FOXO3A genotype is strongly associated with human longevity

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Human longevity is a complex phenotype with a significant familial component, yet little is known about its genetic antecedents. Increasing evidence from animal models suggests that the insulin/IGF-1 signaling (IIS) pathway is an important, evolutionarily conserved biological pathway that influences aging and longevity. However, to date human data have been scarce. Studies have been hampered by small sample sizes, lack of precise phenotyping, and population stratification, among other challenges. Therefore, to more precisely assess potential genetic contributions to human longevity from genes linked to IIS signaling, we chose a large, homogeneous, long-lived population of men well-characterized for aging phenotypes, and we performed a nested-case control study of 5 candidate longevity genes. Genetic variation within the *FOXO3A* gene was strongly associated with human longevity. The OR for homozygous minor vs. homozygous major alleles between the cases and controls was 2.75 ($P = 0.00009$; adjusted $P = 0.00135$). Long-lived men also presented several additional phenotypes linked to healthy aging, including lower prevalence of cancer and cardiovascular disease, better self-reported health, and high physical and cognitive function, despite significantly older ages than controls. Several of these aging phenotypes were associated with *FOXO3A* genotype. Long-lived men also exhibited several biological markers indicative of greater insulin sensitivity and this was associated with homozygosity for the *FOXO3A* GG genotype. Further exploration of the *FOXO3A* gene, human longevity and other aging phenotypes is warranted in other populations.

gene | insulin | healthy aging | disease | disability

Human longevity is a complex phenotype with multiple determinants. While non-genetic factors, including diet, physical activity, health habits, and psychosocial factors are important, up to 50% of the variation in human lifespan might be explained by genetic differences (1–5). Several studies suggest that about 25% of the variation in human lifespan in average-lived populations can be explained by genetic factors, but in populations with larger numbers of exceptional survivors, the genetic contribution to lifespan may be much higher. For example, family studies of nonagenarians and centenarians show that sibling relative risk, a common method for assessing potential genetic contribution to a complex phenotype (6), is particularly high and grows with increasing age of the proband (7–10). However, studies of candidate "longevity-associated" genes in humans, hereafter referred to as "longevity genes," have generally been disappointing. Few replications have been observed across populations, with the exception of the *APOE* gene (3).

In contrast, there have been several robust genetic findings in model organisms of aging (11–13). For example, variation in single genes can result in substantial differences in lifespan in model organisms, particularly with genes that are considered part of the insulin/IGF-1 (IIS) signaling pathway (14–18).

Mutations that increase SIR-2 activity or that decrease insulin/IGF-1 signaling both increase the lifespan of *C. elegans* by activating the DAF-16/FOXO protein (19, 20). In mammalian cells, a Sir2 homolog "SIRT1," influences several downstream transcription events affecting lifespan, including the cellular response to stress. SIRT1 accomplishes this by regulating the FOXO (Forkhead box transcription) factors, a family of proteins that function as sensors in the IIS pathway and influence mammalian longevity (17).

Genetic knock-out models in mammals (and other species) have also supported the IIS hypothesis. For example, mice with a fat-specific insulin receptor knockout (FIRKO) have reduced fat mass, protected against age-related obesity, and have extended longevity (21). Many other mutations in the IIS pathway appear to impact longevity in mice. These include mutations in the IGF-1 receptor (22), IRS-1 (22), IRS-2(23), PAPP-A (24), and the Ames Dwarf mouse mutation (22).

The basic molecular pathway of insulin signaling is conserved through evolution, evidence of which can be seen in yeast, flies, worms, rodents, and humans (25). A key regulator of this pathway in worms is the transcription factor DAF-16 (abnormal DAuer Formation-16), which is required for the large lifespan extension produced in *C. elegans* by inhibiting insulin/IGF-1 signaling (16). A number of factors appear to extend lifespan in *C. elegans* in a DAF-16 dependent manner, such as AMP kinase (26), 14–3-3 proteins (27), the lin-4 microRNA (28), and heat shock factor (29). Homologues of DAF-16 in several species have been linked to aging phenotypes and longevity (30). For example, the stress responsive Jun-N-K terminal kinase (JNK) pathway appears to require FOXO to prolong lifespan in Drosophila (31), and when flies over express dFOXO, the DAF-16 ortholog, it can increase lifespan (32). The convergence of such a diverse array of signals on DAF-16/FOXO suggests that this protein may be an important, evolutionarily conserved "node" in a signaling network that impacts aging and longevity.

