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(54) Benævnelse: **Fremgangsmåde til anvendelse af FOXO3A-polymorfismer og haplotyper til at forudsige og fremme sund aldring og lang levetid**

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DESCRIPTION

FIELD OF INVENTION

[0001] The invention relates to a method of using FOX03A polymorphisms and haplotypes in diagnostics to predict or in planning treatments and interventions to promote healthy aging and longevity.

CROSS-REFERENCE TO RELATED APPLICATION

[0002] This application is in part based on, and claims the benefit of, U.S. Provisional Patent Application No. 61/087,722, filed August 10, 2008.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0003] This invention was made with government support under grant 1 R01 AG027060-01 (Defining the Healthy Aging Phenotype) from the National Institute on Aging. Additional funding was provided by U.S. government support under contract N01-HC-05102 from the National Heart, Lung, and Blood Institute, contract N01 -AG-4-2149 and grants 5 U01 AG019349-05 and K08 AG22788-02 from the National Institute on Aging. The U.S. government has certain rights in the invention. Additional support came under grant 2004-0463 from the Hawaii Community Foundation.

BACKGROUND OF THE INVENTION

[0004] The FOXO3A gene belongs to the forkhead family of transcription factors which are characterized by a distinct forkhead domain. This gene likely functions as a trigger for apoptosis through expression of genes necessary for cell death. Translocation of this gene with the MLL gene is associated with secondary acute leukemia. Alternatively spliced transcript variants encoding the same protein have been observed.

[0005] The FOXO3A gene is one of the human homologs of DAF-16, a gene that has been described to extend lifespan in the model organisms *C. elegans* (Murphy CT (2006) The search for DAF-16/FOXO transcriptional targets: approaches and discoveries. Exp Gerontol 41:910-921) and *D. melanogaster.* (Giannakou ME et al. (2007) Dynamics of the action of dFOXO on adult mortality in Drosophila. Aging Cell 6:429-438).

[0006] The FOXO3A gene is located on human chromosome 6q21, from position 108,987,719 to 109112664 (NCBI ver. 36), is composed of four (4) exons that can be alternately expressed, which results in the same protein (variant #1 is described by file NM_001455.3; variant #2 is described by file NM_201559.2. The FOXO3A protein is composed of 673 amino acids and is 71,277 Da in size. The amino acid sequence of FOXO3A, as defined by the file "NP_963853" at the NCBI is identified as SEQ ID No. 1 and is the following:

MAEAPASPAPLSPLEVELDPEFEPQSRPRSCTWPLQRPELQASPAKPSGETAADSMIP
EEEDDEDDEDGGGRAGSAMAIGGGGGSGTLGSGLLLEDSARVLAPGGQDPGSGPAT
AAGGLSGGTQALLQPQQPLPPPQPGAAGGSGQPRKCSSRRNAWGNLSYADLITRAIE
SSPDKRLTLSQIYEWMVRCVPYFKDKGDSNSSAGWKNSIRHNLSLHSRFMRVQNEG
TGKSSWWIINPDGGKSGKAPRRRAVSMDNSNKYTKSRGRAAKKKAALQTAPESAD
DSPSQLSKWPGSPTSRSSDELDAWTDFRSRTNSNASTVSGRLSPIMASTELDEVQDD
DAPLSPMLYSSSASLSPSVSKPCTVELPRLTDMAGTMNLNDGLTENLMDDLLDNITL
PPSQPSPTGGLMQRSSSFPYTTKGSGLGSPTSSFNSTVFGPSSLNSLRQSPMQTIQENK
PATFSSMSHYGNQTLQDLLTSDSLSHSDVMMTQSDPLMSQASTAVSAQNSRRNVML
RNDPMMSFAAQPNQGSLVNQNLLHHQHQTQGALGGSRALSNSVSNMGLSESSSLGS
AKHQQQSPVSQSMQTLSDSLSGSSLYSTSANLPVMGHEKFPSDLDLDMFNGSLECD
MESIIRSELMDADGLDFNFDSLISTQNVVGLNVGNFTGAKQASSQSWVPG

[0007] FOXO3A interacts with YWHAB/14-3-3-beta and YWHAZ/14-3-3-zeta, UniProt: the Universal Protein Resource

(www.uniprot.org), which is required for cytosolic sequestration. Upon oxidative stress, interacts with STK4, which disrupts interaction with YWHAB/14-3-3-beta and leads to nuclear translocation. The subcellular location of FOXO3A is in the cytoplasm, and cytosol. It translocates to the nucleus upon oxidative stress and in the absence of survival factors. In the presence of survival factors such as IGF-1, FOXO3A is phosphorylated on Thr-32 and Ser-253 by AKT1/PKB. This phosphorylated form then interacts with 14-3-3 proteins and is retained in the cytoplasm. Survival factor withdrawal induces dephosphorylation and promotes translocation to the nucleus where the dephosphorylated protein induces transcription of target genes and triggers apoptosis. Although AKT1/PKB doesn't appear to phosphorylate Ser-315 directly, it may activate other kinases that trigger phosphorylation at this residue. FOXO3A is phosphorylated by STK4 on Ser-209 upon oxidative stress, which leads to dissociation from YWHAB/14-3-3-beta and nuclear translocation.

[0008] 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]

[0009] 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]

[0010] 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 are also regulators of longevity in several mammals.[17]

[0011] 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, protection 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]

[0012] 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 markedly increase lifespan.[32] The remarkable 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.

[0013] The human homologue of DAF-16 includes four FOXOs: FOXO1, FOXO3A, FOXO4 and FOXO6. Therefore, it is tempting to hypothesize that common, natural variation in the form of single nucleotide polymorphisms (SNPs) in FOXO and related genes might influence human longevity. "FOXO3 is synonymous with "FOXO3A" since FOXO3B is a pseudo-gene on chromosome 17.

[0014] This is an appealing hypothesis. 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[33] and although not yet universally accepted, supportive evidence has accumulated over the

years.[34,35] Thus, FOXO may provide a potential branch-point or bridge between insulin signaling, free radicals and human aging/longevity.

[0015] 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]

[0016] 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, in order 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 of aging.[3,4,14-17,36,42] Priority was given to genes that are involved in insulin sensitivity and glucose (energy) homeostasis.

[0017] 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.

SUMMARY OF THE INVENTION

[0018] The challenge in finding genes that have widespread effects on human aging phenotypes and longevity suggests that 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 from these signaling pathways and assessed variations in these candidate genes occurring at a frequency of approximately 10% or greater in the Japanese population. Due to limited resources, only three SNPs were chosen from each gene for analysis. SNPs were selected from regions with linkage disequilibrium (LD), when possible, in order to provide maximal coverage of each gene.

[0019] In general the invention provides compositions and methods for detecting the FOXO3A "GCC" haplotype (e.g. a FOXO3A haplotype associated with an increased longevity, defined herein as the likelihood of a human subject living an additional 15 or more years) as further defined in the claims. In preferred embodiments, the detected FOXO3A haplotypes are associated with either an increased likelihood or a decreased likelihood of living longer, however the invention necessarily encompasses materials and methods for detecting a FOXO3A haplotype associated with neither an increased nor a decreased likelihood of living longer and/or minimizing risk for age-associated diseases (e.g. a "normal" or "wt" genotype). Age-associated diseases refers to coronary heart disease (CHD), also known as coronary artery diseases, stroke, cancer, chronic obstructive pulmonary disease (COPD) or other chronic lung disease, Parkinson's disease, diabetes, obesity, dementia (and general cognitive function), frailty (ability to walk) or other age-related disease or physical and or cognitive impairment. There may also be an association with obesity in humans.

