RESEARCH ARTICLE

Muscle-Related Polymorphisms (MSTN rs1805086 and ACTN3 rs1815739) Are Not Associated with Exceptional Longevity in Japanese Centenarians

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Abstract

Myostatin (MSTN) and α-actinin-3 (ACTN3) genes are potentially associated with preservation of muscle mass and oxidative capacity, respectively. To explore the possible role of these genes in exceptional longevity (EL), the allele/genotype frequency distribution of two polymorphisms in MSTN (rs1805086, K153R) and ACTN3 (rs1815739, R577X) was studied in Japanese centenarians of both sexes (n = 742) and healthy controls (n = 814). The rs1805086 R-allele (theoretically associated with muscle mass preservation at the expense of oxidative capacity) was virtually absent in the two groups, where genotype distributions were virtually identical. Likewise, no differences in allele (p = 0.838 (women); p = 0.193 (men); p = 0.587 (both sexes)) or genotype distribution were found between groups for ACTN3 rs1815739 (p = 0.975 (women), p = 0.136 (men), p = 0.752 (both sexes)). Of note, however, the frequency of the rs1805086 R-allele observed here is the lowest been reported to date whereas that of the ‘highly oxidative/efficient’ rs1815739 XX genotype in Japanese male centenarians (33.3%) or supercentenarians of both sexes (≥110 years) are the highest (32.6%), for a non-American population. No definite conclusions can be inferred in relation to EL owing to its lack of association with both rs1815739 and rs1805086. However, it cannot be excluded that these gene variants could eventually be related to a “healthy” metabolic phenotype in the Japanese population. Further research might determine if such metabolic profile is among the factors that can potentially predispose these individuals to live longer than Caucasians and what genetic variants might be actually involved.
Introduction

As life expectancy in developed countries rises, the number of elderly people and among them those showing exceptional longevity (EL, ≥100 years) steadily climbs. A major problem associated with ageing is the gradual decline in those systems and organs that determine physical fitness, notably the skeletal muscle tissue. Accelerated age loss of muscle strength (sarcopenia) is associated with higher mortality risk and genes potentially associated with preservation of muscle mass and metabolic function could be potentially associated not only with healthy ageing and disability risk, but also with EL [1].

One candidate to modulate muscle mass during aging is the gene encoding myostatin (MSTN, also termed ‘growth differentiation factor 8’; MIM#601788). Myostatin is a highly conserved member of the transforming growth factor-β superfamily that is expressed predominantly in the muscle tissue and mechanistic studies have revealed its role as a negative modulator of muscle mass [2, 3], with over-expression of this molecule being involved in the development of cachexia in cancer patients [4]. Besides its potential role in the modulation of sarcopenia and muscle metabolism, the rs1805086 (2379A>G) polymorphism in exon 2 of MSTN gene, which causes a Lys(K)153Arg(R) substitution, has been linked with a higher likelihood of reaching EL in North-Italian and Spanish cohorts [5].

Progressive mitochondrial dysfunction notably affecting the nervous and muscle tissue, e.g., due to accumulation of deletions in mitochondrial DNA (mtDNA), is a major hallmark of the aging process [6] and thus genetic variants associated with muscle oxidative metabolism could also potentially influence EL. One such candidate is the Arg(R)577Ter(X) (rs1815739) polymorphism in the gene (ACTN3, MIM#102574) encoding α-actinin-3, a sarcomeric protein expressed in skeletal-glycolytic fibers [7], which can potentially affect not only exercise performance [8], but also health-related phenotypes owing to its influence on muscle oxidative metabolism. In this regard, classic studies in artificially selected rats showed that improved oxidative pathways in muscle mitochondria may be a common factor linking physical fitness with decreased disease risk and higher survival [9].

Japan has the longest life expectancy worldwide, as well as the highest number of centenarians. This population represents an interesting model to investigate the genetic factors involved in EL. We aimed to study the potential role of MSTN and ACTN3 genes in EL by analyzing the genotype/allele frequency distribution of MSTN rs1805086 and ACTN3 rs1815739 in a large cohort of Japanese centenarians. We also studied a control group of younger adults, and sex was taken into account in the statistical analyses.

