Original Article

Exome-wide Association Study Identifies CLEC3B Missense Variant p.S106G as Being Associated With Extreme Longevity in East Asian Populations

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Abstract

Life span is a complex trait regulated by multiple genetic and environmental factors; however, the genetic determinants of extreme longevity have been largely unknown. To identify the functional coding variants associated with extreme longevity, we performed an exome-wide association study (EWAS) on a Japanese population by using an Illumina HumanExome Beadchip and a focused replication study on a Chinese population. The EWAS on two independent Japanese cohorts consisting of 530 nonagenarians/centenarians demonstrated that the G allele of CLEC3B missense variant p.S106G was associated with extreme longevity at the exome-wide level of significance (p = 2.33 × 10^-7, odds ratio [OR] = 1.50). The CLEC3B gene encodes tetranectin, a protein implicated in the mineralization process in osteogenesis as well as in the prognosis and metastasis of cancer. The replication study consisting of 448 Chinese nonagenarians/centenarians showed that the G allele of CLEC3B p.S106G was also associated with extreme longevity (p = .027, OR = 1.51), and the p value of this variant reached 1.87 × 10^-8 in the meta-analysis of Japanese and Chinese populations. In conclusion, the present study identified the CLEC3B p.S106G as a novel longevity-associated variant, raising the novel hypothesis that tetranectin, encoded by CLEC3B, plays a role in human longevity and aging.

Keywords: Longevity—Centenarian—Human aging—Human genetics
Life span is a complex trait regulated by multiple genetic and environmental factors. The higher mean life expectancy in the industrialized countries than in the developing countries indicates that environmental factors such as medicine, nutrition, and social welfare impact longevity (1). Nevertheless, twin studies have demonstrated the heritability of longevity to be 20%–30% (2–4). Furthermore, the larger genetic than environmental influence on life span has been observed in exceptionally long-lived families than in normal populations (5,6), suggesting the existence of genetic variants associated with extreme longevity. Identification of longevity-associated genes may help us to understand the mechanism of longevity and aging and also contribute to the discovery of target molecules for prevention of age-related diseases.

Up to date, a number of theories on aging such as insulin signaling (7) and free radical (oxidative stress/mitochondrial) ones (8,9) have been proposed. Researchers therefore have explored longevity-associated genes under the hypothesis that the polymorphisms in the genes associated with these aging theories are determinants of human life span (9–15). However, most single-nucleotide polymorphisms (SNPs) have not been shown to be associated with extreme longevity across populations, except for apolipoprotein E (APOE) (16–22) and forkhead box protein O3 (FOXO3) (16,23,24), which have been implicated in Alzheimer’s disease onset (25,26) and insulin signaling, respectively (27).

Researchers also have explored genetic variants associated with extreme longevity by performing a genome-wide association study (GWAS), which is a powerful approach to identify the genetic loci responsible for complex traits (16–18,28). However, only the APOE locus was associated with longevity at the genome-wide significance level (p < 5.0 × 10−8) in multiple GWASs. Thus, the genetic components explaining the heritability of longevity have been largely unknown, and genetic variants that have not been uncovered by classical GWASs may underlie the heritability of longevity.

Rapid advances in DNA sequencing technology have enabled us to determine individual whole-genome sequences and have prompted researchers to explore the functional variants associated with complex traits, including low-frequency and rare variants in the protein-coding region (29). Several whole-genome sequencing studies on super-centenarians (age ≥ 110 years) have been performed to identify extreme longevity-associated coding variants; however, these studies have found no significant association because of the limited sample size (30,31). Although the sequence-based association study is currently difficult due to the high cost incurred to analyze a large number of samples, the genotyping array-based approach can resolve the cost problem and enables us to detect functional coding variants associated with extreme longevity with increased statistical power.

Here, we performed an exome-wide association study (EWAS) on extreme longevity in Japanese centenarians (age ≥ 100 years) and consecutive autopsy cases including nonagenarians/centenarians (age ≥ 95 years) by using the Infinium HumanExome BeadChip, followed by a replication study in Chinese nonagenarians/centenarians (age ≥ 95 years). By this means, we identified the association of the p.S106G missense variant of the C-type lectin domain family 3, member B (CLEGB) gene encoding tetranectin with extreme longevity, thus providing a novel insight into the genetic basis of human longevity and aging.