The human homologue of DAF-16 includes four FOXOs: FOXO1, FOXO3, FOXO4 and FOXO*6*. We hypothesize that common, natural variation in the form of single nucleotide polymorphisms (SNPs) in FOXO and related genes might influence human aging and longevity. A connection between insulin, FOXO, oxidative stress, and human longevity would be particularly interesting since oxidative stress has long been a favorite putative mechanism of aging. Since 1956, the free radical theory of aging has hypothesized that aging results partly from damage to DNA, cells, and tissues from cumulative exposure to reactive oxygen molecules

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Table 1. Baseline characteristics by case–control status

Variables at baseline examination (1991–1993)	Average lived phenotype (mean attained age 78.5 years) (n = 402)		Longevity phenotype (mean attained age 97.9 years)* (n = 213)		$P^{\dagger}$
	Mean ± SD	Min–Max	Mean ± SD	Min–Max	
Biological, fasting values					
Age at baseline exam, years	74.63 ± 2.05	71–79	85.62 ± 3.12	80–93	<.0001
Body mass index, kg/m^2	23.4 ± 3.17	15.89–32.33	23.0 ± 2.91	15.4–31.1	0.1272
Waist/hip ratio	0.95 ± 0.06	0.78–1.15	0.93 ± 0.06	0.73–1.07	0.0008
Total cholesterol, mg/dl	187.96 ± 34.6	98–303	185.36 ± 32.16	95–304	0.3680
HDL, mg/dl	50.82 ± 14.17	21–129	51.29 ± 13.54	27–100	0.6911
Triglycerides, mg/dl	154.72 ± 118.72	46–1369	140.32 ± 82.23	38–649	0.1178
Log triglycerides‡	4.88 ± 0.51	3.83–7.22	4.81 ± 0.50	3.64–6.48	0.0965
Glucose, mg/dl	117.83 ± 35.9	69–323	108.98 ± 22.55	77–298	0.0012
Insulin, mIU/liter	25.54 ± 82.89	3.3–1164	13.8 ± 11.39	1.5–104	0.0421
Log Insulin‡	2.69 ± 0.74	1.19–7.06	2.44 ± 0.58	0.41–4.64	<0.0001
FOXO3A3 MAF (rs2802292)§	0.255	—	0.371	—	<0.0001
General Health Status					
Self-rated "poor" health, %	41.92	—	31.07	—	0.0163
Disease prevalence					
CHD, %	26.37	—	7.55	—	<0.0001
Stroke, %	7.46	—	3.3	—	0.0394
Cancer, %	20.15	—	13.68	—	0.0468
Diabetes, %	60.55	—	59.81	—	0.8587
Physical/cognitive function					
Lower body (difficulty walking), %	30.59	—	16.83	—	0.0002
Upper body (grip strength), kg	29.85 ± 7.54	0–47	26.37 ± 5.53	8–45	<0.0001
Cognitive score (CASI)§	80.96 ± 19.48	0–100	78.54 ± 13.85	12–98	0.1088

*Cases (longevity phenotype) consisted of all HHP/HAAS participants with DNA samples (living and dead) who had reached the age of 95 years by Aug. 2007: Gp 1: Alive, n = 37, mean age 98.7, range 97–106 years; Gp 2: dead, n = 166, mean death age 97.5, range 95–106 y).
$^{\dagger}P$ value from Student's t test for continuous variables and χ^2 for categorical variables.
‡Log transformation performed for variables not normally distributed.
§MAF, minor allele frequency; CASI, cognitive abilities screening instrument.

(33) and although not yet universally accepted, supportive evidence has accumulated over the years (34, 35). Thus, FOXO may provide a potential forkhead or bridge between insulin signaling, free radicals, and human aging/longevity.

There has been some prior work linking genes in the IIS pathway to human longevity (36, 37) including an interesting recent report by Suh *et al.* (38), which links functionally significant IGF-1 receptor mutations to exceptional longevity, but we have not found any published reports of association between FOXO genes and human longevity. Prior studies have found links between FOXO genes and other aging phenotypes, including 4-year survival and stroke risk (39) as well as premature menopause (40).