Material and Methods

The study was approved by the local ethics Committee (Keio University and National Institute of Health and Nutrition, Japan) and written consent was obtained from all the subjects. Seventy hundred and forty-two centenarians (100–116 years, 623 women) and 814 healthy controls (23–65 years, 601 women), all from the same Asian (Japanese) descent were studied. The centenarians were recruited from two previously described [10] prospective cohorts: The Tokyo Centenarians Study (TCS) and the Semi-Supercentenarians Study in Japan (SSC-J). The TCS cohort includes 304 centenarians randomly selected between July 2000 and May 2002 among those living in the 23 wards of metropolitan Tokyo (representing 17.5% of an estimated 1735 centenarians living in this area in the aforementioned period) [10]. The SSC-J is a nationwide longitudinal survey consisting mainly of individuals aged 105 years or older, which started in 2002 and had a total of sample size of n = 450 by end of November 2011. The
prevalence rates of hypertension, coronary artery disease and dementia in the Japanese centenarians were 63.6%, 28.8% and 59.4%, respectively [11]. Inclusion criteria for the control group, which was recruited during 2007–2012 from people participating in a Nutrition and Exercise Intervention Study (NEXIS, registered at ClinicalTrials.gov, Identifier: NCT00926744), were being a man or woman aged 23–65 years without a history of stroke, cardiovascular disease, chronic renal failure, or walking difficulties related to knee or back pain [12]. People with the aforementioned conditions were excluded from NEXIS because their exercise habits could be partly influenced by their disease or pain.

Total DNA was isolated from venous blood by use of QIAamp DNA Blood Maxi and/or Mini Kit (QIAGEN, Hilden, Germany). The rs1805086 and rs1815739 polymorphisms were genotyped using TaqMan SNP genotyping assays (assay ID, C____282184_30 and C____590093_1_, respectively) and a real-time thermocycler (LightCycler 480, Roche Applied Science, Mannheim, Germany). A total of 5 μL of genotyping mixture containing 2.5 μL of GTXpress™ Master Mix, 0.125 μL of assay mix (40x), and 1.375 μL of distilled water was mixed with 1 μL of genomic DNA (10 ng/μL) in each reaction. PCR 384-well plates were read on the thermocycler using the end-point analysis mode. Allelic discrimination analysis was performed with a LightCycler 480 SW software version 1.5.1.62 (Roche Applied Science, Mannheim, Germany). Four or five negative controls were included on each plate.

Genotype and allele frequencies were compared between groups using the χ² and the Fisher exact test (α set at 0.05).

Results and Discussion

The **MSTN** rs1805086 K-allele was highly predominant in the whole cohort and genotype distribution was virtually identical in centenarians and controls (**Table 1**). The **ACTN3** rs1815739 genotype distribution did not differ between the two groups regardless of sex or model of analysis (co-dominant or dominant, **Table 1**), and no differences were found in allele frequency either (**Fig 1**).

Although no insight on the potential role of the **MSTN** rs1805086 variant on EL can be extrapolated from our data, the highly homogeneous genotype found in the Japanese population is somehow striking. This polymorphism has been previously analyzed in other cohorts of a different ethnic/geographic origin, which showed a higher prevalence of the variant R-allele (**Table 2**). Interestingly, populations from the African continent (which conversely are those with a lower prevalence of the variant **ACTN3** X-allele--see below) show the highest prevalence of the mutant R-allele (**Table 2**).

**In vitro** experiments have recently shown that the variant R-allele in **MSTN** rs1805086, which is potentially linked with lower muscle strength and higher obesity risk, is associated with a reduced circulating myostatin activity [13]. The virtual absence of this allele in the Japanese population is in line with that observed in a cohort of healthy young Han Chinese men (frequency of KK genotype of 93.6%) [14], suggesting a highly conserved myostatin activity (that in turn could modulate a shift towards a more oxidative phenotype) [15]. In this regard, we recently postulated that the m.1382A>C polymorphism located in the mtDNA region encoding the recently discovered mitochondrial open reading frame of the 12S rRNA-c (MOTS-c), which is specific for the Northeast Asian population and is associated with metabolic homeostasis and insulin sensitivity, may be among the putative biological mechanisms explaining the high longevity of Japanese people [16].

No differences were found between controls and centenarians in the **ACTN3** R577X allele/genotype distribution. The ‘null’ XX genotype results in complete protein deficiency and the **Actn3**−/− mouse model shows a shift in the properties of fast fibers towards a more oxidative
phenotype \cite{17}. Therefore, it might be possible that the X-allele could confer some resistance against metabolism-related diseases and thus contribute, at least partly, to extend life expectancy in some individuals. Previous data in a Spanish cohort showed a similar frequency distribution of the $\textit{ACTN3}$ R577X genotype among centenarians and those humans with the highest oxidative capacity, \textit{i.e.}, elite endurance athletes \cite{18}. Further, the frequency of the XX genotype in Spanish centenarians was the highest been reported in non-athletic Caucasian populations \cite{18}. Interestingly, the frequency of the XX genotype reported here in male centenarians ($n = 117$) as well as in supercentenarians of both sexes (110–116 years, $n = 82$ women and 7 men) are the highest values ever reported for a non-American population \cite{8}, \textit{i.e.}, 33.3% and 32.6%, respectively.

