The rs1333049 polymorphism on locus 9p21.3 and extreme longevity in Spanish and Japanese cohorts

Tomàs Pinós · Noriyuki Fuku · Yolanda Cámara · Yasumichi Arai · Yukiko Abe · Gabriel Rodríguez-Romo · Nuria Garatachea · Alejandro Santos-Lozano · Elisabet Miro-Casas · Marisol Ruiz-Meana · Imanol Otaegui · Haruka Murakami · Motohiko Miyachi · David Garcia-Dorado · Kunihiko Hinohara · Antoni L. Andreu · Akinori Kimura · Nobuyoshi Hirose · Alejandro Lucia

Abstract The rs1333049 (G/C) polymorphism located on chromosome 9p21.3 is a candidate to influence extreme longevity owing to its association with age-related diseases, notably coronary artery disease (CAD). We compared allele/genotype distributions of rs1333049 in cases (centenarians) and controls (younger adults, without (healthy) or with CAD) in two independent cohorts: Spanish (centenarians: $n=152$, 128 women, 100–111 years; healthy controls: $n=343$, 212 women, age <50 years; CAD controls: $n=98$, 32 women, age ≤65 years) and Japanese (centenarians: $n=742$, 623 women, 100–115 years; healthy controls: $n=920$, 511 women, < 60 years; CAD controls: $n=395$, 45 women, age ≤65 years). The frequency of the "risk" C-allele tended to be lower in Spanish centenarians (47.0 %) than in their healthy (52.9 %, $P=0.088$) or CAD controls (55.1 %, $P=0.078$), and significant differences were found in genotype distributions ($P=0.034$ and $P=0.045$), with a higher frequency of the GG genotype in cases than in both healthy and CAD

T. Pinós · Y. Cámara · A. L. Andreu
Departament de Patología Mitocondrial i Neuromuscular, Institut de Recerca Hospital Universitari Vall d'Hebron, Barcelona, Spain

N. Fuku
Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

Y. Arai · Y. Abe · N. Hirose
Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

G. Rodríguez-Romo
INEF, Universidad Politécnica, Madrid, Spain

N. Garatachea
Facultad de Ciencias de la Salud y del Deporte, Universidad de Zaragoza, Huesca, Spain

A. Santos-Lozano
Departmento de Ciencias Biomédicas, Universidad de León, León, Spain

E. Miro-Casas · M. Ruiz-Meana · I. Otaegui · D. Garcia-Dorado
Departamento de Cardiología, Hospital Universitari Vall d'Hebron, Barcelona, Spain

controls as well as a lower proportion of the CG genotype compared with healthy controls. In the Japanese cohort, the main finding was that the frequency of the C-allele did not differ between centenarians (46.4 %) and healthy controls (47.3 %, $P=0.602$), but it was significantly lower in the former than in CAD controls (57.2 %, $P<0.001$). Although more research is needed, the present and recent pioneer findings (*Rejuvenation Res* 13:23–26, 2010) suggest that the rs1333049 polymorphism could be among the genetic contributors to exceptional longevity in Southern European populations, albeit this association does not exist in the healthy (CAD-free) Japanese population.

Keywords Centenarians · Genetics · Coronary artery disease · *CDKN2A* · *CDKN2B*

Introduction

Centenarians are the survival tail of the population (with a lifespan at least 15–20 years higher than the average westerner) and a model of healthy aging (Christensen et al. 2008). Such exceptional longevity is a partly heritable phenotype, with several candidate gene variants exerting a potential effect, e.g., polymorphisms associated with the risk of chronic, age-related diseases that are prominent causes of mortality among westerners, notably several types of cancer and coronary artery disease (CAD) (Ruiz et al. 2012).

H. Murakami · M. Miyachi
Department of Health Promotion and Exercise, National Institute of Health and Nutrition,
Tokyo, Japan

K. Hinohara · A. Kimura
Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University,
Tokyo, Japan

A. Lucia (✉)
Universidad Europea and Instituto de Investigación Sanitaria Hospital 12 de Octubre (i+12),
Madrid, Spain
e-mail: alejandro.lucia@uem.es