Human longevity, however, is a complex phenotype that encompasses disease-specific risks as well as the individual rate of aging. The study of its genetic antecedents is challenging. The study of longevity may be affected by small genetic effect sizes, population stratification artifact, population heterogeneity, lack of sufficient numbers of long-lived study participants, and other problems (3, 4, 41). Therefore, to assess potential genetic contributions to human longevity from genes linked to IIS signaling, we chose a large, homogeneous, long-lived population of men well-characterized for aging phenotypes, and we performed a nested-case control study of 5 candidate longevity genes with links to the IIS pathway. These genes were chosen based on prior associations with aging phenotypes principally from gene knockout, transgenic, mutant, and other model organisms (3, 4, 14–17, 36, 42). Priority was for genes involving insulin sensing and glucose (energy) homeostasis.

Results

The baseline characteristics of the HHP/HAAS study population at the 1991–1993 examination are presented in supporting information (SI) Table S1. The mean age was 77.9 years and 100% of the population was male and of Japanese ethnicity. Biological characteristics, general health status, disease prevalence, and functional status are presented.

From this 1991–1993 baseline population, we selected all participants who, by 2007, had survived to age 95 years or more as "longevity" cases (n = 213). We then selected all participants who died before the age of 81 years as "average-lived" controls (n = 402). Baseline characteristics of the cases and controls are presented in Table 1. In terms of biological characteristics, the long-lived cases were older, leaner (lower waist:hip ratio), had lower triglycerides (borderline), lower glucose, lower insulin levels, and higher prevalence of the FOXO3A3 allele at the baseline examination. The cases also had better self-rated health and lower prevalence of cardiovascular disease [coronary heart disease (CHD) and stroke] and cancer. Functionally, they appeared better able to walk but had lower grip strength. There was no difference in cognitive score (43).

Five genes were investigated (*ADIPOQ*, *FOXO1A*, *FOXO3A*, *SIRT1*, and *COQ7*). Minor allele frequencies and other related genetic information for the cases and controls are presented in Table 2. However, only *FOXO3A* genotype was associated with longevity using an initial cut-off value of $P < 0.05$.

Further investigation comparing the genotype frequencies of *FOXO3A3* between cases and controls revealed a highly significant difference $P = 0.00009$ for the Pearson's exact χ^2 statistic (Table 3). Five loci with 3 SNPs within each allele were tested (Table 2). Bonferroni adjustment for multiple comparisons resulted in a corrected p value of $15 \times 0.00009 = 0.00135$. Due to the high LD between the 3 SNPs of *FOXO3A*, we further investigated the FOXO3A3 SNP only (rs 2802292). The OR for homozygous minor vs. homozygous major alleles for FOXO3A3 between the cases and controls was 2.75 (95% CI: 1.51 − 5.02, $P = 0.0007$), and the OR for heterozygous vs. homozygous major alleles between the cases and controls was 1.91 (95% CI: 1.34 − 2.72, $P = 0.0003$). These results suggest an additive effect on longevity.

Table 2. Candidate genes for human longevity and the MAF in cases and controls

Gene name	Symbol	SNP ID	Variable name	MAF		
				Cases	Controls	*P**
Adipo,† C1Q, CDC	*ADIPOQ*	rs1063539	ADIPOQ_1	0.297	0.263	0.20
		rs182052	ADIPOQ_2	0.455	0.493	0.22
		rs266729	ADIPOQ_3	0.195	0.239	0.08
Forkhead Box O1A	*FOXO1A*	rs2755209	FOXO1A1	0.272	0.291	0.48
		rs2721069	FOXO1A2	0.293	0.307	0.62
		rs2755213	FOXO1A3	0.350	0.358	0.77
Forkhead Box O3A	*FOXO3A*	rs2764264	FOXO3A1	0.347	0.248	0.0002
		rs13217795	FOXO3A2	0.340	0.248	0.0006
		rs2802292	FOXO3A3	0.371	0.255	<0.0001
Sirtuin 1	*SIRT1*	rs7069102	SIRT1_1	0.185	0.181	0.84
		rs10823112	SIRT1_2	0.337	0.360	0.44
		rs1885472	SIRT1_3	0.188	0.179	0.71
Coenzyme Q7	*COQ7*	rs8051232	COQ7_1	0.147	0.150	0.90
		rs11074359	COQ7_2	0.153	0.171	0.43
		rs7192898	COQ7_3	0.162	0.170	0.73

*Comparing MAF between cases and controls with χ^2 test.
†Adipocyte, C1Q, and collagen domain containing.