[0020] The "GCC" haplotype encompasses tens of kilobases of DNA. Other SNPs in this region demonstrate linkage disequilibrium with the three SNPs described herein. It is anticipated that additional SNPs will be identified within this GCC haplotype that also have an association with longevity and healthy aging, and may be useful of predicting age-associated diseases. The "GCC haplotype" can serve as a surrogate for other types of alteration of DNA, either within or adjacent to the FOXO3A gene, that is ultimately found to be the "functional variant" that leads to the prediction of exceptional longevity and/or healthy aging. These other alterations may be in the form of inversions, duplications, deletions, and may include other genes or transcripts that were previously unknown, for example, the gene "LOC100130966". LOC100130966 is similar to SMT3 suppressor of mif two 3 homolog 2 has been identified to lie within exon 2 of the FOXO3A gene, which is within the "GCC" haplotype. The DNA

sequence for LOC100130966 is described by the GenBank accession ID# "XM_001725519" and the predicted amino acid sequence of LOC100130966 is described by GenBank file "XP_0017255.71".

[0021] Haplotype analysis may be used to potentially predict which patients would benefit by aggressive wellness or disease prevention/treatment interventions. Haplotype analysis may be provided in a kit form. Risk calculators could use such information for purposes of assessing likelihood of disease, disability or death or determining how many years of survival or disease-free survival a person has. Such information would be important to patients, health insurance companies, long term care insurance companies and physicians or other health care providers in order to provide some guidance as to the patient's long-term needs. Pharmaceuticals could be developed that modify the action of the FOXO3A gene, modify the cellular location of the FOXO3A protein and/or its interactions with other proteins, or modify the amount or type of protein produced by the gene in order to impact health or diseases related to aging.

[0022] Homologous sequences in mice may be associated with premature ovarian failure. Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science. 2003 Jul 11;301(5630):215-8. Consequently, similar haplotype analysis can be useful in veterinary applications.

[0023] Further features of the invention will now become apparent from the following description, by way of example only, with reference to the accompanying Figures and Tables.

BRIEF DESCRIPTION OF THE FIGURES

[0024]

Figure 1 shows the results of an ARMS -PCR assay to detect the FOXO3A G/T variants using the primers and conditions described in Table 10.

Figure 2 illustrates the schematic outline of the ARMS-PCR assay to detect the FOXO3A G/T variants using the primers described in Table 10.

[0025] The following tables are part of the description:

Table 1. Baseline Characteristics of the HHP/HAAS Cohort in 1991-93 (n=3,741)

Table 2. Baseline Characteristics by Case-Control Status

Table 3. Candidate Genes for Human Longevity and the MAF in Cases and Controls

Table 4. FOXO 3A3 Genotype by Case-Control Status

Table 5. Difference in Health Status between Genotype Groups at Baseline

Table 6. Insulin Sensitivity Phenotypes According to FOXO3A Genotype

Table 7. Prevalence of Aging-related Phenotypes in Relation to FOXO3A3 Genotype

Table 8. Genotype Distribution by Maximum Attained Age

Table 9. Primers for Identification of the rs2802292 G-T Polympophism

Table 10. PCR Conditions for Identification of rs2802292 G-T Polymorphism

DETAILED DESCRIPTION OF THE INVENTION

A. HAWAII LIFESPAN STUDY

Study Population

[0026] 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 8,006 Japanese American men that began in 1965. The HHP participants were recruited from 9,877 men with valid contact information who were born between 1900 and 1919 and lived on the island of Oahu in 1965.[61]

[0027] Study participants had both parents from Japan, usually the west and southern parts of Japan (94% from the central region or further west and south); 49% of them had parental origins from the adjacent prefectures of Hiroshima and Yamaguchi.[61,62] Although the 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).

[0028] The HHP cohort recruitment, design, and procedures have been outlined in detail elsewhere.[62] Briefly, at the time of study enrollment (1965-1968), participants were aged 45 to 68 years (mean age, 54 years). From the commencement of the HHP, information on the development of incident coronary heart disease and stroke, as well as mortality from all causes, has been obtained by monitoring obituaries in local newspapers (English and Japanese) and through surveillance of hospital discharge records.[61] A follow-up survey in the 1991-1993 examination found that only 5 men could not be traced for mortality information.[63]

[0029] All participants for the current study were drawn from records of study participants updated to August, 2007. Archived phenotypic data and blood samples from Exam 4 of the HHP (1991-1993), which coincided with the commencement of the Honolulu Asia Aging Study (HAAS), was used as the baseline exam 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 3,741 men aged 71 to 93 at Exam 4 (mean age 77.9 ± 4.7 years), approximately half the number of the original HHP.[64]

[0030] 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. 176 of these individuals 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).

[0031] 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 (approximately 77 years of age). In order to achieve a case: control ratio of approximately 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 cases and controls were ethnic Japanese whose families came mainly from Central-West Japan.[61,62]

[0032] 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

[0033] We chose three SNPs from each of five 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 Figure 1. SNPs were chosen based on their minor allele frequencies reported in the HapMap or JSNP database (snp.ims.u-tokyo.ac.jp).

[0034] Total cellular DNA was isolated using the PureGene system (Gentra Systems, Inc.) quantified using PicoGreen staining (Molecular Probes, Eugene, OR) and SNPs from candidate genes genotyped using allelic discrimination assays. Taq Man® (Applied Biosystems, Inc.) reagents were purchased from ABI and SNPs were chosen with a frequency ≥ ~0.1 in the Japanese population (http://www.ncbi.nlm.nih.gov/projects/SNP/). PCR was amplified under standard conditions using Taq Gold (Perkin-Elmer, Corp) 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, using minor groove binding (MGB) quenchers to enhance detection of assays. PCR products were measured with the ABI Prism 7000 Sequence Detection System.

[0035] Genotype data were managed through an integrated database system (MS Excel, Microsoft, Inc). 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

[0036] SNPs were evaluated for deviation from Hardy-Weinberg equilibrium. The Pearson chi-square 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 (GLM) and analysis of covariance (ANCOVA) 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 Chi square for proportional variables.

RESULTS

[0037] The baseline characteristics of the HHP/HAAS study population at the 1991-1993 examination are presented in Table 1. 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.

Table 1. Baseline Characteristics of the HHP/HAAS Cohort in 1991-93 (n=3,741)

Variables at Baseline Exam (1991-93)	Mean ± SD	Min - Max
Biological Characteristics		
Age (y)	77.88 ± 4.69	71 93
Body Mass Index (BMI) kg/m2	23.43 ± 3.16	12.25 - 39.34
Waist/Hip Ratio	0.94 ± 0.06	0.73 - 1.27
Total Cholesterol (mg/dl)*	189.73 ± 33.16	81 - 382
Triglyceride level (mg/dl)*	148.96 ± 93.85	32 - 1369
HDL (mg/dl)*	50.94 ± 13.36	20 - 129
Glucose (mg/dl)*	113.03 ± 29.4	44 - 399
Insulin (mIU/L)*	16.82 ± 32.48	1.5 - 1164
General Health Status		
Self-Reported "Poor" Health (%)	32.88	--
Disease Prevalence		
CHD (%)	19.38	--
Stroke (%)	4.73	--
Cancer (%)	13.84	--
Diabetes (%)	58.11	--
Physical and Cognitive Function		
Lower Body (Difficulty Walking) (%)	20.47	--
Upper Body (Grip Strength in kg)	30.09 ± 6.88	0 55

Physical and Cognitive Function		
Cognitive Score (CASI)	82.24 ± 16.37	0-100
*Fasting values		

[0038] From this 1991-93 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 2. 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 exam. The cases also had better self rated health and lower prevalence of cardiovascular disease (CHD and stroke) and cancer. Functionally they appeared better able to walk but had lower grip strength. There was no significant difference in cognitive score.