Methods
Ethics Statement
Written informed consent was obtained from all participants or the bereaved family of each of the patients prior to the autopsy examination. The study was approved by the Ethical Committee of Keio University, National Institute for Longevity Sciences, Tokyo Metropolitan Institute of Gerontology, and Kunming Institute of Zoology, Chinese Academy of Sciences.

Study Populations
The discovery EWAS consisted of two independent Japanese cohorts. Cohort 1 consisted of 438 centenarians (mean age at death, 104.3 years; age range, 100–107 years; men, n = 84; women, n = 354) and 3,674 controls (mean age, 61.9 years; age range, 40–79 years; men, n = 1,741; women, n = 1,933). Of these centenarians, 404 were participants in the Tokyo Centenarian Study (32), who had been enrolled mainly in the Tokyo metropolis. The remaining 34 centenarians had been enrolled in Gifu Prefecture, located in the central area of Honshu, the main island of Japan (14). The control group in Cohort 1 consisted of the participants of two longitudinal studies. Of these controls, 2,903 were middle-aged and elderly people who participated in the National Institute for Longevity Sciences Longitudinal Study of Aging (NILS-LSA) and lived in the city of Obu or in the town of Higashirua in Aichi Prefecture, located in the central area of Honshu (33). The remaining 771 controls were elderly people in metropolitan Tokyo who participated in the Itabashi Cohort Study 2011 (ITABASHI11) (34).

Cohort 2 consisted of 2,337 consecutive cases of autopsy performed at the Tokyo Metropolitan Geriatric Hospital from 1995 to 2012 (mean age at death, 80.6 years; age range, 33–104 years; men, n = 1,296; women, n = 1,041). The participants had been enrolled in the Internet database of Japanese SNPs for geriatric research (JG-SNP) (35). Because only 16 participants lived to be 100 years old in this cohort, we set the criteria of case (extreme longevity) participants at 95 years of age (n = 109; mean age at death, 97.3 years; age range, 95–104 years; men, n = 33; women, n = 76). Because individuals who survived for more than 80 years likely had a chance to possess longevity-associated alleles, we removed 1,194 individuals aged 80–94 years, and the data on remaining 1,034 participants were used in the case–control study (mean age at death, 72.8 years; age range, 33–79 years; men, n = 666; women, n = 368).

The replication study (Cohort 3) was performed on a Chinese cohort consisting of 447 nonagenarians/centenarians (mean age, 101.8 years; age range, 95–110 years; men, n = 43; women, n = 404) and 505 younger controls (mean age 37.9 years; age range, 15–79 years; men, n = 256; women, n = 249). All participants had been recruited from Hainan Province of China in 2010, and none of them were reported, following medical examination, to have had any severe disease when they were recruited (36).

Characteristics of each cohort and a histogram of age distribution of control samples (Cohorts 1 and 3) or age at the time of death of autopsy cases (Cohort 2, including individuals who lived to be 80–94 years) are summarized in Table 1 and Supplementary Figures 1–3.

Genotyping and Quality Control
Total DNA was extracted from peripheral blood (Cohorts 1 and 3) or kidney (Cohort 2) by using standard protocols such as phenol–chloroform extraction and spin column ones. DNA quality was evaluated by performing agarose gel electrophoresis and spectrophotometry. Genotyping of SNPs in Japanese populations was conducted with Infinium HumanExome BeadChip version 1.1 (Cohort 2) or version 1.2 (Cohort 1; Illumina, San Diego, CA) according to the manufacturer’s protocol. Genotype calling was performed...
by using the GenTrain clustering algorithm (version 1.0) in the GenomeStudio (version. 2011.1; Illumina). Cluster boundaries were determined by use of the standard cluster files provided by Illumina. A total of 242,901 (version 1.1) or 244,770 (version 1.2) variants were genotyped and subsequently subjected to quality control. SNP genotyping in Cohort 3 was performed by use of the standard Sanger sequencing protocol. The CLEC1B sequences were amplified using a 5′ primer of sequence 5′-CTGGGCGGTTGGCTCTTGG-3′ and a 3′ primer 5′-AGTGTAGTTCTCGCGTTGTT-3′, following the temperature profile of 94°C, 30 seconds; 64°C, 30 seconds; and 72°C, 90 seconds for 35 cycles, then ending with incubation at 72°C for 7 minutes. Polymerase chain reaction products were purified by using Exo-SAP-IT reagent before being subjected to sequencing reactions using a BigDye Terminator kit (Applied Biosystems, Carlsbad, CA). Capillary separation was performed on the ABI 3730xl Genetic Analyzer (Applied Biosystems). Sequences were aligned and edited by use of LaserGene v7.1 software (DNAStar, Madison, WI).