To understand more about the longevity phenotype at younger ages, we compared the proportion of people who were healthy at the baseline examination (1991–1993) for each of the three *FOXO3A* genotype groups using the definition of healthy survival from Willcox *et al.* (44). The differences were highly significant (Table 4). Those who possessed one or more G alleles were more likely to be healthy at baseline than those who were homozygous for the major (TT) allele; 75% of those homozygous for the minor allele were healthy at the baseline examination vs. 57% of those homozygous for the major allele. After adjusting for case-control status, the differences were still marginally significant. This suggests remaining association of the allele with health status in cases and controls.

To assess whether there was a relation between insulin sensitivity, a potential intermediate phenotype of longevity, and genotype, we tested the relation between fasting insulin, glucose, HOMA and genotype (Table 5). For non-normally distributed variables we used log conversion to a normal distribution. There was a significant relation between insulin, log insulin, HOMA and genotype. Homozygosity for the G allele was associated with markedly lower insulin, log insulin and HOMA score, but in controls only.

We also tested for a relation between lifetime prevalence of several chronic diseases and *FOXO3A* genotype (Table 6). A significant protective relation was found for homozygosity for the G allele with regard to prevalence of CHD and a borderline relation for cancer. Finally, we assessed the FOXO3A3 MAF distribution by maximum attained age in all participants combined. The MAF increased markedly with age (Table 7).

Table 3. FOXO3A3 genotype by case–control status

Case-Control Status	FOXO 3A3 Genotype (rs 2802292)		
	TT	TG	GG
Average-Lived Phenotype*	223 (55%)	153 (38%)	26 (6%)
Longevity Phenotype†	81 (38%)	106 (50%)	26 (12%)
p value for Pearson Exact test‡		0.000091	
p value after Bonferroni adjustment		0.00135	

*Number and percentage of subjects from $n = 402$ "average-lived" decreased controls (mean attained age 78.5 years).
†Number and percent of subjects from $n = 213$ "long-lived" cases (mean attained age 97.9 years).
‡From the exact Pearson χ^2 test comparing the genotype frequencies in the cases and controls.

Discussion

The rapid aging of the population will place unprecedented challenges on society due to increased prevalence of chronic disease and disability (45). Better understanding of mechanisms of aging, including biological pathways that may have widespread influence on how we age, could have important implications for lowering our risk for age-related disease and disability. There are many biologically plausible candidate genes for human longevity but only one finding has so far been widely replicated in multiple populations, that of the *APOE* gene (3). This gene has widespread effects on aging phenotypes, particularly cardiovascular disease and dementia, and as such influences the ability to achieve a long and healthy life.

To find other such genes, it may be helpful to use model organisms to identify *a priori* potential candidates before conducting human studies. Therefore, we chose to study several candidate genes within the human insulin/IGF-1 signaling pathway and/or oxidative stress response system on the basis of sequence and/or functional homology with model organisms of aging or prior human studies. We constructed a list of human candidate genes (Table S2) from these signaling pathways and assessed variations in these genes occurring at a frequency of ≥10% in the Japanese population. Three SNPs were chosen from each gene for analysis. SNPs were selected mainly from regions with linkage disequilibrium (LD) for maximal coverage of each gene.