Table 2. Baseline Characteristics by Case-Control Status

Variables at Baseline Examination (1991-93)	Average Lived Phenotype (Mean Attained Age 78.5 y)		Longevity Phenotype (Mean Attained Age 97.9 y)*		
	Mean ± SD	Min- Max	Mean ± SD	Min- Max	P†
Biological‡					
Age at Baseline Exam (y)	74.63 ± 2.05	71 - 79	85.62 ± 3.12	80 - 93	<.0001
Body Mass Index (BMI) kg/m2	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	9 - 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/L)	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 Walk	30.59	--	16.83	--	0.0002
Upper Body (Grip Strength in	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); † p value from Students t test for continuous variables and Chi Square for categorical variables; ‡ Fasting values; § Log transformation performed for variables not normally distributed; ¶ CAS1 (Cognitive Abilities Screening Instrument)[43]

[0039] 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 the Table 3. However, only FOXO3A genotype was associated with longevity using an initial cut-off value of p <0.05.

Table 3. Candidate Genes for Human Longevity and the MAF in Cases and Controls

				Minor allele freq. (MAF)		
Gene Name	**Symbol**	**SNP ID#**	**Variable Name**	**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 Chi-square test; † Adipocyte, C1Q, and Collagen Domain Containing.

[0040] Variant "rs2764264" has previously been referred to as "rs12524491". The DNA Sequence of SNP rs2764264 ("FOXO3A1") identified as SEQ ID No. 2 is:

TATTTCACTGGCCAGGACCTCCAATACATTGTTGAATAGCAGTGGTGAAAGCAGA
GATCCTTACCATTTTTCTCATCTTAAGGGGAAAGCATTCAGTCTTTCACTGTTAAG
TATCATGTTAGGTGTAAGTTTGTCACATATTTCCTTTATCAGGCTGAGGTAGTTTT
CTCTATTCCTATGTGTTGAGTAGTTTTTGTTTTTTAAATTATGAGTGGATATTGAA
TTTTGTCAGATGCTTTTTCCTCACCTGTTGAGAAGATCAGATGGTTTTTCTTTTTC
AGTCTTTTAATATGATGAAATACATTGACTGATTTGCAATGTTAAACCAACCTTA
CATTCCTGGGATAAATCCCACCTGGTCTTGATATGTTACCATGAGATTCAAGTAG
CTAAAATTTTGTTAAGGATTTTTGTGTCTGTCTTCATGAGGAATATTGATCTATAC
ATTTCTTATAATATCTTTGCCTGTTTTTGGTACCAGGGTAATGGTGGTCTTATAA
(C/T)
ATGAGTTGGA AAGTGTTCCC TGTTCTGCTC TGGTAGCACT GTAGTATCTC
TTCCTTAAATGTTTGGTAGAATTCAACGGCAGTTAAGCCATCAGAGCCTGGAGTT
TTTTTGTGTGTGAGGAAATGTTTAACTGCTAATTCAATTTATTTCATAGATACAAT
GCTGTTGGCTTGTCTGTTTCTTCTTGAATGAGTTTTGGTAGTCTGTGTCTTTTAAG
GAATTTGCCCATTTTATTTAAGTTGTCTAATTTATGGGCATAAAGTCATTTATAAT
GTTCTCTTATTATCCTTTTAATAGATATATCATCTGTAGTGATTTCATTTTCATTCC
TGATGTTGATAATTTGTCTTAACTCCCTTTCCCCCTCATTCCTTATCTGTTTAGTGC
CTTGCAATTTCATTGATCTTTTAAACGAATTAACATTTGCTTCCACTGACTTTTCC
CCCGTTACTTTTATGTTTTTACTTCCATTGATTTTTTTTTTCTCTTTTAATCTTTTA

[0041] The DNA Sequence of SNP rs13217795 ("FOXO3A2") is identified as SEQ ID No. 3 is:

CACCACCACCCACTAGACAAATTGCTTAACCTTTCTGCACCTCAGTTTCCTCCTGA
CAGGCTTGTTTAGAAAATAAAATGAGATCAAATTTGTCAAGCACAGAGCATTGG
CCCTGGTAGGCACCACATACATGAATTTCCTTCAGATTGTAGGTGAAGTAGACTT
GATTTGGGATTTCTCTTGTTACCTAGGTGCTTGTGTAGAGGAGACTTTAGAACCA
GAATGTGTTATTTGTGGTTTTGAGTGTGCCTGGGACTCTGAGCCAACTGAATTAC
CAAGTAATGGGGGCCCCATGGCATC
(C/T)
CATGACAGGTGGAGAGCCGGCTCTTCACCCTGGATGGACCTGAAATGCCTGCTA
AGGCCTTGCTCCACCGAGTAGCACACACCCTATCAGTTTGCCCTTCTTTCCATCTC
TTATTCTAGAGACCTTAAAGCCTACTTGTTGGTATATATTTTCAGGTTTTTGGAAA

TTGGGCTGTTTAATTGAAGTTAATACCAGTGATGAGACTTTTCAACCTGAGAACA
ACCTAGATGCTACTTCACATTTTGCAGTGGAAGCTTACTTCCATCTTCACTCATGT
AGGACATTCTTTGGTCTCAATGT

[0042] The DNA Sequence of SNP rs2802292 ("FOXO3A3") identified as SEQ ID No. 4 is:
TGAAGCAGGGCATCAGGGAATGGGAGTTGGTGAGGAAATTACATTAACATTTAT
TGAGCACCATTCTCACTATAAACCTGAACGTAAATATTATTATTATTATTATTATT
ATTATTATTATTATTATTATTATTATTTTAGTAGAGATGAGGCCTTGCTGTGT
TGCCTAGACTGGTCTTGAACTCCTGGGCTCAAGCAATCCTCTCACCTTGGCCTCTC
AAAGTGCCTCTCAAAGGTGTGAGCCACCATGCCCAGCCTATTCGTTTTTAATTTC
TGAAGAAACTGAGGCTAACAGCTGGGTCTGGCCCATGACTGGTTCAGTTGGTATT
TGGTGGACCAAGTTGACCAAGCTCACCCAGCTTCTGAGTGACAGAGTGAATATA
AACCCAGCCTGCTCACTCCATTTCCTAGTTTTCTCACCTCTACCAGGGTCTCTGTT
GCTCACAAGAGCTCAGGGCTGGGA
(G/T)
AAGCCTCTGTGTGACAGATGAAGGGGTCCTGCTGCTCTCTAGGGAAGAATCGGT
CCCAAATTGCTCAAGGGAGTAAGGTGGTTTCGTTGAGGAGCATCAGCTAGGGGG
ATTGATGGGAATAGGTGTCAGGCAGCCAGTGGAAATTTTGTGTGCCCACCTGTGG
CACATGTATTATGCAAATTCATGCAAAAATATATATA

[0043] The "GCC haplotype" can be described using SNPs rs2764264, rs13217795, and rs2802292 and is the allele that contains the following combination of genotypes:

rs2764264	rs13217795	rs2802292
"C"	"C"	"G"

[0044] When viewing these variants from the top of the chromosome (lower nucleotide position on the genetic map) to the bottom (higher nucleotide position on the genetic map) the "GCC haplotype" can be described using SNPs rs2802292, rs2764264, and rs13217795 according to NCBI nomenclature, and is the allele that contains the following combination of genotypes:

SNP ID #	rs2802292	rs2764264	rs13217795
SNP Variable Name from Table 3	FOXO3A3	FOXO3A1	FOXO3A2
Chromosome 6 Nucleotide position	109015211	109041154	109080791
SNP Allele	"G"	"C"	"C"

[0045] The above data are from:

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: 129, NCBI genome build 36.3). Available from: http://www.ncbi.nlm.nih.gov/SNP/[70]

[0046] Further investigation comparing the genotype frequencies of FOX03A3 between cases and controls revealed a highly

significant difference with an exact p value of 0.00009 using the permutation distribution of the Pearson's chi-square statistic. These results are presented in Table 4. There were five loci with 3 SNPs within each allele in this study (Table 3) so Bonferroni adjustment for multiple comparisons resulted in a corrected p value of 15 x 0.00009 = 0.00135. Due to the high link LD between the 3 SNPs of FOXO3A, we further investigated the FOXO3A3 SNP only (rs2802292). The odds ratio (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 4. FOXO 3A3 Genotype by Case-Control Status

	FOXO 3A3 Genotype (rs 2802292)		
Case-Control Status	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 % 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 Chi-square test comparing the genotype frequencies in the cases and controls.