Quality control in the Japanese discovery cohorts was performed according to the following procedure: We first removed the participants with call rates less than 0.98 (157 samples in Cohort 1; 0 samples in Cohort 2) and subsequently excluded the SNPs with call rates less than 0.99 (7,415 SNPs in Cohort 1; 6,919 SNPs in Cohort 2), SNPs with minor allele frequency (MAF) less than 0.001 (200,709 SNPs in Cohort 1; 200,388 SNPs in Cohort 2), SNPs with deviation from the Hardy–Weinberg equilibrium (p < 1 × 10−8) in controls (615 SNPs in Cohort 1; 581 SNPs in Cohort 2), and SNPs in sex chromosomes and mitochondrial genome (872 SNPs in Cohort 1; 856 SNPs in Cohort 2). We also excluded the SNPs showing different call rates between cases and controls by using PLINK—test-missing option (exclusion criteria: p < .001 by chi-squared test; 34 SNPs in Cohort 1; 0 SNPs in Cohort 2). Duplicated samples and relatives were checked on the basis of identity-by-descent (IBD) estimates. We calculated the IBD estimate by adopting the PLINK—genome option (37), and each pair with IBD estimate greater than 0.375 was defined as first-degree relative or duplicated sample, and either one of each pair was removed (114 samples in Cohort 1; 0 samples in Cohort 2). Outliers in the population were also checked based on pairwise identity-by-state (IBS) distance by using the PLINK—neighbor option. The IBS distance between each of five nearest neighbors was transformed into a Z score, with which individuals less than –4 were defined as outliers and excluded (27 samples in Cohort 1; 8 samples in Cohort 2). Both the IBD estimate and IBS distance were calculated based on the subset of modest and high linkage disequilibrium (LD) region-pruned autosomal common SNPs with MAF greater than 0.05. We used the PLINK—indel-pairwise option to exclude SNPs in LD with r² ≥ .2. A window size was set at 50 SNPs, and the number of SNPs to shift the window at each step was 5. Then, 15,271 LD-pruned (Cohort 1) or 15,206 (Cohort 2) SNPs were used to calculate the IBD estimate and IBS distance. We also removed individuals with high rates of autosomal heterozygosity (> or < 3 SD), 82 samples in Cohort 1; 25 samples in Cohort 2). After quality control, 41,213 SNPs and 3,732 individuals consisting of 423 centenarians and 3,309 controls in Cohort 1, and 39,305 SNPs and 1,100 individuals including 107 nonagenarians/centenarians in Cohort 2 were available for analysis. We restricted the analysis to missense, stop gain, stop loss, frameshift, and splice site SNPs; and 25,089 (Cohort 1) or 23,915 (Cohort 2) of these SNPs were finally analyzed by using the single-variant association test (Figure 1). An average genotyping call rate of 99.9% for the remaining samples was achieved in both Cohorts 1 and 2.

Variant Annotation

Variant annotation was performed by using SNPnexus based on the RefSeq database (human genome build GRCh37; (38)). The variants were functionally annotated as missense, stop gain, stop loss, frameshift, and splice site.

Statistical Analysis

We performed principal component analyses (PCA) with EIGENSTRAT version 6.0.1 to account for potential population stratification in the statistical analysis for discovery EWAS (39). For PCA, 20,234 LD region-pruned autosomal (Cohort 1) or 20,114 (Cohort 2) SNPs with MAF greater than 0.01 were used; and the first 10 principal components (PCs) in each cohort were computed. Population structures of Cohorts 1 and 2 identified by PCA are shown in Supplementary Figures 4 and 5, respectively. Population stratification was evaluated by calculating genomic inflation factor λ, from uncorrected or PC-corrected Cochran–Armitage trend χ² statistics by using EIGENSTRAT.

