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Title:
rs2802292 polymorphism in the FOXO3A gene and exceptional longevity in two ethnically distinct cohorts

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Highlights

- The evidence regarding an association of FOXO3A rs2802292 with extreme longevity is conflicting.
- We found no significant association in two ethnically diverse populations.
- The results were unchanged in sex-based analyses.
- Further resequencing efforts of the FOXO3A gene are needed.

ABSTRACT

Objectives: Previous studies have indicated that the rs2802292 polymorphism in the human forkhead box O3A (FOXO3A) gene might be associated with exceptional longevity (EL, i.e., living 100+ years), although the results are conflicting. Study design and main outcome measures: Using a case-control design, we investigated the distribution of the rs2802292 polymorphism in two ethnically distinct cohorts of centenarians (cases) and younger adults (controls). The first cohort included Japanese individuals (733 centenarians and 820 controls) and the second was from Northern Italy (79 disease-free centenarians and 316 controls). Results: No statistically significant association was found between the rs2802292 polymorphism and EL in either cohort (either examined in their entirety or in a sex-based analysis). Conclusions: In light of our negative findings, further research and resequencing efforts are needed to shed more light on the potential association between EL and FOXO3A polymorphisms.

ABBREVIATIONS: EL, exceptional longevity; GWAS, genome-wide association studies; HWE, Hardy-Weinberg equilibrium; IGF-1, insulin-like growth factor-1; IIS, insulin and IGF-1 signaling; NGS, next-generation sequencing; SNP, single nucleotide
polymorphism; SSC-J, semi-supercentenarians study in Japan; TCS, Tokyo centenarians study.

**KEYWORDS:** FOXO3A, polymorphism, longevity, aging, genetics, centenarian

1. **INTRODUCTION**

Human life expectancy is a complex function that is affected by a number of environmental and genetic factors. Age at death during adulthood has a heritability of ~25% [1]. The genetic contribution to lifespan may, however, play a more important role in populations with a high number of long-lived individuals, among which Japan is a paradigm [2]. The heritability of longevity also increases at advanced age, with estimations of 0.33 in women and 0.48 in men who reach exceptional longevity (EL, i.e., living 100+ years) [3]. Advances in genome-wide association studies (GWAS) have improved our understanding of the pathophysiological basis of complex diseases in general. By contrast, GWAS of EL have generally yielded poorer results, with single nucleotide polymorphisms (SNPs) in the apolipoprotein E gene being the only variants that achieved genome-wide significance [1]. Nevertheless, candidate genetic association studies have shown that other genetic variants—including those in the gene encoding the protein forkhead box O3A (FOXO3A) [1]—can potentially be associated with EL. In addition, we have recently reviewed the genetics of exceptional longevity based on the twenty most widely investigated SNPs on centenarians [4].

Several mechanisms may link genetic variations in FOXO3A or other related genes with EL. Dysregulation of the nutrient-sensing somatotrophic axis (comprising growth hormone and its secondary mediator, insulin-like growth factor-1 [IGF-1]) is a major hallmark of human aging [5]. The intracellular IGF-1 signaling pathway is
elicited by insulin, which informs cells of the presence of glucose. Consequently, IGF-1 and insulin signaling collectively represent the “insulin and IGF-1 signaling” (IIS) pathway. Among the multiple targets of the IIS pathway are the FOXO family of transcription factors, which are also involved in aging and show a striking evolutionary conservation [6-8].

Growing evidence indicates that genetic polymorphisms in FOXO3A can be associated with EL, especially the rs2802292 SNP in men [9]. Moreover, Willcox et al. [10] reported a strong association between the SNP rs2802292 and EL in a case-control study of American men of Japanese ancestry, including 213 “longeves cases” (aged 95+ years) and 420 controls (who died at a mean age of ~79 years). A subsequent case-control study replicated this association in a cohort from Southern Italy consisting of 281 male centenarians and 195 sex-matched controls [11]. Additional case-control studies on the association between the rs2802292 FOXO3A variant and EL yielded statistically significant results in both sexes for Chinese people of Han origin [12], in Chinese subjects living in the southern part of the country (Red River Basin) [13], as well as in Danish men [14]. By contrast, no association was observed in German individuals [15]. A recent meta-analysis of five independent studies including a total of 2,521 cases (aged 90+ years) and 2,537 middle-aged controls of different ethnic/geographic origins indicated a significant association of the rs2802292 polymorphism with EL (odds ratio [OR]=1.36, 95% confidence interval (CI)=1.10, 1.69; $P=0.005$). However, sex-based analyses revealed that the association remained significant in men only [16]. These results were essentially corroborated by a more recent meta-analysis, although the association was stronger in Asians than in Europeans [13].
In light of such discrepancies, we designed the present study to replicate the association of rs2802292 with EL (in both sexes, in combination or separately) in a large cohort of Japanese centenarians. We also included a second replication cohort of different geographic and ethnic origin (Northern Italy) consisting of disease-free centenarians, i.e., ‘dodgers’ [17-19].