[0047] In order to understand more about the longevity phenotype at younger ages, we compared the proportion of people who were healthy at the baseline exam (1991-93) for each of the three FOXO3A genotype groups using the definition of healthy survival from Willcox et al. (2006).[44] The differences were highly significant (Table 5). Those who possessed one or more G alleles were much more likely to be healthy at baseline than those who were homozygous for the major (TT) allele. Approximately 75% of those homozygous for the minor allele were healthy at the baseline exam versus only about 57% of those homozygous for the major allele. After adjusting for case-control status, the differences were still marginally significant. This suggests that there was remaining association of the allele with health status within the categories of long term survivors (cases) and controls.

Table 5. Difference in Health Status between Genotype Groups at Baseline

	% Healthy at Baseline*			p 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" defined as absence of six major chronic diseases (CHD; stroke, cancer, PD, COPD and treated Type 2 DM; high physical function (can walk ½ mile) and high cognitive function (CASI score >74).

[0048] In order 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 6). 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.

Table 6. 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±4.1	0.73
Fasting Insulin (mIU/L)				
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/L)				
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.				

[0049] We also tested for a relation between lifetime prevalence of several chronic diseases and FOXO3A genotype in study participants (Table 7).

Table 7. Prevalence of Aging-related Phenotypes in Relation to FOXO3A3 Genotype

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

[0050] A significant protective relation was found for homozygosity for the G allele with regard to prevalence of CHD and a borderline relation for cancer and cognitive function. Finally, we assessed the FOXO3A3 minor allele frequency (MAF) distribution by maximum attained age in all participants combined (cases and controls). The MAF increased markedly with age, as expected by earlier case-control analyses (Table 8).

Table 8. 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
* 37 "long-lived" cases were still alive; mean age of 98.7 y (range 97-106).		

[0051] The 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. The odds ratio (OR) for being homozygous minor vs. homozygous major for FOXO3A3 allele (rs 2802292) 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 between the cases and controls was 1.91 (95% CI: 1.34 - 2.72, p=0.0003). These results suggest an additive effect of the FOXO3A3 G allele on longevity. (i.e., two copies of the G allele conferred about twice the protective effect). Consistent with this, the minor allele frequency rose markedly with age of the study participants, from septuagenarian to centenarian ages (Table 8).

[0052] Second, all three SNPs that were assessed in the FOXO3A gene, which were in tight linkage disequilibrium (LD), were strongly correlated with the longevity phenotype. This indicates that the finding was unlikely due to chance. Third, those who possessed one or more of the minor (G) alleles were much more likely to be healthy at the baseline exam, approximately 15 years prior, than those homozygous for the major (TT) allele. About 75% of those homozygous for the minor allele were healthy at baseline exam versus only about 57% of those homozygous for the major (TT) allele (Table 5).

[0053] In fact, the baseline exam 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 that suggested higher insulin sensitivity at younger ages, with lower waist to hip ratio, lower glucose levels, lower insulin levels and lower HOMA values (Tables 2 and 6). Several phenotypes were associated with variation in FOXO3A genotype.

[0054] 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 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 include the fact that all participants were tested for diabetes by several different clinical tests and at several prior examinations making detection more likely.

[0055] Of note, 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.

[0056] 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.

[0057] While FOXO was clearly associated longevity we did not observe a strong effect of genotype on insulin sensitivity in cases-just 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 that 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.

[0058] 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.

[0059] 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-1 signaling 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.

[0060] To date, there has 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.

[0061] One of the major advantages of the current study is that it employed 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. Recall bias, where study results are less accurate due to difficulty in remembering past events, can be a significant challenge with older adults.

[0062] 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.

[0063] 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 of aging 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 a highly homogenous cohort and there was no population stratification detected in our study participants.

[0064] 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, sub analyses 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. Another possible drawback is that this study was conducted in only one population and thus should be replicated in other populations in order to assess its generalizability.

[0065] In summary, we found that common, natural genetic variation within the FOXO3A gene was strongly associated with human longevity. The prevalence of the protective allele increased markedly with age. Long-lived cases were also more likely to possess several additional phenotypes linked to healthy aging, including lower prevalence of cancer and cardiovascular disease,

better self-reported health, high functional status and they exhibited several biological markers suggestive of greater insulin sensitivity at the baseline exam. Finally, particular variants within the FOXO3A gene were also associated with several of these aging phenotypes, including insulin sensitivity, a putative intermediate phenotype of longevity.

B. DETECTION OF ALLELES IN PATIENTS (HUMAN AND NON-HUMAN)

[0066] Many methods are available for detecting specific alleles at polymorphic loci. The preferred method for detecting a specific polymorphic allele will depend, in part, upon the molecular nature of the polymorphism. For example, the various allelic forms of the polymorphic locus may differ by a single base-pair of the DNA. Such single nucleotide polymorphisms (or SNPs) are major contributors to genetic variation, comprising some 80% of all known polymorphisms, and their density in the human genome is estimated to be on average 1 per 1,000 base pairs. SNPs are most frequently biallelic --occurring in only two different forms (although up to four different forms of an SNP, corresponding to the four different nucleotide bases occurring in DNA, are theoretically possible). Nevertheless, SNPs are mutationally more stable than other polymorphisms, making them suitable for association studies in which linkage disequilibrium between markers and an unknown variant is used to map disease-causing mutations. In addition, because SNPs typically have only two alleles, they can be genotyped by a simple plus/minus assay rather than a length measurement, making them more amenable to automation.

[0067] A variety of methods are available for detecting the presence of a particular single nucleotide polymorphic allele in an individual. Advancements in this field have provided accurate, easy, and inexpensive large-scale SNP genotyping. Most recently, for example, several new techniques have been described including dynamic allele-specific hybridization (DASH), microplate array diagonal gel electrophoresis (MADGE), pyrosequencing, oligonucleotide-specific ligation, the TaqMan system as well as various DNA "chip" technologies such as the Affymetrix SNP chips. These methods require amplification of the target genetic region, typically by PCR. Still other newly developed methods, based on the generation of small signal molecules by invasive cleavage followed by mass spectrometry or immobilized padlock probes and rolling-circle amplification, might eventually eliminate the need for PCR. Several of the methods known in the art for detecting specific single nucleotide polymophisms are summarized below. The method of the present invention is understood to include all available methods.

[0068] Several methods have been developed to facilitate analysis of single nucleotide polymorphisms. In one embodiment, the single base polymorphism can be detected by using a specialized exonuclease-resistant nucleotide, as disclosed, e.g., in Mundy, C. R. (U.S. Pat. No. 4,656,127). According to the method, a primer complementary to the allelic sequence immediately 3' to the polymorphic site is permitted to hybridize to a target molecule obtained from a particular animal or human. If the polymorphic site on the target molecule contains a nucleotide that is complementary to the particular exonuclease-resistant nucleotide derivative present, then that derivative will be incorporated onto the end of the hybridized primer. Such incorporation renders the primer resistant to exonuclease, and thereby permits its detection. Since the identity of the exonuclease-resistant derivative of the sample is known, a finding that the primer has become resistant to exonucleases reveals that the nucleotide present in the polymorphic site of the target molecule was complementary to that of the nucleotide derivative used in the reaction. This method has the advantage that it does not require the determination of large amounts of extraneous sequence data.