Single-variant associations with longevity case–control status were assessed with PLINK version 1.07 (37). In the discovery EWAS, we used logistic regression analyses adjusted for sex and the first 10 (Cohort 1) or 6 (Cohort 2) PCs, assuming additive, dominant, and recessive genetic models. A logistic regression analysis adjusted for sex was also used for the replication study on Cohort 3. We also performed a gene-based burden test to examine the association between the set of only rare and low-frequency variants with MAF less than

| Table 1. Characteristics of the Study Cohort |
| --- |
| **Case/Control Genotyping Platform** | Number | Number of Men/Women | Current Age/Age at Death (range) |
| Discovery cohort (Japanese) Case | Infinium HumanExome | 438 | 84/354 | 104.3 (100–107) |
| Control | BeadChip ver.1.2 | 3,674 | 1,741/1,933 | 61.9 (40–79) |
| Cohort 1 | Infinium HumanExome | 109 | 33/76 | 97.3 (95–104) |
| Control | BeadChip ver.1.2 | 1,034 | 666/368 | 72.8 (33–79) |
| Cohort 2 | Infinium HumanExome | 447 | 43/404 | 101.8 (95–110) |
| Control | BeadChip ver.1.1 | 505 | 256/249 | 37.9 (15–79) |

Note: ITABASHI11, Itabashi Cohort Study 2011; NILS-LSA, National Institute for Longevity Sciences Longitudinal Study of Aging.
Figure 1. Flow diagram of the study.

0.05 and extreme longevity. We tested 3,374 (Cohort 1) or 3,132 (Cohort 2) genes including at least two such variants by use of the sequence kernel association optimal test (SKAT-O) (40). Gene-based association analyses were adjusted for sex and the first 10 or 6 PCs as well as for single-variant association. We set the exome-wide significance level at $p < 1.99 \times 10^{-6}$ for the single-variant association and $p < 1.48 \times 10^{-5}$ for the gene-based burden test according to the number of tested SNPs or genes in Cohort 1. The statistical power of the single-variant test was estimated by using the CaTS power calculator (http://www.sph.umich.edu/csg/abecasis/CaTS/index.html).

Meta-analysis of the results from single-variant association analyses in Cohorts 1–3 was performed with METAL (41). A total of 22,536 SNPs commonly included in Infinium HumanExome BeadChip version 1.1 and 1.2 were analyzed in the combined analysis of Japanese Cohorts 1 and 2. The combined $p$ values were calculated by using the inverse-variance method from the beta coefficient and standard error obtained in each cohort. Heterogeneity of allelic effects between the discovery and replication cohorts was assessed with Cochran’s $Q$ statistics using METAL. Regional association results from combined analysis of Japanese populations were plotted by using LocusZoom (42). We also examined the sex-specific allelic effects by performing the Cochran–Armitage trend test; then the heterogeneity between men and women was assessed by use of the above-described meta-analysis procedures. Meta-analysis of the results from the SKAT-O test was carried out by use of MetaSKAT (43).

To verify that the SNPs identified as being associated with extreme longevity were also associated with overall life span, we performed Kaplan–Meier survival analysis of 2,274 quality control–passed consecutive autopsy cases including the individuals who lived to be 80–94 years by using the R package “survival” (http://cran.r-project.org/web/packages/survival/). Difference in survival curves between genotypes was compared by use of the log-rank test, assuming genotypic (two degrees of freedom), dominant, and recessive genetic models.

To identify the gene sets/pathways associated with extreme longevity, we performed a gene set enrichment analysis (GSEA) with MAGENTA version 2.4 (44) using the $p$ values of 22,536 SNPs from combined analysis of Japanese Cohorts 1 and 2. Briefly, MAGENTA creates a gene association score from the smallest $p$ value of the set of SNPs assigned to the genes, which are adjusted for confounders such as gene size, density of SNPs per gene, and LD. Subsequently, MAGENTA tests whether any of the gene sets are enriched for highly ranked gene association scores that are above a predefined cutoff value, and the $p$ values for GSEA are generated by 10,000–1,000,000 permutations of gene sets. We tested 3,216 gene sets/pathways annotated from Gene Ontology, PANTHER, Ingenuity, KEGG, REACTOME, and BIOMICARTA databases. We adopted the 95th percentile of the gene association scores as the cutoff and set the significance level at $p < 0.05$ and the false-discovery rate (FDR) at less than 0.2.