2. METHODS

2.1. Study population

2.1.1. Japanese cohort

A total of 1,553 individuals were studied, divided into 733 cases (centenarians aged 100–115 years; 615 women, 118 men) and 820 controls (aged 23–88 years; 602 women, 218 men). Cases were gathered from two cohorts that have been described in detail elsewhere [20], i.e., the Tokyo Centenarians Study (TCS) and the Semi-Supercentenarians Study in Japan (SSC-J). The controls were recruited from the Nutrition and EXercise Intervention Study (NEXIS) registered on ClinicalTrials.gov (Identifier: NCT 00926744). All controls were healthy Japanese individuals living in Tokyo and the surrounding areas. All subjects gave written informed consent before their inclusion in the study. The study protocol was approved by the ethics committee of the Keio University and National Institute of Health and Nutrition and was conducted according to the Declaration of Helsinki.

2.1.2. Italian cohort

The Italian cohort has been previously described in detail [21]. In brief, cases were 79 centenarians aged 100–104 years (40 women, 39 men) in good physical health (i.e.,
“dodgers”). A total of 316 young healthy subjects (156 women, 160 men) aged 29–50 years served as controls. For all participants, age was formally verified by checking official documents (personal identity cards). All subjects in the Italian cohort were Caucasian whites and originated from Northern Italy (mainly from the Lombardy and Piedmont regions). Centenarians were identified in the community through general practitioners and none of them presented common age-related disorders, including cancer, renal failure, severe cognitive decline, frailty, and cardiovascular disorders. However, reduced auditory and visual acuity was observed in some centenarians. Control individuals were required to meet the following criteria: 1) being a Caucasian individual originating from Northern Italy; 2) being apparently healthy; and 3) showing willingness to undergo venous blood sampling. The study protocol was approved by the local ethics committee and all participants provided their written informed consent.

2.2. Genotyping

2.2.1. Japanese cohort

Total DNA was isolated from venous blood with the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany). The rs2802292 SNP was genotyped on a Real Time Thermocycler with the end-point analysis mode (LightCycler 480, Roche Applied Science, Mannheim, Germany) using TaqMan® SNP Genotyping Assay (Assay ID: C___1841568_10). Allelic discrimination analysis was performed with LightCycler 480 SW software version 1.5.1.62 (Roche Applied Science).

2.2.2. Italian cohort

Genomic DNA was extracted from venous blood samples using the QiaAmp DNA Mini Kit (Qiagen). Genotyping was performed using a TaqMan® rs2802292 genotyping assay (Applied Biosystems, Foster City, CA). All analyses were performed by personnel
blinded to the case/control status of the sample being examined. In addition, a random 20% of the samples underwent repeated genotyping for quality control. No discrepant results were observed in repeated samples.

2.3. Statistical analysis

All calculations were performed with the SPSS v.22 statistical software (IBM, Somers, NY, USA). Hardy–Weinberg equilibrium (HWE) was tested in each cohort with the $\chi^2$ test. The same test was used to compare allele and genotype frequencies in cases and controls (with box sexes studied both in combination and separately). To minimize the risk of type I error, the stringent Bonferroni method was applied, i.e., the threshold $P$-value was obtained dividing 0.05 by the numbers of comparisons. Thus, the $P$-value was set at 0.004 ($=0.05/12$).

3. RESULTS

3.1. Japanese cohort

Genotyping success was 100% in both cases and controls. The distribution of rs2802292 genotypes was consistent with the HWE both in centenarians and controls ($P>0.05$). Table 1 shows the genotype and allele distributions in the Japanese subjects. In the entire cohort, the frequencies of the rs2802292 genotypes did not differ significantly between centenarians and control subjects in the recessive, dominant or additive models (all $P>0.05$). In addition, the distribution of allele frequencies did not differ between groups ($P>0.05$), and no significant differences were found when analyses were repeated separately by sex.

3.2. Italian cohort
Genotyping was successful in 100% of cases and controls. The distribution of rs2802292 genotypes was consistent with the HWE both in centenarians and controls ($P>0.05$). Table 2 shows the genotype and allele distributions in the Italian subjects. In the entire cohort, the frequencies of the rs2802292 genotypes did not differ significantly between centenarians and control subjects in the recessive, dominant or additive models (all $P>0.05$). In addition, the distribution of allele frequencies did not differ between groups ($P>0.05$), and no significant differences were observed in sex-based analyses.