[0069] In another embodiment of the invention, a solution-based method is used for determining the identity of the nucleotide of a polymorphic site. Cohen, D. et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087). As in the Mundy method of U.S. Pat. No. 4,656,127, a primer is employed that is complementary to allelic sequences immediately 3' to a polymorphic site. The method determines the identity of the nucleotide of that site using labeled dideoxynucleotide derivatives, which, if complementary to the nucleotide of the polymorphic site will become incorporated onto the terminus of the primer.

[0070] An alternative method, known as Genetic Bit Analysis or GBA™ is described by Goelet, P. et al. (PCT Appln. No. 92/15712). The method of Goelet, P. et al. uses mixtures of labeled terminators and a primer that is complementary to the sequence 3' to a polymorphic site. The labeled terminator that is incorporated is thus determined by, and complementary to, the nucleotide present in the polymorphic site of the target molecule being evaluated. In contrast to the method of Cohen et al. (French Patent 2,650,840; PCT Appln. No. WO91/02087) the method of Goelet, P. et al. is preferably a heterogeneous phase assay, in which the primer or the target molecule is immobilized to a solid phase.

[0071] Recently, several primer-guided nucleotide incorporation procedures for assaying polymorphic sites in DNA have been described (Komher, J. S. et al., Nucl. Acids. Res. 17:7779-7784 (1989); Sokolov, B. P., Nucl. Acids Res. 18:3671 (1990); Syvanen, A.-C., et al., Genomics 8:684-692 (1990); Kuppuswamy, M. N. et al., Proc. Natl. Acad. Sci. (U.S.A.) 88:1143-1147 (1991); Prezant, T. R. et al., Hum. Mutat. 1:159-164 (1992); Ugozzoli, L. et al., GATA 9:107-112 (1992); Nyren, P. et al., Anal. Biochem. 208:171-175 (1993). These methods differ from GBA™ in that they all rely on the incorporation of labeled

deoxynucleotides to discriminate between bases at a polymorphic site. In such a format, since the signal is proportional to the number of deoxynucleotides incorporated, polymorphisms that occur in runs of the same nucleotide can result in signals that are proportional to the length of the run (Syvanen, A. -C., et al., Amer. J. Hum. Genet. 52:46-59 (1993)).

[0072] For mutations that produce premature termination of protein translation, the protein truncation test (PTT) offers an efficient diagnostic approach (Roest, et. al., (1993) Hum. Mol Genet. 2:1719-21; van der Luijt, et. al., (1994) Genomics 20:1-4). For PTT, RNA is initially isolated from available tissue and reverse-transcribed, and the segment of interest is amplified by PCR. The products of reverse transcription PCR are then used as a template for nested PCR amplification with a primer that contains an RNA polymerase promoter and a sequence for initiating eukaryotic translation. After amplification of the region of interest, the unique motifs incorporated into the primer permit sequential in vitro transcription and translation of the PCR products. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis of translation products, the appearance of truncated polypeptides signals the presence of a mutation that causes premature termination of translation. In a variation of this technique, DNA (as opposed to RNA) is used as a PCR template when the target region of interest is derived from a single exon.

[0073] Any cell type or tissue may be utilized to obtain nucleic acid samples for use in the diagnostics described herein. In a preferred embodiment, the DNA sample is obtained from a bodily fluid, e.g, blood, obtained by known techniques (e.g. venipuncture) or saliva. Alternatively, nucleic acid tests can be performed on dry samples (e.g. hair or skin). When using RNA or protein, the cells or tissues that may be utilized must express an FOXO3A gene.

[0074] Diagnostic procedures may also be performed in situ directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents may be used as probes and/or primers for such in situ procedures (see, for example, Nuovo, G. J., 1992, PCR in situ hybridization: protocols and applications, Raven Press, N.Y.).

[0075] In addition to methods which focus primarily on the detection of one nucleic acid sequence, profiles may also be assessed in such detection schemes. Fingerprint profiles may be generated, for example, by utilizing a differential display procedure, Northern analysis and/or RT-PCR.

[0076] A preferred detection method is allele specific hybridization using probes overlapping a region of at least one allele of an FOXO3A haplotype and having about 5, 10, 20, 25, or 30 nucleotides around the mutation or polymorphic region. In a preferred embodiment of the invention, several probes capable of hybridizing specifically to other allelic variants are attached to a solid phase support, e.g., a "chip" (which can hold up to about 250,000 oligonucleotides). Oligonucleotides can be bound to a solid support by a variety of processes, including lithography. Mutation detection analysis using these chips comprising oligonucleotides, also termed "DNA probe arrays" is described e.g., in Cronin et al. (1996) Human Mutation 7:244. In one embodiment, a chip comprises all the allelic variants of at least one polymorphic region of a gene. The solid phase support is then contacted with a test nucleic acid and hybridization to the specific probes is detected. Accordingly, the identity of numerous allelic variants of one or more genes can be identified in a simple hybridization experiment.

[0077] These techniques may also comprise the step of amplifying the nucleic acid before analysis. Amplification techniques are known to those of skill in the art and include, but are not limited to cloning, polymerase chain reaction (PCR), polymerase chain reaction of specific alleles (ASA), ligase chain reaction (LCR), nested polymerase chain reaction, self sustained sequence replication (Guatelli, J. C. et al., 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. et al., 1989, Proc. Natl. Acad. Sci. USA 86:1173-1177), and Q-Beta Replicase (Lizardi, P. M. et al., 1988, Bio/Technology 6:1197).

[0078] Amplification products may be assayed in a variety of ways, including size analysis, restriction digestion followed by size analysis, detecting specific tagged oligonucleotide primers in the reaction products, allele-specific oligonucleotide (ASO) hybridization, allele specific 5' exonuclease detection, sequencing, hybridization, and the like.

[0079] PCR based detection means can include multiplex amplification of a plurality of markers simultaneously. For example, it is well known in the art to select PCR primers to generate PCR products that do not overlap in size and can be analyzed simultaneously. Alternatively, it is possible to amplify different markers with primers that are differentially labeled and thus can each be differentially detected. Of course, hybridization based detection means allow the differential detection of multiple PCR products in a sample. Other techniques are known in the art to allow multiplex analyses of a plurality of markers.

[0080] In a merely illustrative embodiment, the method includes the steps of (i) collecting a sample of cells from a patient (from saliva, cheek swab, blood or other body fluid or component), (ii) isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, (iii) contacting the nucleic acid sample with one or more primers which specifically hybridize 5' and 3' to at least one allele of an FOXO3A haplotype under conditions such that hybridization and amplification of the allele occurs, and (iv)

detecting the amplification product. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

[0081] In a preferred embodiment of the subject assay, the allele of an FOXO3A haplotype is identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis.

[0082] In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the allele. Exemplary sequencing reactions include those based on techniques developed by Maxim and Gilbert ((1977) Proc. Natl Acad Sci USA 74:560) or Sanger (Sanger et al (1977) Proc. Nat. Acad. Sci USA 74:5463). It is also contemplated that any of a variety of automated sequencing procedures may be utilized when performing the subject assays (see, for example Biotechniques (1995) 19:448), including sequencing by mass spectrometry (see, for example PCT publication WO 94/16101; Cohen et al. (1996) Adv Chromatogr 36:127-162; and Griffin et al. (1993) Appl Biochem Biotechnol 38:147-159). It will be evident to one of skill in the art that, for certain embodiments, the occurrence of only one, two or three of the nucleic acid bases need be determined in the sequencing reaction. For instance, A-track or the like, e.g., where only one nucleic acid is detected, can be carried out.