**Confirmation of MAF in the General Population**

To confirm the MAF of EWAS-identified variants in the general population, we utilized the SNP data of individuals who had participated in the Inabe Health and Longevity Study and lived in Mie Prefecture, located in the central area of Honshu (45). SNP data on 4,499 individuals (mean age, 62.0 years; age range, 17–79 years; men, $n = 1,429$; women, $n = 3,070$) genotyped with Infinium HumanExome BeadChip version 1.2 were available for confirmation.

**Results**

An EWAS on extreme longevity was conducted on quality control–passed individuals of two Japanese cohorts. Genomic inflation factor $\lambda$ based on the median Cochran–Armitage trend $\chi^2$ statistics showed slight population stratification ($\lambda = 1.105$ in Cohort 1; $\lambda = 1.093$ in Cohort 2; Supplementary Figure 6A and 6C); therefore, we performed PCA to correct for potential population stratification. Genomic inflation factor $\lambda$ was minimized when $\chi^2$ statistics were corrected by the first 10 PCs in Cohort 1 ($\lambda = 1.046$; Supplementary Figure 6B) or 6 PCs in Cohort 2 ($\lambda = 1.068$; Supplementary Figure 6D); and further correction using the genomic control method was not performed. For the single-variant association test, we used logistic regression analyses adjusted for sex and the first 10 PCs (Cohort 1) or 6 PCs (Cohort 2), assuming additive, dominant, and recessive genetic models. None of the SNPs reached the exome-wide significance ($p < 1.99 \times 10^{-6}$) in Cohort 1 or Cohort 2 alone in all of the genetic models (Supplementary Tables 1–3).

We next performed meta-analysis of the results from Cohorts 1 and 2 consisting of 530 nonagenarians/centenarians and 4,312 controls. The combined analysis of Japanese populations identified that $CLEC3B$ p.S106G was associated with extreme longevity at the exome-wide significance level in the additive model (odds ratio $[OR] = 1.50$, $p = 2.33 \times 10^{-7}$; Figure 2A and Table 2). The statistical power to detect an OR of 1.50 for the G allele of $CLEC3B$ p.S106G was 82% with $\alpha = 1.99 \times 10^{-4}$ when the survival rate of individuals at 95 years of age was postulated to be 16% (average of men and women) according to a statistical survey conducted by the Ministry of Health, Labor, and Welfare in Japan (http://www.mhlw.go.jp). $CLEC3B$ p.S106G was associated with extreme longevity with comparable effect size between Cohorts 1 and 2, and the results of the Cochran Q test indicated no significant heterogeneity of the allelic effect of $CLEC3B$ p.S106G between these cohorts ($p_{het} = .416$). The regional plot of $CLEC3B$ locus is shown in Supplementary Figure 7, which clearly demonstrated that $CLEC3B$ p.S106G was the lead SNP in this locus. In the combined analysis of the Japanese populations, $APOE$ p.R176C, which defines the $APOE$ ε2 haplotype previously reported to be associated with Alzheimer’s disease and longevity (20,22,26), also reached the exome-wide significance level.
in the dominant model (OR = 1.98, \( p = 1.18 \times 10^{-4} \); Figure 2B and Table 2). The statistical power to detect an OR of 1.98 for the GG or AG genotype of APOE p.R176C was 72\% with \( \alpha = 1.99 \times 10^{-4} \). No cohort-specific allelic effects of APOE p.R176C were observed (\( p_{het} = .694 \)). Moreover, no SNPs except for CLEC3B p.S106G and APOE p.R176C reached exome-wide significance in the combined analysis of the Japanese populations.

Our EWAS in Japanese cohorts demonstrated that CLEC3B p.S106G and APOE p.R176C were associated with extreme longevity at the exome-wide significance level in the Japanese population. Because the significant association of APOE p.R176C with longevity was previously reported in multiple studies by the candidate gene approach (20,22), we therefore sequenced only CLEC3B p.S106G in the Chinese population and performed a replication study to elucidate whether the variant was associated with longevity across east Asian populations. The replication study showed that the minor G allele (glycine 106) of CLEC3B p.S106G was more frequent in the Chinese nonagenarians/centenarians than in the younger controls with comparable size effect to that in the Japanese population (\( p = .027, OR = 1.51 \)). The p value of the association with CLEC3B p.S106G reached the exome-wide significance level (\( p < 1.81 \times 10^{-8}, OR = 1.50 \)) in the meta-analysis of Japanese and Chinese populations.