Analysis of five candidate genes demonstrated that one gene clearly stood out from the others in terms of a potential human longevity gene—*FOXO3A*. That this gene might be important to human longevity is supported by several lines of evidence. First, in nested case-control analyses, variation within this gene was strongly associated with longevity. Furthermore, two copies of the G allele conferred about twice the protective effect (suggesting an additive

Table 4. Difference in health status between genotype groups at baseline

	Healthy at baseline,* %			*P* value for trend	
	Homo. Major	Heter.	Homo. Minor	Unadjusted	Adj. for Case-Control Stat
FOXO3A1	57.41	69.48	75.51	0.01	0.065
FOXO3A2	57.37	69.35	77.08	0.01	0.035
FOXO3A3	57.89	68.34	75.00	0.02	0.097

*"Healthy" is defined as absence of 6 major chronic diseases (CHD, stroke, cancer, PD, COPD and treated type 2 diabetes; high physical function (can walk one-half mile) and high cognitive function (CASI score > 74).

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Table 5. Insulin sensitivity phenotypes according to *FOXO3A* genotype

	FOXO3A Genotype (rs 2802292)			
	TT	TG	GG	*P**
Fasting glucose, mg/dl				
Average-lived	118.4 ± 34.0	117.4 ± 38.0	115.9 ± 40.1	0.80
Long-lived	108.3 ± 20.7	109.1 ± 23.7	110.5 ± 24.1	0.73
Fasting insulin, mIU/liter				
Average-lived	23.7 ± 81.2	30.4 ± 91.9	13.2 ± 5.9	0.004
Long-lived	13.5 ± 9.0	14.1 ± 13.4	13.3 ± 9.3	0.77
Log fasting insulin, mIU/liter				
Average-lived	2.68 ± 0.67	2.73 ± 0.85	2.47 ± 0.48	0.03
Long-lived	2.45 ± 0.55	2.43 ± 0.61	2.44 ± 0.52	0.99
HOMA IR Score				
Average-lived	9.1 ± 53.0	10.0 ± 32.2	3.8 ± 2.4	0.03
Long-lived	3.7 ± 2.8	4.0 ± 4.3	3.6 ± 2.2	0.55

*P value for Student's t test comparing mean values between GG genotype and other groups within cases and controls.

effect), roughly tripling the odds of living close to a century. The minor allele frequency also rose markedly from septuagenarian to centenarian ages (Table 7).

Second, all three SNPs that we assessed in the FOXO3A gene, which were in tight LD, were strongly correlated with the longevity phenotype. This indicates that the finding was unlikely to be due to chance. Third, carriers of the minor (G) alleles were healthier at the baseline examination, ≈15 years prior (Table 4).

In fact, the baseline examination suggested that cases were markedly healthier than controls despite the fact that cases were, on average, 11 years older. The cases possessed significantly less age-related disease, including less prevalent CHD, stroke, and cancer. They also had better self-rated health and generally had high physical function, including less difficulty walking. Interestingly, despite being more than a decade older than controls, the longevity cases had similar levels of cognitive function. This supports the existence of a "healthy aging" phenotype where individuals somehow delay or avoid major clinical disease and disability until late in life. The healthy aging phenotype that we observed in cases is similar to the healthy aging phenotypes reported in centenarians at younger ages when compared to their age-matched birth cohorts (46–48) and in centenarian offspring (49). Long-lived cases also had metabolic profiles suggesting higher insulin sensitivity at younger ages, with lower waist to hip ratio, lower glucose, insulin, and HOMA values (Tables 1 and 5). Several phenotypes were associated with variation in *FOXO3A* genotype.

Table 6. Prevalence of aging-related phenotypes in relation to FOXO3A3 genotype

	FOXO3A3 Genotype			
	TT	TG	GG	*P*
CHD prevalence, %				
Average-lived	32.3	18.3	23.1	0.010
Long-lived	7.4	7.6	7.7	0.998
All	25.7	14.0	15.4	0.002
Stroke prevalence, %				
Average-lived	6.7	8.5	7.7	0.813
Long-lived	4.9	1.9	3.8	0.510
All	6.3	5.8	5.8	0.974
Cancer prevalence, %				
Average-lived	22.4	18.3	11.5	0.326
Long-lived	17.3	12.4	7.7	0.400
All	21.1	15.9	9.6	0.075
Diabetes prevalence, %				
Average-Lived	60.6	62.3	50.0	0.498
Long-Lived	57.5	64.1	50.0	0.368
All	59.8	63.0	50.0	0.212

P values based on χ^2 test comparing frequency of GG genotype to other genotypes for average lived controls (n = 402), long lived cases (n = 213), and all subjects (n = 615).