The $\textit{ACTN3}$ XX genotype is present in \textit{~}18% of the population of European descent \cite{7}, while much lower and higher frequencies have been reported in African and Asian populations, respectively \cite{19, 20}. Such latitude/ethnic heterogeneity has raised the possibility that the X-allele is one of the very few variants associated with gene loss-of-function which have been positively selected over human evolution \cite{7}. This mutation probably preceded the appearance of anatomically modern humans in Europe and Asia (~40,000–60,000 years ago) \cite{8}. Owing to its effect on skeletal muscle metabolism, the X-allele probably provided some sort of functional advantage to modern humans adapting to the novel, colder Eurasian environment that required survival despite scarce food availability.

A main limitation of genetic association studies using a case/control design as the present one is selection of controls. A first potential confounder is differences in date of birth, \textit{e.g.}, the centenarians and controls of our study were born in the early 1900s and after 1940, respectively. In this regard, the risk of death depends on the interaction of genetic and environmental/lifestyle risk factors; notably, the pattern of exposure to such risk factors is related to year of

\begin{table}
\centering
\caption{Genotype distributions of the myostatin ($\textit{MSTN}$) rs1805086 and $\alpha$-actinin-3 ($\textit{ACTN3}$) rs1815739 variations in Japanese centenarians and controls.}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{MSTN} & & & & & & & & & & & & \\
\textbf{K153R (rs1805086)} & \textbf{Women} & \textbf{Men} & \textbf{All together} & \textbf{Controls} & \textbf{Centenarians} & \textbf{Centenarians} & \textbf{Controls} & \textbf{Controls} & \textbf{Centenarians} & \textbf{Centenarians} & \textbf{Controls} & \textbf{Controls} \\
\hline
Codom & & & & & & & & & & & & \\
\textbf{KK} & 615 & 99.8 & 601 & 100.0 & 117 & 100.0 & 213 & 100.0 & 732 & 99.9 & 814 & 100.0 \\
\textbf{KR} & 1 & 0.2 & 0 & 0.0 & 0 & 0.0 & 0 & 0.0 & 1 & 0.1 & 0 & 0.0 \\
\textbf{RR} & 0 & 0.0 & 0 & 0.0 & 0 & 0.0 & 0 & 0.0 & 0 & 0.0 & 0 & 0.0 \\
\hline
Dom & & & & & & & & & & & & \\
\textbf{KK/KR} & 615 & 99.8 & 601 & 100.0 & 117 & 100.0 & 213 & 100.0 & 732 & 99.9 & 814 & 100.0 \\
\hline
\textbf{ACTN3} & & & & & & & & & & & & \\
\textbf{R577X (rs1815739)} & \textbf{Women} & \textbf{Men} & \textbf{All together} & \textbf{Controls} & \textbf{Centenarians} & \textbf{Centenarians} & \textbf{Controls} & \textbf{Controls} & \textbf{Centenarians} & \textbf{Centenarians} & \textbf{Controls} & \textbf{Controls} \\
\hline
Codom & & & & & & & & & & & & \\
\textbf{RR} & 124 & 20.3 & 118 & 19.8 & 28 & 23.9 & 53 & 24.9 & 0.975 & 152 & 20.9 & 171 & 21.1 \\
\textbf{RX} & 319 & 52.1 & 311 & 52.2 & 50 & 42.7 & 110 & 51.6 & 50.6 & 211 & 26.8 & 421 & 52.0 \\
\textbf{XX} & 169 & 27.6 & 167 & 28.0 & 39 & 33.3 & 50 & 23.5 & 0.136 & 208 & 28.5 & 217 & 26.8 \\
\hline
Dom & & & & & & & & & & & & \\
\textbf{RR/RX} & 429 & 72.0 & 443 & 72.4 & 78 & 66.7 & 163 & 76.5 & 0.898 & 521 & 71.5 & 592 & 73.2 \\
\textbf{XX} & 167 & 28.0 & 169 & 27.6 & 39 & 33.3 & 50 & 23.5 & 0.069 & 208 & 28.5 & 217 & 26.8 \\
\hline
\end{tabular}
\end{table}
Second, we cannot infer whether controls will eventually reach the age of 100 + years in the future. In this regard, however, EL remains a rare phenotype worldwide, even in Japan only 25000 centenarians were alive in 2006 [10]. Therefore, the probability of having one potential centenarian in our control cohort is low. In addition, functional studies in model organisms shall be undertaken to unveil the role on EL of the genes that we studied here. We are aware that the cross-sectional nature of our design precludes conclusions on causality. In addition, we focused on EL as a categorical trait (i.e., centenarians versus non-centenarians) and life-span was not considered as a continuous variable (implying that some information could be lost). Finally, caution should be exercised when attempting to extrapolate our findings as being representative to the entire Japanese population. Convenience sampling was used (notably of healthy controls), which is prone to bias (because of population stratification).

