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Genetic association of *FOXO1A* and *FOXO3A* with longevity trait in Han Chinese populations

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FOXO1A and *FOXO3A* are two members of the *FoxO* family. *FOXO3A* has recently been linked to human longevity in Japanese, German and Italian populations. Here we tested the genetic contribution of *FOXO1A* and *FOXO3A* to the longevity phenotype in Han Chinese population. Six tagging SNPs from *FOXO1A* and *FOXO3A* were selected and genotyped in 1817 centenarians and younger individuals. Two SNPs of *FOXO1A* were found to be associated with longevity in women ($P = 0.01–0.005$), whereas all three SNPs of *FOXO3A* were associated with longevity in both genders ($P = 0.005–0.001$). One SNP from *FOXO1A* was found not to be associated with longevity. In haplotype association tests, the OR (95% CI) for haplotypes TTG and CCG of *FOXO1A* in association with female longevity were 0.72 (0.58–0.90) and 1.38 (1.08–1.76), $P = 0.0033$ and 0.0063, respectively. The haplotypes of *FOXO3A* were associated with longevity in men [GTC: OR (95% CI) = 0.67 (0.51–0.86), $P = 0.0014$; CGT: OR (95% CI) = 1.48 (1.12–1.94), $P = 0.0035$] and in women [GTC: OR (95% CI) = 0.75 (0.60–0.94), $P = 0.0094$; CGT: OR (95% CI) = 1.47 (1.16–1.86), $P = 0.0009$]. The haplotype association tests were validated by permutation analysis. The association of *FOXO1A* with female longevity was replicated in 700 centenarians and younger individuals that were sampled geographically different from the original population. Thus, we demonstrate that, unlike *FOXO3A, FOXO1A* is more closely associated with human female longevity, suggesting that the genetic contribution to longevity trait may be affected by genders.

INTRODUCTION

Human longevity is a complex trait affected by both genetic and environmental factors (1,2). How the genetic determinants contribute to longevity has provoked great interest over the past decades. A twin-based study suggested that genetic components may account for ~25% of the variation in the longevity trait (3). Later, it was found that genetic factors have increasing effects, particularly after the age of 60 (4). Though longevity may not correlate perfectly with health

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status (5), a recent study has demonstrated that the offspring of centenarians have a lower risk of cardiovascular disease and live significantly longer than those of non-centenarians (6). This suggests that genetic contributions to longevity are, at least in part, due to protective effects against disease. Therefore, the search for longevity-associated genes should lead not only to an understanding of the fundamental mechanisms of human aging but also to the identification of new potential targets beneficial for medical care.

Despite growing evidence for genetic determinants of longevity, the identification of longevity-predisposing loci or genes remains very difficult because of strong environmental effects and the complexity of the longevity trait. Previously, genome-wide linkage analysis provided a link between the marker D4S1564 at chromosome 4 and the exceptional longevity phenotype (7,8). However, this locus has been debated in a recent study (9). Several loci or genes, except for *APOE*, were found to be associated with longevity in some studies, but not reproduced by others (10). A recent genome-wide association study in the Framingham population suggested that eight SNPs are strongly associated with the age of death. Among these eight, two SNPs, rs4943794 and rs10507486 located in the *FOXO1A* gene, present the strongest association tested in the generalized estimating equation (GEE) model (11). In addition, *FOXO3A*, the other *FoxO* member, has recently been associated with human longevity in Japanese, German and Southern Italian (12–14). FOXO1A and FOXO3A are critical downstream molecules of AKT1 and are inactivated by AKT1-mediated insulin signaling pathways that regulate the cell cycle, apoptosis, stress resistance and metabolism (15–18). It has been shown that the modification of *FoxO* in *C. elegans* (*DAF-16*) or in *D. melanogaster* (*dFOXO*) significantly affects their maximum lifespan (19–21), suggesting that *FoxOs* play an important role in the aging process. FOXO1A is recognized as a longevity factor (22). Although some controversies remain, these studies provide promising findings in the association of genetic factors with the human longevity trait (12,23,24).