We next examined the sex-specific effects of EWAS-identified CLEC3B p.S106G on extreme longevity (Table 3). Although CLEC3B p.S106G was associated with extreme longevity with comparable OR between men and women in Cohort 1, no significant association (\( p < .05 \)) was observed in men from Cohorts 2 and 3. However, meta-analysis of Cohorts 1–3 showed no significant heterogeneity of the allelic effect between men and women (\( p_{het} = .463 \)), suggesting no sex-specific allelic effects of CLEC3B p.S106G on extreme longevity.

The MAF of CLEC3B p.S106G was 0.264 in the control samples of Japanese cohorts did not deviate from that in the general Japanese population. On the other hand, the MAF of CLEC3B p.S106G in the Chinese population of the present study was lower than that of the Japanese population (0.103 in the cases and 0.066 in the controls, respectively). We confirmed that the MAF of CLEC3B p.S106G in HapMap CHB was almost the same as that of the control participants in Cohort 3 (MAF = 0.073, \( n = 82 \), http://hapmap.ncbi.nlm.nih.gov).

Although we revealed that CLEC3B p.S106G was associated with extreme longevity defined as survival to 95 years of age in the Japanese population, it remained unknown whether this SNP would influence the overall life span. We therefore performed survival analysis of consecutive autopsy cases in Cohort 2. The CLEC3B p.S106G genotype was associated with age at the time of death in 2,274 consecutive autopsy cases including individuals who lived to be 80–94 years in any of genetic model (\( p_{genotypic} = .025; p_{dominant} = .020; p_{recessive} = .047 \), Supplementary Figure 8). However, when we analyzed the data from 2,168 individuals who lived to be less than 95 years of age, no association between the CLEC3B p.S106G genotype and age at the time of death was observed in any genetic model (\( p_{genotypic} = .731; p_{dominant} = .510; p_{recessive} = .551 \)).

The single-variant association test of rare and low-frequency functional variants has insufficient statistical power even in the sample size of the present study. We therefore performed a gene-based burden test using the SKAT-O test to identify the set of rare and low-frequency variants with MAF less than 0.05 associated with extreme longevity in the Japanese population. However, none of the genes reached the exome-wide significance level in either Cohort 1 or 2 alone, and meta-analysis using MetaSKAT also did not identify the genes associated with extreme longevity (Supplementary Table 4).

Finally, we performed a GSEA to identify the gene sets/pathways associated with extreme longevity. A GSEA using MAGENTA identified 14 gene sets/pathways possibly associated with extreme longevity (\( p < .05 \) and FDR < 0.2; Table 4).

**Discussion**

We performed the first study exploring functional coding variants associated with extreme longevity by use of an exome array. Our EWAS on Japanese nonagenarians/centenarians from two independent cohorts and the replication study on Chinese nonagenarians/centenarians identified CLEC3B missense variant p.S106G as being associated with extreme longevity in east Asian populations.

The CLEC3B gene encodes tetranectin, which is a trimeric Ca\(^{2+}\)-binding protein belonging to the family of C-type lectins and is mainly found in plasma and extracellular matrix (46,47). Tetranectin binds to plasminogen kringle 4, to sulfated polysaccharides, and to extracellular matrix proteins, enhancing plasminogen activation in the presence of tissue plasminogen activator (46–48). Therefore, tetranectin is thought to participate in plasminogen activator-related biological processes such as tissue degradation/remodeling and extracellular proteolysis.

It has been suggested that tetranectin has a potential role in the mineralization process in osteogenesis (49). An earlier animal study demonstrated that delayed fracture healing in tetranectin-deficient mice was due to delayed soft tissue and callus formation, but not due to decreased capacity for cartilage and bone formation (50). Another animal study reported that ovariectomized mice, a postmenopausal osteoporosis model, exhibited a 50 times higher level of plasma tetranectin than sham mice, and authors suggested tetranectin to be a candidate biomarker reflecting bone metabolic turnover (51).
### Table 2. Association of CLEC3B p.S106G With Extreme Longevity