4. DISCUSSION

Our results indicate that the rs2802292 SNP in the FOXO3A gene is not associated with EL either in non-disease free centenarians from Japan or in a unique cohort of disease-free centenarians from Northern Italy. This study was conducted in subjects born and living in Japan and ascertained to be of Japanese descent. No study on this polymorphism has been previously conducted in a group of disease-free centenarians, i.e., “dodgers”, such as those enrolled in the Italian cohort. Although the current results are negative, we believe that our data may add significantly to the available literature in the field. To estimate the overall association between SNPs and phenotypes, meta-analysis preferentially takes into account studies yielding statistically significant results, simply because trials with positive findings are published more often than trials with negative findings [22]. This may overestimate the actual magnitude of some genetic associations. Furthermore, studies combining different populations in meta-analyses may generate considerable confusion since potential differences in environmental effects are not taken into account. This effect can be even greater when a complex phenotype, such as EL, is considered.
FOXO3A is a DNA-binding transcription factor. It has been studied extensively as a pivotal protein involved in the regulation of several cellular functions, including defense against oxidative stress, apoptosis, cell cycle regulation, immunity, and inflammation [23]. Thus, there is strong rationale for postulating that FOXO3A should be a potential target in the study of EL. rs2802292 associates with a small but significant difference in messenger RNA levels in functional studies [24]. Nevertheless, more extensive studies are required to determine whether this SNP has a significant effect on the function of FOXO3A. Of note, the lack of association between rs2802292 and EL does not necessarily preclude that the IIS pathway can have an important impact on this phenotype. However, we believe that further investigations are required to confirm these findings. Replication studies like ours draw attention to the need for fine-mapping and sequencing analyses to identify the potential causal variants. Next-generation sequencing (NGS) has demonstrated the value of performing deep sequencing analysis to identify rare or low-frequency variants associated with susceptibility to complex diseases [25, 26], providing a powerful approach for pinpointing causal variants in candidate genes.

The identity of the longevity-associated functional variant of FOXO3 remains unresolved. The region containing the FOXO3A gene displays high linkage disequilibrium of SNPs, thereby making the identification of possible causal variants challenging. Moreover, almost all the identified common variants in FOXO3 are noncoding variations, likely because FOXO3 is a crucial transcription factor [27]. Resequencing of the FOXO3A locus, or the complete sequencing of intron 2, could help clarify the nature of the genetic variation that is tracked by rs2802292, to identify candidate variants for functional study of FOXO3 in human aging.
On the other hand, several limitations in our design should be kept in mind, which precludes making solid conclusions. These include differences in year of birth between centenarians and controls [28]. Another potential confounder is population stratification, with differences between and within cohorts in gender distribution among cases and controls. In addition, the sample size of our cohorts is relatively small compared to other studies finding a significant association between FOXO3A and human longevity [13].

5. CONCLUSIONS

While keeping in mind the aforementioned limitations, our study failed to replicate previous results suggesting an association between the rs2802292 SNP in FOXO3A and EL in two distinct cohorts of non-healthy centenarians from Japan and healthy centenarians from Northern Italy. Further research and resequencing efforts are needed in order to gain a deeper understanding of the potential contribution of FOXO3A to longevity.

CONTRIBUTORS AND THEIR ROLE

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

Study conception and design. Díaz-Peña, Fuku, Emanuele and Lucía.

Acquisition of data. Arai, Abe, Zempo, Naito, Murakami, Miyachi, Venturini, Ricevuti and Nobuyoshi.

Analysis and interpretation of data. Pareja-Galeano, Sanchis-Gomar and Santos-Lozano.
Contributors
Díaz-Peña, Fuku, Emanuele and Lucía were responsible for study conception and design.
Arai, Abe, Zempo, Naito, Murakami, Miyachi, Venturini, Ricevuti and Nobuyoshi were responsible for acquisition of data.
Pareja-Galeano, Sanchis-Gomar and Santos-Lozano were responsible for analysis and interpretation of data.
All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

Conflict of interest
No conflict of interest is reported by any of the authors.

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Ethical approval
The study protocol was approved by the local ethics committees and all participants provided their written informed consent, according to the Declaration of Helsinki.

Provenance and peer review
This article has undergone peer review.
REFERENCES

Table 1. Genotype and allele distribution for rs2802292 in Japanese centenarians and controls

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Values are numbers (%). The study cohort consisted of 733 centenarians (615 women and 118 men) and 820 controls (602 women and 218 men).
Table 2. Genotype and allele distribution for rs2802292 in Italian centenarians and controls

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