[0083] In a further embodiment, protection from cleavage agents (such as a nuclease, hydroxylamine or osmium tetroxide and with piperidine) can be used to detect mismatched bases in RNA/RNA or RNA/DNA or DNA/DNA heteroduplexes (Myers, et al. (1985) Science 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type allele with the sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to base pair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton et al (1988) Proc. Natl Acad Sci USA 85:4397 ; and Saleeba et al (1 992) Methods Enzymol. 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

[0084] In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes). For example, the mutY enzyme of E. coli cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu et al. (1994) Carcinogenesis 15:1657-1662). According to an exemplary embodiment, a probe based on an allele of an FOXO3A locus haplotype is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like See, for example, U.S. Pat. No. 5,459,039.

[0085] In other embodiments, alterations in electrophoretic mobility will be used to identify an FOXO3A locus allele. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) Proc Natl. Acad. Sci USA 86:2766 , see also Cotton (1993) Mutat Res 285:125-144; and Hayashi (1992) Genet Anal Tech Appl 9:73-79). Single-stranded DNA fragments of sample and control FOXO3A locus alleles would be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) Trends Genet 7:5).

[0086] In yet another embodiment, the movement of alleles in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al. (1985) Nature 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing agent gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) Biophys Chem 265:12753).

[0087] Examples of other techniques for detecting alleles include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known

mutation or nucleotide difference (e.g., in allelic variants) is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki et al. (1986) Nature 324:163); Saiki et al (1989) Proc. Natl Acad. Sci USA 86:6230). Such allele specific oligonucleotide hybridization techniques may be used to test one mutation or polymorphic region per reaction when oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations or polymorphic regions when the oligonucleotides are attached to the hybridizing membrane and hybridized with labelled target DNA.

[0088] Alternatively, the allele specific amplification technology, which depends on selective PCR amplification, may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation or polymorphic region of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al (1989) Nucleic Acids Res. 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) Tibtech 11:238. In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini et al (1992) Mol. Cell Probes 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) Proc. Natl. Acad. Sci USA 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

[0089] In another embodiment, identification of the allelic variant is carried out using an oligonucleotide ligation assay (OLA), as described, e.g., in U.S. Pat. No. 4,998,617 and in Landegren, U. et al. ((1988) Science 241:1077-1080). The OLA protocol uses two oligonucleotides which are designed to be capable of hybridizing to abutting sequences of a single strand of a target. One of the oligonucleotides is linked to a separation marker, e.g., biotinylated, and the other is detectably labeled. If the precise complementary sequence is found in a target molecule, the oligonucleotides will hybridize such that their termini abut, and create a ligation substrate. Ligation then permits the labeled oligonucleotide to be recovered using avidin, or another biotin ligand. Nickerson, D. A. et al. have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson, D. A. et al. (1990) Proc. Natl. Acad. Sci. USA 87:8923-27). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA.

[0090] Several techniques based on this OLA method have been developed and could be used to detect alleles of an FOXO3a locus haplotype. For example, U.S. Pat. No. 5,593,826 discloses an OLA using an oligonucleotide having 3'-amino group and a 5'-phosphorylated oligonucleotide to form a conjugate having a phosphoramidate linkage. In another variation of OLA described in Tobe et al. ((1996) Nucleic Acids Res 24: 3728), OLA combined with PCR permits typing of two alleles in a single microtiter well. By marking each of the allele-specific primers with a unique hapten, i.e. digoxigenin and fluorescein, each OLA reaction can be detected by using hapten specific antibodies that are labeled with different enzyme reporters, alkaline phosphatase or horseradish peroxidase. This system permits the detection of the two alleles using a high throughput format that leads to the production of two different colors.

[0091] Another embodiment of the invention is directed to kits for detecting a likelihood for long life or the need for wellness or diagnostic intervention in the near future. This kit may contain one or more oligonucleotides, including 5' and 3' oligonucleotides that hybridize 5' and 3' to at least one allele of a FOXO3A locus haplotype. PCR amplification oligonucleotides should hybridize between 25 and 2500 base pairs apart, preferably between about 100 and about 500 bases apart, in order to produce a PCR product of convenient size for subsequent analysis.

[0092] Particularly preferred primers include nucleotide sequences described in SEQ IDs Nos. 2 - 9. Suitable primers for the detection of a human polymorphism in these genes can be readily designed using this sequence information and standard techniques known in the art for the design and optimization of primers sequences. Optimal design of such primer sequences can be achieved, for example, by the use of commercially available primer selection programs such as Primer 2.1, Primer 3 or GeneFisher.

[0093] An example of a simple method for the detection of the "GCC haplotype" involves the use of allele-specific primers that amplify the specific nucleotide of interest, similar to that described in paragraph [0067]. This method exploits the fact that oligonucleotide primers must be perfectly annealed at their 3' ends for a DNA polymerase to extend these primers during PCR. By designing oligonucleotide primers that match only a specific DNA point difference, such as that found in the rs2802292 polymorphisms-primers that do not bind the T-type allele-such primers can distinguish between polymorphic alleles. It is necessary to set up a control reaction in the same tube as the amplification refractory mutation system reaction (ARMS) to ensure that lack of product generation from a given sample is not simply due to failure of the PCR reaction rather than absence of the "G" variant that the assay is probing for. Oligonucleotides used for this purpose included forward outer ("rs2802292_FO"), 5'-GAAACTGAGGCTAACAGCTGGGTCTGGCCC-3' identified as SEQ ID No. 5; reverse outer ("rs2802292_RO"), 5'-AGCTGATGCTCCTCAACGAAACCACCTTAC-3' identified as SEQ ID No. 6; reverse G-specific ("rs2802292 RG"), 5'-

GGACCCCT-FCATCTGTCAC-ACAGAGGCTcC-3' identified as SEQ ID No.7; and forward T-specific ("rs2802292_FT"), 5'-CTGTTGCTCACAAGAGCTCAGGGCTGGGcT-3' identified as SEQ ID No.8, where the underlined final base in the latter two primers anneals at the site of the G-T difference, whereas the 2nd bp from the 3' end (lowercase) is intentionally mismatched to maximize allelic specificity. The four primers in this illustrative example are set forth in Table No. 9.

Table 9. Primers for Identification of the rs2802292 G-T Polymporphism

Primer	Sequence
forward outer "rs2802292_FO"	5'-GAAACTGAGGCTAACAGCTGGGTCTGGCCC-3'
reverse outer "rs2802292_RO"	5'-AGCTGATGCTCCTCAACGAAACCACCTTAC-3'
reverse G-specific "rs2802292_RG"	5'-GGACCCCTTCATCTGTCACACAGAGGCTcC-3'
forward T-specific "rs2802292_FT"	5'-CTGTTGCTCACAAGAGCTCAGGGCTGGGcT-3'
(Table 9 discloses SEQ ID NOS 5-8 respectively, in order of appearance)	

[0094] The DNA Sequence of PCR Product Denoting Primers and G/T Variants (source Genbank AL391646.12) is as follows:
GAAACTGAGGCTAACAGCTGGGTCTGGCCCATGACTGGTTCAGTTGGTATTTGGT

GGACCAAGTTGACCAAGCTCACCCAGCTTCTGAGTGACAGAGTGAATATAAACC
CAGCCTGCTCACTCCATTTCCTAGTTTTCTCACCTCTACCAGGGTCT*CTGTTGCTCA*
*CAAGAGCTCAGGGCTGGGA(T/G)AAGCCTCTGTGTGACAGATGAAGGGGTCC*TGCTG
CTCTCTAGGGAAGAATCGGTCCCAAATTGCTCAAGGGAGTAAGGTGGTTTCGTTG
AGGAGCATCAGCT, identified as SEQ ID No. 9.