| Chromosome | Position | SNP      | Gene   | Amino Acid Change | Major | Minor | Cohort            | Case MAF | Control MAF | OR    | 95% CI         | p       | $p_{het}$ |
|------------|----------|----------|--------|-------------------|-------|-------|-------------------|----------|-------------|-------|---------------|---------|----------|
| 3          | 45077123 | rs13963  | CLEC3B | Missense p.S106G  | A     | G     | Cohort 1 (Japanese) | 0.266    | 0.198       | 1.45  | 1.22–1.72    | $1.20 \times 10^{-5}$ | .002     |
|            |          |          |        |                   |       |       | Cohort 2 (Japanese) | 0.257    | 0.177       | 1.70  | 1.21–2.39    | .002    | .416     |
|            |          |          |        |                   |       |       | All Japanese       | 1.50     | 1.28–1.74   | 2.33 x 10^{-7} | .416     |
|            |          |          |        |                   |       |       | Cohort 3 (Chinese)  | 0.103    | 0.066       | 1.51  | 1.05–2.17    | .027    | .718     |
|            |          |          |        |                   |       |       | All east Asian     | 1.50     | 1.30–1.72   | 1.87 x 10^{-6} | .718     |

Notes: Data were analyzed by performing logistic regression analysis assuming the additive genetic model. Genotype counts for each SNP are listed in Supplementary Tables 1–3. 95% CI = 95% confidence interval; MAF = minor allele frequency; OR = odds ratio for the minor allele; $p_{het}$ = $p$ values for heterogeneity between the cohorts; SNP = single-nucleotide polymorphism.

### Table 3. Association of CLEC3B p.G106S With Extreme Longevity in Men and Women

| Chromosome | Position | SNP      | Gene | Allele | MAF | Men | MAF | Women | MAF |
|------------|----------|----------|------|--------|-----|-----|-----|-------|-----|
| 3          | 45077123 | rs13963  | CLEC3B | A      | G   |     |     |       |     |
|            |          |          |        |        |     |     |     |       |     |

Notes: Data were analyzed by performing Cochran–Armitage trend test. 95% CI = 95% confidence interval; MAF = minor allele frequency; OR = odds ratio for the minor allele; $p_{het}$ = $p$ values for heterogeneity between the cohorts; SNP = single-nucleotide polymorphism.
In humans, although no association of the circulating tetranectin level with the bone phenotype has been reported, previous studies suggested that CLEC3B genetic variations were associated with susceptibility to osteoarthritis and osteoporosis. Valdes and colleagues genotyped CLEC3B p.S106G in 603 knee osteoarthritis patients and demonstrated that the major A allele (serine 106) of the CLEC3B p.S106G, the frequency of which was lower in the nonagenarians/centenarians in our study, was associated with an increased risk of knee osteoarthritis in both men and women (men: OR = 1.49; women: OR = 1.45; 52). Furthermore, a linkage analysis of 1,094 pedigrees identified quantitative trait loci for bone mineral density at 3p21, which contains the CLEC3B gene (53). Considering that knee osteoarthritis and osteoporosis increase the risk of impaired mobility (54,55), which increases the risk of mortality (56,57), the minor G allele (glycine 106) of CLEC3B p.S106G may be associated with extreme longevity in part via the promotion of bone and articular health.

Tetranectin plays roles in tissue degradation and extracellular proteolysis, which are activities associated with tumor invasion (58). Therefore, it has been investigated as a potential biomarker for prognosis and metastasis of cancer. Previous studies demonstrated that a low level of circulating tetranectin is associated with a poor treatment response to anticancer drugs and survival from various types of cancer (59–61). Low levels of circulating tetranectin were also observed in patients with multiple tumors or metastases (61–63). Furthermore, the expression level of tetranectin in tumor tissue is decreased in patients with poor survival from cancer (64). These findings suggest that tetranectin is a potent inhibitor of tumor invasion and metastasis.

The present study demonstrated that CLEC3B p.S106G was not significantly associated with age at death in the Japanese autopsy cases that lived to be less than 95 years old, suggesting that CLEC3B p.S106G was only associated with whether or not people could survive until 95 years of age in the Japanese population. A statistical survey conducted by the Ministry of Health, Labor and Welfare in Japan (http://www.mhlw.go.jp) indicates that the main cause of death is quite different according to whether the individuals live to be 95 years old or not. The survey examining the cause of death in each 5-year age group showed that cancer is the leading or second cause of death in Japanese people who died at 30–94 years of age. Indeed, 28.9% of Japanese people died due to cancer. In contrast, cancer is the fifth cause of death in individuals older than 95 years; and the mortality rate from cancer is dramatically decreased to 8.0%. These statistics suggest that long-lived individuals have a genetic advantage against death from cancer, which may be due to the presence of CLEC3B p.S106G.