Table 7. Genotype distribution by maximum attained age

Age at death (years)*	*n*	MAF of FOXO3A3
72–74	17	0.21
75–79	277	0.25
80–81	108	0.26
95–99	185	0.37
100–106	28	0.39

*Thirty-seven cases were still alive; mean age 98.7 years (range 97–106).

Surprisingly, there was no significant difference in diabetes prevalence between cases and controls. However, since the cases were more than a decade older than controls, and diabetes tends to increase markedly with age, it is noteworthy that prevalence of diabetes was not significantly different. In fact, both cases and controls had a high prevalence of diabetes (near 60%), despite relatively low BMI. Why Type 2 diabetes tends to be more prevalent in Japanese at a relatively low BMI is not completely understood (50). However, there may be metabolic differences in Japanese (and some other Asians) with higher visceral fat in Asians at lower BMI than in whites and blacks (51, 52). Indeed, Japan national guidelines reflect such population differences and consider Japanese obese at a BMI of 25 (53). Other contributing factors to the high prevalence of diabetes in the HHP/HAAS cohort are that participants were tested for diabetes by several clinical methods and at several examinations making detection more likely.

Of note, the *FOXO3A* genotype was significantly associated with plasma insulin levels as well as CHD, cancer, and Type 2 diabetes prevalence. This is consistent with a known role for FOXO as a mediator of the effects of insulin and insulin-like growth factors on diverse physiological functions, including cell proliferation, apoptosis, and metabolism (17, 54). Genetic studies in C. *elegans* and *Drosophila* have shown that FOXO proteins are ancient targets of insulin-like signaling that regulate metabolism and longevity. Additional work in mammalian cells has shown that FOXO proteins are the targets of protein kinases, influence cell cycle progression, and regulate resistance to oxidative stress *in vitro* (54). *In vivo* studies have shown that FOXO modifies hepatic glucose output in response to insulin and mediates other metabolic actions (54). This strengthens the evidence that FOXO proteins may mediate insulin effects on metabolism and influence longevity in humans.

Overall, the totality of the evidence supports a potential role of FOXO3A in human health, aging, and longevity. The association of FOXO with diverse aging phenotypes, including insulin sensitivity, CHD, cancer, type 2 diabetes, and longevity, is suggestive of a "gatekeeper" role in the IIS pathway. An important downstream mechanism whereby FOXO3A might influence human aging is through modification of oxidative stress—a long held theory of how we age (33), although we have no direct evidence for this in the current study. However, since FOXO genes are the closest human homologues of *C. elegans* DAF-16, which protects cells from oxidative stress, this is a plausible mechanism of action for modification of human aging (17). In *C. elegans*, DAF-16 increases the expression of manganese superoxide dismutase (SOD2), which converts superoxide to less damaging hydrogen peroxide and is a potent endogenous protector against free radicals (55), among other "anti-aging" effects. *In vivo* studies show that oxidative lesions in DNA, proteins, and other tissues accumulate with age and feeding calorically restricted diets (a potent insulin sensitizer) to rodents (56) and humans (57) mitigates this damage.

While FOXO was clearly associated with longevity we did not observe a strong effect of genotype on insulin sensitivity in cases—only in controls. However, the GG genotype demonstrated similarly

low plasma insulin levels in both cases and controls, consistent with a modulating effect of genotype on insulin levels in both groups. It is tempting to speculate that since the cases showed greater insulin sensitivity no matter what their genotype, they have multiple mechanisms to maintain insulin sensitivity other than FOXO. This would be consistent with the hypothesis that most longevity genes have modest or small effect sizes. It is also possible that small sample size limited our ability to detect differences in the cases. On the other hand, long-lived mice carrying mutations in either IRS-1 (58) or IRS-2 (23) are actually insulin resistant, so insulin sensitivity is not a necessary condition for mutations in the IIS pathway to be able to confer greater longevity.

However, it is interesting to note that in *C. elegans,* several genes that by themselves may have small effects on lifespan are influenced by the transcription regulating "master gene" DAF-16 (59). Small differences in *FOXO3A* that may be otherwise difficult to detect, could theoretically modify several downstream genes related to DNA binding, protein–protein interactions, cell cycle progression, apoptosis, and metabolism. In this manner, a small modifying effect by *FOXO3A* potentially has larger, additive downstream effects on aging phenotypes and longevity.