[0095] When amplicons generated in this way are resolved in an agarose gel, the G-type primers can be shown to have generated a 186-bp product, whereas the T-type primers give a 132-bp product. The outside primers generate a 288-bp product that must be present in every reaction in order to guarantee the reaction has proceeded accurately.

[0096] Representative reagents and conditions for the amplification are shown in Table 10.

Table 10. PCR conditions for Identification of rs2802292 G-T Polymorphism

Reagent	Final Concentration	Vendor
AmpliTaq Gold PCR Buffer	1 X	Perkin-Elmer
dNTPs	200 µM	Perkin-Elmer
$MgCl_2$	1.5 mM	Perkin-Elmer
"rs2802292 FO"	1.0 µM	see above
"rs2802292_RO"	1.0 µM	see above
"rs280229.2_RG"	0.5 µM	see above
"rs2802292_FT"	0.5 µM	see above
"AmpliTaq Gold"	0.6 U	Perkin-Elmer
Human DNA	5-10 ng/µL	
H_2O	to volume	
The PCR conditions include 15 minutes at 94° C followed by 30 cycles of 94° C for 30 seconds, 60° C for 30 seconds, 72° C for 30 seconds, then a final incubation at 72° for 7 minutes. The results shown in the example were performed on an MJ Research model "PTC200" thermocycler.		

[0097] The amplified fragments can be resolved on a 3% agarose gel as shown in Figure 1. Figure 1 gives the results of an ARMS -PCR assay to detect the FOXO3A G/T variants using the primers and conditions described above. Track 1 shows a subject homozygous for the "T" allele (132 bp); tracks 2 and 3 show subjects who are homozygous for the "G" allele (186 bp); and tracks 4 and 5 show subjects who are heterozygous for the "T" and "G" alleles (132 + 186 bp) and M is the 100 bp DNA ladder (Invitrogen, Paisley, United Kingdom).

[0098] In summary, Figure 2 shows a schematic outline of the assay. Primers "rs2802292_FO" and "rs2802292_RO" flank the polymorphic locus rs2802292 and should generate a control 288-bp band in all cases. Primers "rs2802292_FO" and "rs2802292

RG" generate a 186-bp G-specific product and primers "rs2802292_FT" and "rs2802292_RO" generate a 132-bp T-specific product.

[0099] For use in a kit, oligonucleotides may be any of a variety of natural and/or synthetic compositions such as synthetic oligonucleotides, restriction fragments, cDNAs, synthetic peptide nucleic acids (PNAs), and the like. The assay kit and method may also employ labeled oligonucleotides to allow ease of identification in the assays. Examples of labels which may be employed include radio-labels, enzymes, fluorescent compounds, streptavidin, avidin, biotin, magnetic moieties, metal binding moieties, antigen or antibody moieties, and the like.

[0100] The kit may, optionally, also include DNA sampling means. DNA sampling means are well known to one of-skill in the art and can include, but not be limited to substrates, such as filter papers, the AmpliCard™ (University of Sheffield, Sheffield, England S10 2J F; Tallow, J W, et al., J of Invest. Dermatol. 103:387-389 (1994)) and the like; DNA purification reagents such as Nucleon™ kits, lysis buffers, proteinase solutions and the like; PCR reagents, such as reaction buffers, thermostable polymerase, dNTPs, and the like; and allele detection means such as the HinfI restriction enzyme, allele specific oligonucleotides, degenerate oligonucleotide primers for nested PCR from dried blood.

C. PHARMACOGENOMICS

[0101] Knowledge of the particular alleles associated with a susceptibility to developing a particular disease or condition, alone or in conjunction with information on other genetic defects contributing to the particular disease or condition allows a customization of the prevention or treatment in accordance with the individual's genetic profile, the goal of "pharmacogenomics". Thus, comparison of an individual's FOXO3A profile to the population profile for healthy aging, permits the selection or design of drugs or other therapeutic regimens that are expected to be safe and efficacious for a particular patient or patient population (i.e., a group of patients having the same genetic alteration).

[0102] Knowledge of the particular alleles described in this invention can be used to examine differences in cell behavior in cell cultures and tissue systems and measure the response of the cells to chemicals or biological agents that are added to the cell or tissue culture systems. Differences in cell behavior and responses can be compared between the genotypes in order to identify drugs or other pharmacologic agents that may be implemented in the desire to improve health or extend lifespan or to test new compounds for toxicity or potential effects on genes or gene expression.

[0103] In addition, the ability to target populations expected to show the highest clinical benefit, based on genetic profile can enable: 1) the repositioning of already marketed drugs; 2) the rescue of drug candidates whose clinical development has been discontinued as a result of safety or efficacy limitations, which are patient subgroup-specific; and 3) an accelerated and less costly development for candidate therapeutics and more optimal drug labeling (e.g. since measuring the effect of various doses of an agent on the causative mutation is useful for optimizing effective dose).

[0104] The treatment of an individual with a particular therapeutic agent can be monitored by measuring the level of expression for a gene associated with longevity. The level of expression can be measured by determining protein (e.g. FOXO3A), mRNA and/or transcriptional level. Depending on the level detected, the therapeutic regimen can then be maintained or adjusted (increased or decreased in dose). In a preferred embodiment, the effectiveness of treating a subject with an agent comprises the steps of: (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level or amount of a protein, mRNA or genomic DNA in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject after administration of the therapeutic agent; (iv) detecting the level of expression or activity of the protein, mRNA or genomic DNA in the post-administration sample; (v) comparing the level of expression or activity of the protein, mRNA or genomic DNA in the pre-administration sample with the corresponding protein, mRNA or genomic DNA in the post-administration sample, respectively; and (vi) altering the administration of the agent to the subject accordingly.

[0105] Cells of a subject may also be obtained before and after administration of a therapeutic to detect the level of expression of genes other than a FOXO3A gene to verify that the therapeutic does not increase or decrease the expression of genes which could be deleterious. This can be done, e.g., by using the method of transcriptional profiling. Thus, mRNA from cells exposed in vivo to a therapeutic and mRNA from the same type of cells that were not exposed to the therapeutic could be reverse transcribed and hybridized to a chip containing DNA from numerous genes, to thereby compare the expression of genes in cells treated and not treated with the therapeutic.

[0106] The "GCC" haplotype can be used in risk calculators to aid in the prediction of death and age-associated diseases (heart

disease, stroke, cancer, COPD or other chronic lung disease, Parkinson disease, and diabetes and dementia) and future physical function (ability to walk, cognitive function). This information is of interest to the public, physicians, health care companies and insurance companies. Examples of known risk calculators include the system and method disclosed in Perls, U.S. Patent Application Publication No. US 2007/0118398 A1, published on May 24, 2007. Risk calculators can be provided in for example, a physician's office, as a handheld or online. An individual, health-care professional, insurance company, health care organization interested in predicting how long someone will live may enter his/her genotype into a computer and obtains a risk score for aging-related disease, number of healthy years of life left, and number of total remaining years of life.

[0107] Based on a particular score, a physician or health professional may advise the patient on healthy living or risk reduction for the above diseases and death, particularly for persons with the less protective versions of the FOX03A gene. Some exemplary options include: advice concerning food choices (e.g. red wine, soy products, and other foods that contain compounds that may affect the activity of the FOXO3A gene) or intensive risk factor modification such as weight loss or increased physical activity.

[0108] The identification of FOXO3A and in particular the GCC haplotype as predictors of healthy aging and longevity provides a probable source of useful biologics and targets for pharmaceutical screens and testing. For example, one may take the gene product or a synthetic version of the protein or other active compound produced by FOXO3A gene for anticipated health benefits in reduction of age-related diseases. Means of taking the gene product may include ingestion, injection, transdermal administration:and other methods well known in the pharmaceutical arts. Compounds can be screened to find those that affect the type, activity, or the amount of the gene product produced by FOXO3A, in particular, the GCC haplotype.