In summary, our data showed a virtual absence of the variant (K) allele in MSTN rs1805086 in Japanese population, and no differences in allele/genotype frequencies in ACTN3 rs1815739 among centenarians and healthy controls of this country. More research is needed to unveil the role of these two genes involved in muscle mass conservation and metabolic efficiency, as well as in other gene variants involved in the evolutionary adaptive processes towards a more...
efficient, oxidative metabolic profile that might characterize the Japanese population, especially its most long-lived individuals.

Acknowledgments
This work was supported in part by grants from the Grant-in-Aid for Scientific Research (B) (15H03081 to N.F.) program of the Ministry of Education, Culture, Sports, Science and Technology and by a grant-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of Japan (to M.M.). Research in the field by A. Lucia is supported by Fondo de Investigaciones Sanitarias (FIS, grant # PI15/00558) and Fondos Feder as well as by Cátedra Real Madrid-Universidad Europea (grant # 2015/02RM). H. Pareja-Galeano is supported by a grant from Cátedra Real Madrid-Universidad Europea (grant # 2016/RM02). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References

1. Garatachea N, Lucia A. Genes and the ageing muscle: a review on genetic association studies. Age (Dordr). 2013; 35(1):207–33. Epub 2011/11/01. doi: 10.1007/s11357-011-9327-0 PMID: 22037866

2. McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature. 1997; 387(6628):83–90. Epub 1997/05/01. doi: 10.1038/37083a0 PMID: 919826

3. Lee SJ, McPherron AC. Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci U S A. 2001; 98(16):9306–11. Epub 2001/07/19. doi: 10.1073/pnas.151270098 PMID: 11459935

4. Lournaye A, de Barsy M, Nachit M, Lause P, Frateur L, van Maanen A, et al. Role of Activin A and myostatin in human cancer cachexia. J Clin Endocrinol Metab. 2015; 100(5):2030–8. Epub 2015/03/10. doi: 10.1210/jc.2014-4318 PMID: 25751105

5. Garatachea N, Pinos T, Camara Y, Rodriguez-Romo G, Emanuele E, Ricevuti G, et al. Association of the K153R polymorphism in the myostatin gene and extreme longevity. Age (Dordr). 2013; 35(6):2445–54. Epub 2013/01/29. doi: 10.1007/s11357-013-9513-3 PMID: 23354683

6. Garatachea N, Pareja-Galeano H, Sanchis-Gomar F, Santos-Lozano A, Fiuza-Luces C, Moran M, et al. Exercise attenuates the major hallmarks of aging. Rejuvenation Res. 2015; 18(1):57–89. Epub 2014/11/29. doi: 10.1089/rej.2014.1623 PMID: 25431878

7. North KN, Yang N, Wattanasirichaloong D, Mills M, Easteal S, Beggs AH. A common nonsense mutation results in alpha-actinin-3 deficiency in the general population. Nat Genet. 1999; 21(4):353–4. Epub 1999/04/07. doi: 10.1038/7675 PMID: 10192379

8. MacArthur DG, Seto JT, Raftery JM, Quinlan KG, Huttley GA, Hook JW, et al. Loss of ACTN3 gene function alters mouse muscle metabolism and shows evidence of positive selection in humans. Nat Genet. 2007; 39(10):1261–5. Epub 2007/09/11. doi: 10.1038/ng2122 PMID: 17828264

9. Wisloff U, Najjar SM, Ellingsen O, Haram PM, Swoap S, Al-Share Q, et al. Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. Science. 2005; 307(5708):418–20. Epub 2005/01/22. doi: 10.1126/science.1108177 PMID: 15662013

10. Takayama M, Hirose N, Aral Y, Gondo Y, Shimizu K, Ebihara Y, et al. Morbidity of Tokyo-area centenarians and its relationship to functional status. J Gerontol A Biol Sci Med Sci. 2007; 62(7):774–82. Epub 2007/07/20. PMID: 17634326

11. Garatachea N, Emanuele E, Calero M, Fuku N, Arai Y, Abe Y, et al. ApoE gene and exceptional longevity: Insights from three independent cohorts. Exp Gerontol. 2014; 53:16–23. Epub 2014/02/19. doi: 10.1016/j.exger.2014.02.004 PMID: 24334555