Supportive evidence is beginning to accumulate for a role of insulin-signaling in human aging and longevity, but the genes that might mediate these effects are not known. Prior studies have found over or under representation of single nucleotide polymorphisms (SNPs) from the insulin-IGF-1signaling pathway in long-lived humans of Italian (36), Japanese (37, 42), Dutch (60), and Ashkenazi Jewish (38) ethnicity, with links to several aging phenotypes. While some of these findings have been limited by small effect sizes and marginal statistical significance, the study by Suh *et al.* (38) also demonstrated that functionally significant mutations in the IGF-1 receptor exist in some long-lived humans, such as centenarians.

To date, there has been little study of FOXO genes and phenotypes of aging in humans. Two recent studies suggest that FOXO genes deserve further scrutiny. First, a longitudinal study of elderly Dutch men and women found that a *FOXO1A* haplotype predicted 4-year survival and that a *FOXO3A* haplotype predicted stroke risk (39). Second, the Framingham Study, in a genome-wide association analysis, found that a *FOXO3A* SNP was strongly associated with age at natural menopause in women ($P = 0.00003$). However, the Dutch findings were not statistically significant when accounting for multiple comparisons and both studies need replication. The present study is supportive and extends the associations of FOXO3A to human longevity and insulin sensitivity.

One of the major advantages of the current study is that it used a nested case-control design. This study design selects cases and controls from an ongoing cohort study with longitudinally collected data. Therefore, several phenotypes of interest (e.g., disease prevalence, health status, function) were obtained by direct clinical examination when the participants were younger, making the data less subject to recall bias.

Indeed, studies of exceptional survivors, such as centenarians, that have found evidence for phenotypes suggestive of slower aging (46–48) could potentially suffer from significant recall bias. That is, older participants may not recall precisely their past medical history and their past functional status. However, in the current study, major diseases were adjudicated by a morbidity and mortality committee and performance-based measures of physical and cognitive function were used to supplement self-reports, and evidence was found for such a healthy aging phenotype. This lends prospective support to previous retrospective work.

There are several other strengths to this study. First, the candidate genes selected for analysis were chosen *a priori* based on hypothesis-driven criteria. That is, studies of models organisms of aging employing various methods, particularly knockouts, have shown that the IIS pathway is important for aging and longevity. And many functions appear to be evolutionarily conserved. Second, the findings are strong, highly significant, and include several adjacent SNPs in the *FOXO3A* gene. Third, the findings are biologically plausible and support the prior findings in animal models and also support the limited prior human studies. Fourth, the case-control associations with longevity were detected using a nested case-control analysis with a high event rate (deaths) during a long period of follow-up. Fifth, the HHP cohort is highly homogenous and no population stratification was detected.

A possible drawback is that since the cases and controls had an average age difference of 11 years we cannot exclude birth cohort as a confounder. But this is unlikely since there was a maximum 19-year difference in birth years between participants. Also, subanalyses revealed no differences in education and occupation (data not shown) between cases and controls. Moreover, it was the participants who were older at baseline who were more likely to have lived to 95-plus years and thus obtain the longevity phenotype. Most cohort effects show health advantages for younger cohorts.

In summary, we found that common, natural genetic variation within the *FOXO3A* gene was strongly associated with human longevity and was also associated with several phenotypes of healthy aging. Further study of FOXO genes and aging phenotypes is warranted in other populations.

Subjects and Methods

Study Population. This nested-case control study was conducted as part of the Hawaii Lifespan Study, an embedded cohort study of healthy aging drawn from the original population of the Honolulu Heart Program (HHP) and Honolulu Asia Aging Study (HAAS). The HHP is a population-based, prospective study of cardiovascular disease among 8006 Japanese American men that began in 1965. The HHP participants were recruited from 9877 men with valid contact information who were born from 1900–1919 and living on the island of Oahu (61).