In addition to genetic and environmental factors, gender also has strong effects on the human longevity phenotype. It is well-known that females have a longer life expectancy than males (25). A number of studies have documented that females account for a much larger percentage of the centenarian population (26). In the 1998 baseline survey of the Chinese Longitudinal Healthy Longevity Study (CLHLS), we found that the ratio of males to females in centenarians is 1:4 (27). On the other hand, gender effects on longevity can be modified by both genetic and environmental factors (28,29), but how genetic determinants interact with gender and play a role in longevity is not fully understood (30). Previously, it was shown that increased dFOXO, a form of *FoxOs* in *D. melanogaster*, extends female lifespan and reduces fecundity (21). In human, *FOXO3A* has been associated with expended lifespan in both genders. It remains to be determined whether *FOXO1A* is associated with human longevity.

In the present study, we examined the association of *FOXO1A* and *FOXO3A* with the longevity phenotype in Han Chinese. We demonstrated that *FOXO1A* is more closely associated with female than with male longevity, whereas *FOXO3A* is associated with longevity in both genders. Thus, our study provides a new insight into the genetic mechanism of human longevity.

RESULTS

Characterization of population

Two pairs of cases (centenarians) and controls (younger individuals) were collected among the Han Chinese. One pair (Population 1) was from southern China whereas the other (Population 2) was from northern China. The mean ages of centenarians in Populations 1 and 2 were 102.3 ± 0.14 and 100.8 ± 0.19 years (mean $\pm$ SEM). The male-to-female ratio was about 1:3 in centenarians and 1.8:1 in controls in Population 1. But in Population 2, only females were collected. Body mass index (BMI) and systolic and diastolic blood pressures of both populations are presented in Table 1. The differences between case and control groups for the listed parameters were evaluated by Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

Single SNP association analysis

Six SNPs (rs17630266, rs2755209 and rs2755213 for *FOXO1A*; rs2253310, rs2802292 and rs4946936 for *FOXO3A*) were genotyped by direct sequencing in all selected individuals. No significant deviation from the Hardy–Weinberg equilibrium test was found for all six SNPs in both populations. In sex-combined centenarians and controls, except for SNP rs17630266, the minor allele frequencies (MAFs) of both rs2755209 and rs2755213 of *FOXO1A* were significantly lower in the centenarian group than in control.

Table 1. Characteristics of populations

Items	Population 1 Case ($n = 761$)	Control ($n = 1056$)	*P*	Population 2 Case ($n = 350$)	Control ($n = 350$)	*P*
Gender (m/f)	183/578	682/374		0/350	0/350	
Age (year)	102.3 ± 0.14	47.1 ± 0.22	<0.001	100.8 ± 0.19	41.4 ± 0.48	<0.001
BMI (kg/m^2)	19.9 ± 0.2	24.9 ± 0.1	<0.001	18.5 ± 0.3	21.3 ± 0.3	<0.001
SBP (mmHg)	146 ± 0.9	129 ± 0.5	<0.001	149 ± 1.4	115 ± 0.8	<0.001
DBP (mmHg)	82 ± 0.5	84 ± 0.3	0.010	83 ± 0.7	73 ± 0.5	<0.001

The data are presented as mean $\pm$ SEM (standard error of the mean). *P*-values are calculated from *t*-test comparing case and control groups within population. Case, centenarian; Control: individuals from general population; m/f, male/female; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 2. Allelic association of *FOXO1A* and *FOXO3A* with longevity (sex-combined in Population 1)