[0109] Methods of modulating FOXO3A to prevent or treat age-related diseases as well as methods for treating or preventing a disease or condition in which FOXO3A is implicated, e.g. age-related diseases or enhancing longevity in a subject, are possible. "Subject," as used herein, refers to human and non-human animals. The term "non-human animals" includes all vertebrates, e.g., mammals, such as non-human primates (particularly higher primates), farm mammals such as horses, cows, bison, buffalo, goats, pigs and sheep, chicken, ducks and geese, companion animals such as dogs, cats, rabbits, guinea pigs, rodents, and reptiles, and laboratory animals. In a preferred embodiment, the subject is human. In another embodiment, the subject is an experimental animal or transgenic animal suitable as a disease model. Methods of modulating and treatment are well known to those skilled in the art as set forth in Geesaman metal., U.S. Patent Application Publication No. US 2007/0105109 A1, published on May 10, 2007.

[0110] Many other diagnostic and therapeutic uses of the sequences or gene products of the allelic variations taught by this invention will be evident to those skilled in the art. Some examples include use in small molecule screens, antisense oligonucleotides, double stranded small interfering RNAs (siRNAs) will be evident to those skilled in the art. Several approaches to developing diagnostic and therapeutic uses concerning FOXO activity are described in Goldberg et al., U.S. Patent Application No. US 2006/0069049 A1, published on March 30, 2006, and concerning related pathways in Tissenbaum et al., U.S. Patent Application No. US 2006/0272039, published on November 30, 2006.

[0111] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The skilled worker knows, or can identify by using simply routine methods, a large number of equivalents of the specific embodiments of the invention. These equivalents are intended to be included in the patent claims below. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

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[0112]

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SEQUENCE LISTING

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<120> USE OF FOXO3A POLYMORPHISMS AND HAPLOTYPES TO PREDICT AND PROMOTE HEALTHY AGING AND LONGEVITY

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PATENTKRAV

1. Fremgangsmåde til påvisning af en prædisposition for lang levetid i en vævsprøve fra et individ, hvilken fremgangsmåde omfatter enhver valgt fra gruppen bestående af:

Bestemmelse i vævprøven fra dette individ af, hvorvidt individet besidder mindst en "G"-allel af locus/polymorfisme ved position 467 i nukleinsyresekvensen af FOXO3A-genet vist i SEQ ID NO:4;

Bestemmelse i vævprøven fra dette individ af, hvorvidt individet besidder mindst en "C"-allel af locus/polymorfisme ved position 501 i nukleinsyresekvensen af FOXO3A-genet vist i SEQ ID NO:2; og

Bestemmelse i vævprøven fra dette individ af, hvorvidt individet besidder mindst en "C"-allel af locus/polymorfisme ved position 301 i nukleinsyresekvensen af FOXO3A-genet vist i SEQ ID NO:3.

2. Fremgangsmåde ifølge krav 1, hvori lang levetid er at leve mindst yderligere 15 år og prædispositionen endvidere inkluderer en prædisposition for frihed for mindst en kronisk sygdom, der er forbundet med aldring.

3. Fremgangsmåde ifølge krav 2, hvori mindst en kronisk sygdom, der er forbundet med aldring, er valgt fra gruppen bestående af diabetes, koronararteriesygdom og cancer.

4. Fremgangsmåde til påvisning af en prædisposition for lang levetid i en vævsprøve fra et individ, hvilken fremgangsmåde omfatter bestemmelse i vævsprøven fra dette individ af, hvorvidt individet besidder en "GCC"-haplotype, idet "GCC"-haplotypen er en "G"-allel ved position 467 i nukleinsyresekvensen vist i SEQ ID NO:4, en "C"-allel ved position 501 i nukleinsyresekvensen vist i SEQ ID NO:2 og en "C"-allel ved position 301 i nukleinsyresekvensen vist i SEQ ID NO:3 i et FOXO3A-gen.

5. Kit omfattende en første nukleinsyre af tilstrækkelig længde til at hybridisere til en første målnukleinsyresekvensposition valgt fra gruppen bestående af en "G"-allel af locus/polymorfisme ved position 467 i nukleinsyresekvensen af FOXO3A-genet vist i SEQ ID NO:4, en "C"-allel af locus/polymorfisme ved position 501 i nukleinsyresekvensen af FOXO3A -genet vist i SEQ ID NO:2; og en "C"-allel af locus/polymorfisme ved position 301 i nukleinsyresekvensen af FOXO3A-genet vist i SEQ ID NO:3, hvori den første nukleinsyre hybridiserer til den første målnukleinsyresekvens ved positionen, eller komplementen af den første målnukleinsyresekvens, i en eller flere beholdere samt anvendelsesinstruktioner.

6. Fremgangsmåde til testning i en celleprøve af et individ for tilstedeværelsen af mindst en allel af en "GCC"-FOXO3A-haplotype omfattende trinnene:

(a) isolering af en nukleinsyreprøve fra celleprøven;

(b) at bringe nukleinsyreprøven i kontakt med mindst et primerpar, som specifikt hybridiserer 5' og 3' til mindst en allel af en FOXO3A-haplotype under sådanne betingelser, at der optræder hybridisering og amplifikation af allelen; og

(c) påvisning af amplifikationsproduktet ved allelpåvisningsmidler, hvori mindst en primer er valgt fra gruppen bestående af SEQ ID NOs:5-8;

hvori "GCC"-haplotypen er en "G"-allel ved position 467 i nukleinsyresekvensen vist i SEQ ID NO:4, en "C"-allel ved position 501 i nukleinsyresekvensen vist i SEQ ID NO:2 og en "C"-allel ved position 301 i nukleinsyresekvensen vist i SEQ ID NO:3.

7. Anvendelse af tilstedeværelse eller fravær af "GCC"-FOXO3A-haplotypen i en risikoberegning til hjælp til forudsigelse af død, aldersforbundne sygdomme eller fremtidig fysisk funktion, idet "GCC"-haplotypen er en "G"-allel ved position 467 i nukleinsyresekvensen vist i SEQ ID NO:4, en "C"-allel ved position 501 i nukleinsyresekvensen vist i SEQ ID NO:2 og en "C"-allel ved position 301 i nukleinsyresekvensen vist i SEQ ID NO:3.

DRAWINGS

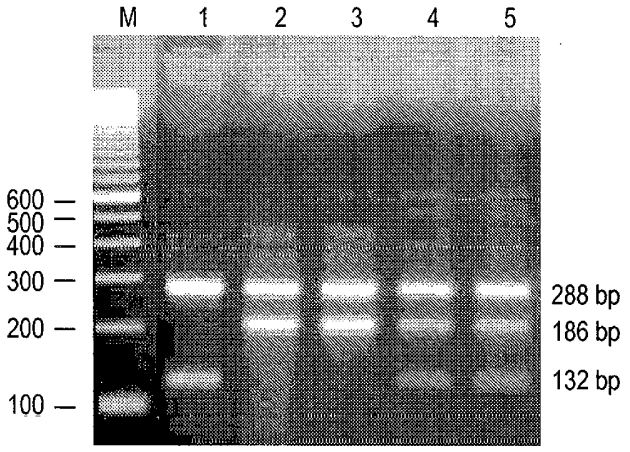

FIG. 1

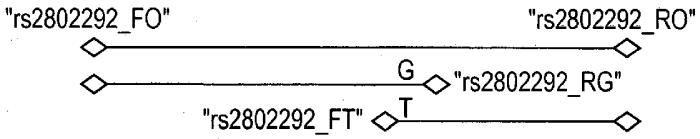

FIG. 2