Study participants had both parents from Japan, usually the west and southern parts of Japan (61, 62). Although most participants were born in Hawaii (88%), there is a theoretical possibility of confounding of case control status with allele frequencies due to geographic origin. Therefore, for certain analyses, cases and controls were stratified by parental prefecture of origin using conditional logistic regression models. Analyses showed no evidence for population stratification in the dataset (data not shown). The HHP cohort has been described elsewhere (62). Briefly, surveillance of hospital records, obituaries, and national databases has resulted in extensive morbidity and mortality data (adjudicated by an expert committee) and near complete follow-up (61–63).

All participants for the current study were drawn from records of study participants updated to August, 2007. Archived phenotypic data and blood samples from Examination 4 of the HHP (1991–1993), which coincided with the commencement of the Honolulu Asia Aging Study (HAAS), was used as the baseline examination for this nested case-control study. The HAAS was begun as an expansion of the HHP for the study of neurodegenerative diseases, cognitive function, and other aging phenotypes in elderly persons (64). Participants included 3741 men aged 71 to 93 at Examination 4 (mean age 77.9 $\pm$ 4.7 years), approximately half the number of the original HHP (64).

For the purposes of the current nested case-control study, ''cases'' (longevity phenotype) were defined as all HHP participants who had survived to at least the upper 1% of the 1910 U.S. birth cohort specific survival (minimum 95 years of age) from the time of recruitment (65, 66). A total of 213 individuals who had survived to at least 95 years of age, as of August 2007, were studied. Of these individuals, 176 had died (mean death age 97.5; SD 2.1; range of 95–106 years) and 37 individuals were still alive (mean age 98.7, SD 2.1; range 97–106 years).

The controls consisted of 402 individuals from the HHP/HAAS cohort who died near the mean death age for the 1910 U.S. birth cohort specific survival for middle-aged men ($\approx$77 years of age). To achieve a case: control ratio of $\approx$1:2, we sampled the HHP/HAAS study population for controls who died up to the age of 81 years. The mean age at death for our control population was 78.5 years (SD 1.8, range 73–81 years). This is slightly higher that of the U.S. male population, but consistent with the high average life expectancy of Japanese-American men in Hawaii, which was 3.5 years longer than white males at last report (67). All were ethnic Japanese (61, 62).

Procedures were performed according to institutional guidelines and approved by the Institutional Review Board of Kuakini Medical Center. Written informed consent was obtained from all study participants or from family representatives if participants could not provide consent.

Genotyping. We chose 3 SNPs from each of 5 candidate genes. We chose genes that have well-described influences on aging pathways in model organisms. All genes were chosen based on hypothetical links to the IIS pathway and potential

links to energy homeostasis, glucose, and/or lipid metabolism [see Table S2]. SNPs were chosen based on their minor allele frequencies reported in the HapMap or JSNP database (snp.ims.u-tokyo.ac.jp).

Total cellular DNA was isolated using the PureGene system (Gentra Systems, Inc.) quantified using PicoGreen staining (Molecular Probes) and SNPs from candidate genes genotyped using allelic discrimination assays. TaqMan (Applied Biosystems) reagents were purchased from ABI and SNPs were chosen with a frequency $\geq \sim 0.1$ in the Japanese population (www.ncbi.nlm.nih.gov/projects/SNP). PCR was amplified under standard conditions using Taq Gold (Perkin-Elmer) and detection of PCR products with Taq Man assay, using a 6-FAM-labeled FRET probe for one allele and a VIC-labeled probe for the other allele and using minor groove binding (MGB) quenchers to enhance detection of assays. PCR products were measured with the ABI Prism 7000 Sequence Detection System.

Genotype data were managed through an integrated database system (MS Excel, Microsoft). All positive controls on each genotyping plate were also evaluated for consistency. Positive markers were tested for deviation from Hardy-Weinberg equilibrium. Call rates exceeded 98%.

Statistical Analysis. SNPs were evaluated for deviation from Hardy-Weinberg equilibrium. The Pearson χ^2 test was used to compare the cases and controls for equal genotype frequencies using the software program StatXact (68). For estimates of strength of association, odds ratios were estimated using logistic regression models from SAS (69). General linear model and analysis of covariance were further used to compare proportion of healthy study participants by FOXO3A genotype. For the analysis of aging phenotypes in case and controls, Student's t test for comparing distribution of continuous variables and χ^2 for proportional variables.

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