SNP	Allele (major/minor)	Function	MAF (case/control)	P_{HWT}	OR (95% CI)	*P*	b*P*	PW ($\alpha = 0.05$)
rs17630266	G/T	Intronic	0.392/0.372	0.50	1.09(0.95–1.25)	0.221	0.221	NC
rs2755209	C/T	Intronic	0.242/0.286	0.86	0.80(0.69–0.93)	4.6×10^{-3}	9.0×10^{-3}	0.83
rs2755213	C/T	Intronic	0.352/0.420	0.53	0.75(0.65–0.86)	7.4×10^{-5}	3.0×10^{-4}	0.98
rs2253310	G/C	Intronic	0.303/0.244	0.70	1.35(1.16–1.57)	7.9×10^{-5}	2.3×10^{-4}	0.97
rs2802292	T/G	Intronic	0.302/0.240	0.74	1.36(1.18–1.60)	2.9×10^{-5}	1.5×10^{-4}	0.98
rs4946936	C/T	3′-UTR	0.283/0.221	0.40	1.40(1.19–1.64)	1.8×10^{-5}	1.1×10^{-4}	0.99

MAF, minor allele frequency; P_{HWT}, *P*-value from Hardy–Weinberg equilibrium test; OR (95% CI), odds ratio with 95% confidence interval; *P*, *P*-value calculated from χ^2 test; b*P*, corrected *P* by Bonferroni step-down method; PW, power to reach an alpha level of 0.05 with a given population size; NC, not calculated; 3′-UTR, 3-untranslated region.

Table 3. Gender effects on allelic association of *FOXO1A* and *FOXO3A* with longevity trait in Population 1

SNP	Male				Female			
	MAF (case/control)	OR (95% CI)	*P*	b*P*	MAF (case/control)	OR (95% CI)	*P*	b*P*
rs17630266	0.357/0.360	0.99(0.77–1.27)	0.914	0.91	0.402/0.392	1.04(0.86–1.27)	0.680	0.680
rs2755209	0.245/0.281	0.83(0.63–1.09)	0.166	0.33	0.243/0.294	0.77(0.62–0.95)	0.014	0.028
rs2755213	0.371/0.423	0.80(0.63–1.03)	0.073	0.22	0.348/0.413	0.76(0.62–0.92)	0.005	0.020
rs2253310	0.327/0.248	1.47(1.13–1.90)	0.003	0.014	0.296/0.235	1.37(1.10–1.71)	0.004	0.022
rs2802292	0.328/0.242	1.53(1.17–1.98)	0.001	0.006	0.295/0.236	1.36(1.09–1.69)	0.005	0.016
rs4946936	0.298/0.225	1.46(1.11–1.91)	0.004	0.017	0.280/0.212	1.44(1.15–1.82)	0.001	0.008

MAF, minor allele frequency; OR (95% CI), odds ratio with 95% confidence interval; *P*, *P*-value from χ^2 test; b*P*, corrected *P* by Bonferroni step-down method.

The adjusted *P* for multiple comparison by the Bonferroni step-down procedure (b*P*) was from 9.0×10^{-3} to 3×10^{-4}. Conversely, the MAFs of all SNPs of *FOXO3A* were much greater in the centenarian group than in control ($bP = 2.3 \times 10^{-4}$–1.1×10^{-4}). For the given sample size, the statistical power was 0.83–0.99 in the setting of an α level of 0.05 (Table 2).

Because the sex ratios were significantly different between the centenarian and control, we compared the frequencies of each SNP between case and control within genders. In males, none of the three SNPs from *FOXO1A* showed a statistic difference in MAF distributions between the case and control groups; whereas the three SNPs from *FOXO3A* presented much higher MAFs in the centenarians than in the younger population ($bP = 0.017$–0.006) with a power of 0.79–0.89 (Table 3). In comparison of the female groups, two SNPs of *FOXO1A*, rs2755209 and rs2755213, had reduced MAFs in the centenarian group versus the younger group ($bP = 0.028$–0.02). The MAFs of all SNPs from *FOXO3A* were higher in centenarians than in the younger population in both genders ($bP = 0.022$–0.008, Table 3).

Genotype association analysis

To test the inheritance models through which *FOXO1A* and *FOXO3A* are associated with longevity, we performed a genotype association test with dominant, recessive and additive models using logistic regression with adjustment for BMI and SBP. For *FOXO1A*, two of its SNPs, rs2755209 and rs2755213, were associated with female longevity in a dominant and additive model ($P = 0.01$–0.005; Supplementary Material, Table S1). For *FOXO3A*, all three SNPs presented strong associations with longevity in both the dominant and additive models for males ($P = 0.005$–0.001) and females ($P = 0.005$–0.0002; Supplementary Material, Table S1).