12. Murakami H, Iemitsu M, Fuku N, Sanada K, Gando Y, Kawakami R, et al. The Q223R polymorphism in the leptin receptor associates with objectively measured light physical activity in free-living Japanese. Physiol Behav. 2014; 129:199–204. Epub 2014/03/19. doi: 10.1016/j.physbeh.2014.02.053 PMID: 24631298

13. Szlama G, Trexler M, Buday L, Pothal L, Katlmyr K. K153R polymorphism in myostatin gene increases the rate of promyostatin activation by furin. FEBS Lett. 2015; 589(3):295–301. Epub 2014/12/30. doi: 10.1016/j.febslet.2014.12.011 PMID: 25543063

14. Li X, Wang SJ, Tan SC, Chew PL, Liu L, Wang L, et al. The A55T and K153R polymorphisms of MSTN gene are associated with the strength training-induced muscle hypertrophy among Han Chinese men. J Sports Sci. 2014; 32(9):883–91. Epub 2014/02/01. doi: 10.1080/02640414.2013.865252 PMID: 24479661

15. Mouisel E, Relizani K, Mille-Hamard L, Denis R, Hourde C, Agbulut O, et al. Myostatin is a key mediator between energy metabolism and endurance capacity of skeletal muscle. Am J Physiol Regul Integr Comp Physiol. 2014; 307(4):R444–54. Epub 2014/06/27. doi: 10.1152/ajpregu.00377.2013 PMID: 24965795

16. Fuku N, Pareja-Galeano H, Zempo H, Alis R, Aral Y, Lucia A, et al. The mitochondrial-derived peptide MOTS-c: a player in exceptional longevity? Aging Cell. 2015. Epub 2015/08/21. doi: 10.1111/acel.12389 PMID: 26289118

17. MacArthur DG, Seto JT, Chan S, Quinlan KG, Raftery JM, Turner N, et al. An Actn3 knockout mouse provides mechanistic insights into the association between alpha-actinin-3 deficiency and human athletic performance. Hum Mol Genet. 2008; 17(8):1076–86. Epub 2008/01/08. doi: 10.1093/hmg/ddm380 PMID: 18178581

18. Fiuza-Luces C, Ruiz JR, Rodriguez-Romo G, Santiago C, Gomez-Gallego F, Yvert T, et al. Are ‘endurance’ alleles ‘survival’ alleles? Insights from the ACTN3 R577X polymorphism. PLoS One. 2011; 6(3):e17558. Epub 2011/03/17. doi: 10.1371/journal.pone.0017558 PMID: 21407828
19. Yang N, MacArthur DG, Wolde Onywera VO, Boit MK, Lau SY, et al. The ACTN3 R577X polymorphism in East and West African athletes. Med Sci Sports Exerc. 2007; 39(11):1985–8. Epub 2007/11/08. doi: 10.1249/mss.0b013e31814844c9 PMID: 17986906

20. Amorim CE, Acuna-Alonzo V, Salzano FM, Bortolini MC, Hunemeier T. Differing evolutionary histories of the ACTN3*R577X polymorphism among the major human geographic groups. PLoS One. 2015; 10(2):e0115449. Epub 2015/02/24. doi: 10.1371/journal.pone.0115449 PMID: 25706920

21. Lewis SJ, Brunner EJ. Methodological problems in genetic association studies of longevity—the apolipoprotein E gene as an example. Int J Epidemiol. 2004; 33(5):962–70. Epub 2004/08/21. doi: 10.1093/ije/dyh214 PMID: 15319409

22. Ferrell RE, Conte V, Lawrence EC, Roth SM, Hagberg JM, Hurley BF. Frequent sequence variation in the human myostatin (GDF8) gene as a marker for analysis of muscle-related phenotypes. Genomics. 1999; 62(2):203–7. Epub 1999/12/28. doi: 10.1006/geno.1999.5984 PMID: 10610713

23. Tosun Tasar P, Sahin S, Karaman E, Oz A, Ulusoy MG, Duman S, et al. Myostatin Gene Polymorphism in an Elderly Sarcopenic Turkish Population. Genet Test Mol Biomarkers. 2015; 19(8):457–60. Epub 2015/06/06. doi: 10.1089/gtmb.2015.0033 PMID: 26046327

24. Fernandez-Santander A, Valveny N, Harich N, Kandil M, Luna F, Martin MA, et al. Polymorphisms influencing muscle phenotypes in North-African and Spanish populations. Ann Hum Biol. 2012; 39(2):166–9. Epub 2012/02/14. doi: 10.3109/03014460.2012.657243 PMID: 22324844