Another significant finding of the present study was that the APOE ε2 and extreme longevity were observed at the exome-wide significance level in the Japanese population. APOE p.R176C defines the APOE ε2 haplotype, which is known as a protective allele against Alzheimer’s disease, and several candidate gene approaches showed a higher frequency of APOE ε2 in long-lived individuals (20,22,26). Because APOE p.R176C is not covered by most conventional SNP arrays, previous GWASs on longevity only identified and replicated the SNPs in LD with APOE p.C130R, which defines the APOE ε4 haplotype (1,16,17,26). The present study demonstrated that not only APOE ε4 but also APOE ε2 was a stronger genetic determinant of extreme longevity than other genetic components except for CLEC3B p.S106G. Nevertheless, a previous study examining the APOE haplotype of centenarians in various ethnic populations showed that significant associations between APOE ε2 and extreme longevity were observed only in the Italian and Japanese cohorts that shared 404 centenarians with our discovery cohort (20). Replication studies on other ethnic populations are needed to elucidate whether APOE p.R176C is associated with life span regardless of ethnicity.
Our GSEA analysis demonstrated that 14 enriched gene sets/pathways were possibly associated with extreme longevity in the Japanese population. Interestingly, several gene sets/pathways, such as those involved in inflammation/oxidative stress (GATA3 pathway, antioxidant activity, and NOS1 pathway), lipid metabolism (chylomicron, lipid homeostasis, and very-low-density lipoprotein particle), and brain function (associative learning, visual learning, and inner ear development), have been proposed to be associated with longevity (8,63) or age-associated diseases (eg, coronary heart diseases and Alzheimer’s disease) (66–68). The coding variants in these gene sets/pathways may interactively regulate the variability of the human life span even though each variant alone has a small effect on longevity. Nevertheless, our findings should be replicated in other populations to confirm that the coding variants in these gene sets/pathways determine extreme longevity.

Whole-genome and exome sequencing data from more than 1,000 individuals have revealed that numerous low-frequency and rare disease-causing variants exist in general populations (69,70), thus raising the hypothesis that long-lived individuals who are resistant to chronic diseases have fewer disease-causing variants than does the general population. However, we did not identify low-frequency and rare coding variants with MAF less than 0.05 associated with extreme longevity by either single-variant association analysis or gene-based burden testing except for APOE p.R176C with MAF equal to 0.048 due to the limited sample size of cases. The statistical power did not reach 80% even for the APOE p.R176C in the combined analysis of Cohorts 1 and 2. Association analysis using a larger sample size is required to conclude that low-frequency and rare coding variants contribute to the genetics of extreme longevity. Furthermore, because the 244,770 markers in the Infinium HumanExome BeadChip were adopted based on the whole-genome and exome sequencing data mainly obtained from individuals of European ancestry, only 25,089 functional variants were polymorphic and available for the association analysis of the Japanese population (Cohort 1). Thus, we might overlook the association of Japanese population-specific low-frequency and rare variants with extreme longevity. Today, large-scale whole-genome sequencing of 1,070 healthy individuals has identified approximately 12 million novel rare variants in the Japanese population (71), thus allowing us to explore Japanese population-specific low-frequency and rare variants associated with extreme longevity in the near future.

In conclusion, EWAS on Japanese nonagenarians/centenarians from two independent cohorts and a replication study on Chinese nonagenarians/centenarians identified the CLEC3B missense variant p.S106G, encoding tetranectin, as being associated with human longevity in east Asian populations. Our findings prompt us to posit the novel hypothesis that tetranectin encoded by CLEC3B plays a role in longevity. Further replication studies on the life span in multi-ethnic populations as well as functional studies on the CLEC3B p.S106G polymorphism should eventually elucidate the genetic basis of human longevity and aging.

Supplementary Material
Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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Conflict of Interest
The authors do not have any conflicts of interest to disclose.

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