Linkage disequilibrium and haplotype association analysis

Linkage disequilibrium (LD) and haplotype blocks ($D' \geq 0.90$) were defined and visualized by the solid spine of LD method (Supplementary Material, Fig. S1). The haplotypes with a frequency of $\geq$5% were subjected to an association test with longevity. We tested the distributions of seven selected haplotypes, five from *FOXO1A* and two from *FOXO3A*, in female centenarians and controls. The haplotypes TTG and CCG of *FOXO1A* as well as GTC and CGT of *FOXO3A* presented significant differences in their distributions between case and control with statistical power >0.96. For *FOXO1A*, the frequency of haplotype TTG was significantly lower in centenarians than in controls, while CCG showed the opposite. A permutation test was used to correct multiple comparisons. The permutation *P*-values were 0.024 and 0.046, respectively (Supplementary Material, Table S2). For *FOXO3A*, GTC lost its statistical difference in permutation analysis. CGT appeared more frequently in centenarians than in controls (permutation *P*-values = 0.009; Supplementary Material, Table S2).

We then tested the association of haplotypes from *FOXO3A* with male longevity, and found that, opposite to CGT, GTC had a reduced frequency in centenarians compared with controls. Statistical powers for significant haplotype association in the χ^2-test were >0.99. The statistical differences remained in the permutation test (Supplementary Material, Table S3).

Table 4. Validation of allelic association of *FOXO1A* with female longevity in Chinese populations

SNP	Population 1 MAF (case/control)	P_{HWT}	OR (95% CI)	*P*	a*P*	Population 2 MAF (case/control)	P_{HWT}	OR (95% CI)	*P*	a*P*
rs17630266	0.402/0.392	0.81	1.04(0.86–1.27)	0.680	0.838	0.407/0.376	0.20	1.14(0.91–1.42)	0.240	0.203
rs2755209	0.243/0.294	0.32	0.77(0.62–0.95)	0.014	0.011	0.220/0.268	0.80	0.77(0.60–0.99)	0.039	0.025
rs2755213	0.348/0.413	0.84	0.76(0.62–0.92)	0.005	0.003	0.324/0.389	0.97	0.75(0.60–0.94)	0.011	0.009

MAF, minor allele frequency; P_{HWT}, *P*-value from Hardy–Weinberg equilibrium test; OR (95% CI), odds ratio with 95% confidence interval; *P*, *P*-value from χ^2 test; a*P*, adjusted *P* for BMI.

Table 5. Ethnic effects on association of *FOXO3A* with longevity trait

SNPs	Han Chinese (this study) MAF (case/control)	*P*	SNPs	Japanese (12) r^2	MAF (case/control)	*P*	SNPs	German (13) r^2	MAF (case/control)	*P*
rs2253310	0.303/0.244	7.9×10^{-5}	rs2802292	1	0.371/0.255	<0.0001	rs768023	1	0.44/0.382	0.007
			rs2764264	0.87	0.347/0.248	0.0002	rs2802288	1	0.445/0.385	0.006
			rs13217795	0.93	0.340/0.248	0.0006	rs2802290	1	0.444/0.387	0.01
							rs9400239	0.93	0.366/0.295	0.0007
rs2802292	0.302/0.240	2.9×10^{-5}	rs2764264	0.87	0.347/0.248	0.0002	rs768023	1	0.440/0.382	0.007
			rs13217795	0.93	0.340/0.248	0.0006	rs2802288	1	0.445/0.385	0.006
							rs2802290	1	0.444/0.387	0.01
							rs9400239	0.93	0.366/0.295	0.0007
rs4946936	0.283/0.221	1.8×10^{-5}	rs2764264	0.8	0.347/0.248	0.0002	rs9400239	0.87	0.366/0.295	0.0007
			rs13217795	0.86	0.340/0.248	0.0006	rs1268170	0.81	0.397/0.347	0.02

MAF, minor allele frequency; *P*, *P*-value from allelic association study; r^2, pairwise linkage disequilibrium value in CHB from HapMap II between SNPs used in this and others (Japanese and German groups).

