
	AA	78	0.066	1.61	0.97-2.67	84	0.47	1.21	0.72-2.04	39	0.10	1.78	0.90-3.52	1.48	1.07-2.03	0.018

OR and 95% CI from a genotypic model are shown in men and women combined, adjusted for sex.

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Table 5

Association of genotype with cause of death in SOF and CHS.

Cause of death	Cohort	Significance	AKT1 rs3803304	FOXO3A rs1935949	FOXO3A rs4946935
Atherosclerosis	SOF	P	0.069	**0.013**	**0.017**
		OR (95% CI)	1.33 (0.98-1.81)	**0.68 (0.51-0.92)**	**0.69 (0.51-0.94)**
	CHS	P	**0.040**	0.49	0.73
		OR (95% CI)	**1.38 (1.02-1.89)**	1.10 (0.84-1.45)	1.05 (0.79-1.40)
	Meta-analysis	P	**0.0062**	0.25	0.17
		OR (95% CI)	**1.36 (1.09-1.69)**	0.89 (0.73-1.09)	0.87 (0.70-1.07)
Cancer	SOF	P	0.087	**0.045**	0.12
		OR (95% CI)	1.28 (0.97-1.67)	**0.76 (0.58-0.99)**	0.81 (0.62-1.06)
	CHS	P	0.37	0.38	0.16
		OR (95% CI)	1.14 (0.85-1.53)	0.89 (0.70-1.15)	0.83 (0.64-1.08)
	Meta-analysis	P	0.064	**0.044**	**0.037**
		OR (95% CI)	1.21 (0.99-1.48)	**0.83 (0.69-0.99)**	**0.82 (0.68-0.99)**
Other	SOF	P	0.10	0.32	0.48
		OR (95% CI)	1.30 (0.95-1.78)	0.86 (0.64-1.16)	0.90 (0.66-1.21)
	CHS	P	**0.036**	**0.035**	**0.048**
		OR (95% CI)	**1.34 (1.02-1.75)**	**0.76 (0.58-0.98)**	**0.76 (0.58-1.00)**
	Meta-analysis	P	**0.0079**	**0.025**	0.051
		OR (95% CI)	**1.32 (1.08-1.62)**	**0.80 (0.66-0.97)**	0.82 (0.67-1.00)

For this analysis, the case group comprises participants who died of specific causes and the control group comprises long-lived participants. Results for an additive model are shown. Analyses in SOF are adjusted for self-reported ethnicity; analyses in CHS are adjusted for sex.