Replication study

Three hundred fifty female centenarians and 350 geographically matched younger female individuals were referred to as 'Population 2' (Table 1) and used for replicating the finding that *FOXO1A* is associated with female longevity. We found that the MAFs of SNPs rs2755209 and rs2755213 from *FOXO1A* were significantly reduced in the centenarian group compared with control (adjusted $P = 0.025$ and 0.009, respectively) while that of SNP rs17630266 was not distinct between the centenarian and control groups (Table 4). These results suggest that *FOXO1A* is associated with female longevity in Population 2.

Effects of ages and ethnicities on the MAF distributions of longevity-associated SNPs and haplotypes

The ages when blood samples were collected were classified into three groups in both male and female. Decreased MAFs of SNPs rs2755209 and rs2755213 with age were found for *FOXO1A* in females (Supplementary Material, Table S4). On the other hand, the MAFs of all three SNPs from *FOXO3A* were enriched with increased age in both genders (Supplementary Material, Table S4). This enrichment was also reported for the SNPs from *FOXO3A* in Japanese and German longevity studies; which are in the same haplotypes and associated with the longevity trait (Table 5).

DISCUSSION

In this study, we demonstrated that *FOXO1A* is strongly associated with human female longevity and validated a previous finding that *FOXO3A* is associated with the longevity phenotype in the Han Chinese population. The association of *FOXO1A* with male longevity is not statistically significant in our study. It may be possible that the small male centenarian population in this study does not allow to unveil a weak association between *FOXO1A* genotypes and male longevity. But it appears true that a female longevity trait is more susceptible to the genetic variations of *FOXO1A*.

That women live longer than men has been recognized. However, studies on identifying the genetic causes for this phenomenon are very limited and preliminary (30). Previously, Barbieri *et al*. (31) found that an exonic SNP replacing the amino acid Pro with Ala in peroxisome proliferators-activated receptor *(PPAR) γ-2* has different distributions between long-lived males and controls ($P = 0.035$), but not between female groups. Recently, it was found that an SNP (A/G)-308 in tumor necrosis factor has sex-dependent distributions, and allele A is specifically associated with male life expectancy ($P = 0.019$) (32). In an animal model, it has been shown that the increased expression of *dFOXO* is associated only with female lifespan (21). Since *D. melanogaster* has only one form of *FoxOs*, it is impossible to know which form of *FoxOs* in mammals could be responsible for female longevity. Our study identified that *FOXO1A* is strongly associated with human female longevity.

Over the past few years, whether *FOXO1A* is associated with human longevity has been debated (12,23,24). This is likely due, at least in part, to difficulties in collecting sufficient numbers of long-lived and well-controlled individuals from both genders who can be used for a genetic association study. To understand the possible reasons for the debate in the literature, we compared our study with others and found that, in addition to ethnicity, the sample sizes varied significantly. More importantly, we noted that none of the previous studies mentioned sexual dimorphism in the association of *FoxOs* with human longevity. The total numbers of centenarians were 761 in our original study and 350 in the replication, but only 122, 218 and 213 in the other three studies (12,23,24). It is possible that a small sample size, or improper sex combination, produces insufficient statistical power, therefore reducing the probability of identifying *FOXO1A* associated with human female longevity. Here, we took full advantage of the large centenarian populations to determine the association of *FOXO1A* and *FOXO3A* with longevity within genders. We validated the association of *FOXO3A* with human longevity previously found in Japanese, German and Italian population-based studies (12–14) and replicated the finding that *FOXO1A* is not associated with male longevity (12). More importantly, we demonstrate that *FOXO1A* is associated with female longevity, providing a new insight into how a genetic factor contributes to human longevity. The association of both *FOXO1A* and *FOXO3A* with the human longevity trait appears in additive and dominant models in Han Chinese. Interestingly, Han Chinese, Japanese, German and Italian share the same longevity-associated haplotypes of *FOXO3A*. This excludes the possible influences of population stratification on association studies.

FOXO1A and FOXO3A, members of the forkhead transcription factors of the FoxO family, serve as the direct downstream signaling molecules of AKT1 in insulin/insulin-like growth factor signaling pathways. *In vivo*, FOXO1A and FOXO3A regulate the cell cycle and growth, apoptosis, DNA damage responses and angiogenesis (33–36). Malfunctions of FOXO1A or FOXO3A are involved in various cancers, insulin resistance, altered immune responses and organ damage (15,22,37–40). In the cardiovascular system, for example, FOXO1A and/or FOXO3A are/is important for the onset of diabetic cardiomyopathy (41,42), cardiac hypertrophy (43,44) and ischemic heart disease (45,46). It is likely that *FOXO1A* and *FOXO3A* affect longevity through multiple pathways, such as insulin resistance, stress responses or proneness to disease. Earlier studies have provided several lines of evidence that both *FOXO1A* and *FOXO3A* are associated with HbA1c level and fasting plasma insulin (12), (47,48), suggesting that their contributions to human longevity may be due to balancing insulin sensitivity and insulin resistance through insulin/insulin-like growth factor signaling pathways. The question remains why *FOXO1A* is more closely associated with human female longevity.

It has been reported that insulin sensitivity is highly sex-differentiated at different developmental stages or under different stresses in both humans and animal models (49,50). In female rats, for example, a high-fat or high-sugar diet does not induce insulin-resistance as seen in males, indicating that females have a gender-dependent protective effect (50–53). But when suffering from diseases such as diabetes, the aged female shows more increased insulin resistance and susceptibility to ischemic injury in the heart than the male (54). These suggest that gender-related human longevity may be associated with sexual dimorphism in insulin resistance. Although FOXO1A and FOXO3A are both direct downstream molecules of AKT1 in insulin/insulin-like growth factor signaling pathways, their functions are not identical. For instance, mice without *FOXO1A* are embryonic lethal, but they are viable without *FOXO3A*, suggesting that *FOXO1A* is indispensable (35,55) and acts as a main factor mediating the insulin signaling pathway (56). Over-expression of *dFOXO* in *D. melanogaster*, an effect mimicking an impaired insulin signaling pathway, only protects females from paraquat, increasing their lifespan (21). These imply that FOXO1A plays a role in female longevity by regulating sex-dependent insulin sensitivity.

In addition, *FOXO1A* is highly expressed in the female reproductive system, including ovaries and uterus, whereas *FOXO3A* is more ubiquitously expressed *in vivo*. Several studies suggest that FOXO1A plays a major role in the regulation of female decidualization (57–59). In women, delayed menopause is associated with age of death. For instance, death is often postponed for a few more decades if menopause occurs after the age of 50 (60). It is also known in *D. melanogaster* that over-expression of dFOXO reduces female fecundity and extends lifespan (21). Thus, it will be of great interest to know whether FOXO1A affects female longevity by regulating reproduction. But whether this is sufficient to explain why *FOXO1A* shows association with human female longevity needs to be determined.

In summary, we demonstrate in this study that *FOXO1A* is associated with female longevity while *FOXO3A* is associated with longevity in both genders. The association of both *FOXO1A* and *FOXO3A* with the human longevity trait is inherited in additive or dominant fashions. Han Chinese shares the same longevity-associated haplotypes of *FOXO3A* with Japanese and German. Although the finding that *FOXO1A* is not associated with male longevity has been replicated in Han Chinese and Japanese populations, the numbers of male centenarians are relatively small in both studies. Therefore, it needs to be cautious. The association of *FOXO1A* and *FOXO3A* with human longevity needs to be validated in more ethnic groups and in larger populations.

MATERIALS AND METHODS

Study subjects

We performed the baseline survey of the CLHLS reported previously (61). In the Survey, we interviewed 9093 oldest-olds aged 80–116 with a questionnaire containing 404 questions and physical tests throughout 85% of the regions of China. Among these 9093, 8441 were Han Chinese. In this study, a total of 761 centenarians (long-lived group) and 1056 unrelated younger individuals (control group) from southern China were selected and used as Population 1 for initial screening of all six SNPs. We also took 350 female centenarians and 350 younger individuals from northern China for the necessary replication of associations found in the initial

screening. All participants were Han Chinese. Finger-prick blood samples from centenarians were spotted onto S&S no. 903 filter paper (Schleicher & Schuell, Germany) and stored at 4°C after the spots were completely dried. Two to three milliliters of blood were obtained from each younger individual. Written informed consent was obtained from all participants or their representative family members in cases of centenarians who were incapable of signing. BMI was calculated based on the formula (body weight/height^2). The basic characteristics of the studied populations are presented in Table 1. The study protocol was approved by the Institutional Review Board, Institute of Molecular Medicine at Peking University. The study conformed to the principles outlined in the Declaration of Helsinki.

Genotyping of SNPs

Human genomic DNA was isolated from a 5-mm diameter punch-out from each blood spot or EDTA-anticoagulated blood using the proteinase K methods described previously (62). Based on the HapMap (CHB+JPT), the tagging SNPs rs17630266, rs2755209 and rs2755213 were selected for *FOXO1A*, and rs2253310, rs2802292 and rs4946936 for *FOXO3A*. DNA fragments of 200–350 bp containing SNPs were amplified by PCR from 10 ng of genomic DNA from each participant, with the primers listed below. The amplified DNA fragments were purified and used for genotyping by direct-sequencing with a BigDye v1.1 kit and running on ABI 3130XL. Based on the GenBank numbers NT_024524 for *FOXO1A* and NT_025741 for *FOXO3A*, the pairs of PCR primers for amplification of rs17630266, rs2755209 and rs2755213 for *FOXO1A* as well as rs2253310, rs2802292 and rs4946936 for *FOXO3A* were 5′-GGTGATGGCAGTGACTGTCTC-3′/5′-GTGGGTACAG CAGACAAGGCT-3′; 5′-GATCAGCTGGCATTCCCAG -3′/5′-CAGTGCCACTGTGTCTCTG-3′; 5′-TGTATATTC AAGGTATGTTCC-3′/5′-CTTAGTAAACAGACTATGTAT CC-3′; 5′-GAGCTTGCTTTGGAGATGCA-3′/5′-CCCAGT CACTCACATAGTCCT-3′; 5′-CTGAGGCTAACAGCT GGGTCT-3′/5′-CACTGGCTGCCTGACACCTAT-3′; and 5′-GGGTCCTGAGAACTTCTGAGT-3′/5′-GACATTCTGT AAGACATTCTGCCT-3′, respectively.

Statistical methods

Allele and genotype frequencies for single SNPs were calculated and tested for departure from Hardy–Weinberg equilibrium using the χ^2 test. Differences in allele and genotype distribution between cases (centenarians) and controls (younger population) were analyzed using logistic regression adjusted for non-genetic covariates under various genetic models that were defined as 1 (aa + Aa) versus 0 (AA) for dominant, 1 (aa) versus 0 (AA + Aa) for recessive, and 0 (AA) versus 1 (Aa) versus 2 (aa) for additive (A: major allele; a: minor allele). A Bonferroni step-down method was used for multiple comparison correction in allele association tests (63).

Linkage disequilibrium and haplotype blocks ($D' \geq 0.90$) were defined and visualized by the solid spine of LD method using Haploview 4.0 (http://www.broad.mit.edu/haploview/haploview). The haplotypes with frequency ≥5% were subjected to an association test with longevity. The permutation *P*-value was obtained by simulating 100 000 times in haplotype association analysis (64).

The two-tailed *P*-values, odds ratios and 95% confidence intervals are presented for all association tests. Statistical power was calculated with the *sampsi* command in STATA (StataCorp LP) under a given sample size and significance level ($\alpha = 0.05$).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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