T. Pinós · Y. Cámara · A. L. Andreu
CIBER de Enfermedades Raras (CIBERER),
Barcelona, Spain

One candidate to influence exceptional longevity is the single nucleotide polymorphism (SNP) rs1333049 (G/C) located on chromosome 9p21.3, a locus outside of annotated genes that confers susceptibility to CAD and myocardial infarction as shown with recent genomewide scanning (McPherson 2010; Palomaki et al. 2010; Samani et al. 2007; Wellcome Trust Case Control Consortium 2007). This increased risk is independent of traditional risk factors including gender, age, obesity, smoking, hypertension, and hyperlipidemia (Schunkert et al. 2008). The C-allele (or the CC genotype) of rs1333049 has been associated with the risk or progression of CAD (Angelakopoulou et al. 2012; Bressler et al. 2010; Dandona et al. 2010; Ellis et al. 2010; Karvanen et al. 2009; McPherson 2010; Mendonca et al. 2011; Muendlein et al. 2009; O'Donnell et al. 2011; Palomaki et al. 2010; Plichart et al. 2012; Samani et al. 2009; Samani et al. 2007; Schunkert et al. 2008; Wellcome Trust Case Control Consortium 2007), and related disease phenotypes such as myocardial infarction (Buysschaert et al. 2010; Helgadottir et al. 2007; Koch et al. 2011; Scheffold et al. 2011; Shen et al. 2008b), atherosclerosis progression (Ye et al. 2008), peripheral arterial disease (Cluett et al. 2009), atherothrombotic manifestations (Arlestig and Rantapaa-Dahlqvist 2012), abdominal aortic aneurysm (Biros et al. 2010; Bown et al. 2008; Helgadottir et al. 2008), ischemic stroke (Karvanen et al. 2009; Smith et al. 2009), vascular dementia (besides Alzheimer's disease) (Emanuele et al. 2011) or type 2 diabetes (Saxena et al. 2007). The aforementioned associations were obtained essentially in populations of Caucasian descent (North Americans or individuals from various European locations) and have been recently replicated in Asiatic cohorts, e.g., the rs1333049 SNP has been linked with CAD in Asian Indians (Maitra et al. 2009) and Japanese (Hinohara et al. 2008), with CAD (Wang et al. 2010), myocardial infarction (Peng et al. 2009), and coronary plaque progression in Chinese people (Yu et al. 2010) and with myocardial infarction in Japanese (Hiura et al. 2008), South Koreans (Shen et al. 2008a) and Pakistanis (Saleheen et al. 2010).

In old Caucasians (>71 years), the C-allele has been associated with excess mortality independent of CAD risk factors (Dutta et al. 2011). A recent candidate–gene association study by Emanuele et al. reported a lower frequency of the risk C-allele of rs1333049 in 80 Italian centenarians compared to both healthy or

diseased (affected with acute myocardial infarction) young controls and thus provided preliminary evidence of association between this polymorphism and extreme longevity (Emanuele et al. 2010). Of note, the centenarians of the pioneer report by Emanuele et al. were free of CAD and dementia. It thus remains to be more clearly determined to what extent the potential effect of rs1333049 on extreme longevity depends mainly on its association with CAD/dementia risk or on other potential mechanisms. This question can be answered, at least partly, by studying centenarians who are not free of these two conditions and both healthy and diseased younger controls. In addition, it is important to corroborate previous findings obtained in candidate–gene association studies using replication, independent cohorts of different ethnic/geographic origins. It was therefore the purpose of our study to determine, using a case–control design, the association of the rs1333049 SNP and extreme longevity in a Spanish (Caucasian) cohort and an independent (Japanese) cohort. Cases were "normal" centenarians (i.e., not necessarily free of CAD or dementia), and we studied two control groups of younger adults, i.e., without (*healthy*) or with CAD. Based on the aforementioned studies and in recent research with centenarians from Southern Europe (Emanuele et al. 2010), we hypothesized that the risk C-allele rs1333049 would be less frequent in centenarians compared with ethnically matched younger controls, at least if these controls have CAD.

Methods

Participants—Spanish cohort

Written consent was obtained from each participant. The study protocol was approved by the institutional ethics committee (Universidad Europea de Madrid, Spain) and was in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2008). All the study participants were of the same Caucasian (Spanish) descent for three or more generations; the majority (~90 %) of them were born and lived most of their lives in the same areas of Spain (Meseta Castellana, ~600 m altitude).

Cases (centenarians, n= 175) During 2009–2012, we obtained DNA from saliva samples in centenarians of

both genders (31 men, 144 women; age range 100–111 years) living mainly in nursing residencies of the Spanish central area (Meseta Castellana). This cohort included the oldest European individual (111 years old) alive in June 2012 (http://www.grg.org/Adams/E.HTM), and ~9 % of the cohort was aged ≥105 years. The most prevalent diseases were osteoarthritis (67 %), hypertension (57 %), dementia (49 %), and CAD (29 %). Two centenarians were free of any diagnosed disease. Approximately 13 % of the centenarians were virtually independent during daily living without assistance from other persons, i.e., their Barthel score was ≥80 and their score in the mini-mental state evaluation was >23 (Christensen et al. 2008).

Healthy controls (n= 362) Inclusion criteria for this group were (1) man or woman aged ≤50 years and (2) free of any diagnosed cardiometabolic disease. We extracted genomic DNA from saliva samples of 143 men and 219 women during the years 2008–2010.

Controls with CAD (n= 99) Inclusion criteria for this group were (1) man or woman aged ≤65 years and (2) with diagnosed CAD. We extracted genomic DNA from patients' (66 men, 33 women) saliva samples in the cardiology department of two Spanish hospitals (Vall d'Hebron, Barcelona and Gregorio Marañón, Madrid) during the summer of 2013.

Participants—Japanese cohort

Written consent was obtained from each participant. The study protocol was approved by the corresponding institutional ethics committee (National Institute of Health and Nutrition and Medical Research Institute, Tokyo Medical Dental University, Japan, for controls and Keio University, Japan, for centenarians) and was in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2008). All of the study participants were of the same Asian (Japanese) descent.

Cases (centenarians, n= 754) The Japanese cohort of cases was gathered from two prospective cohorts: the Tokyo Centenarians Study (TCS) and the Semi-supercentenarians Study in Japan (SSC-J). A detailed description of population-based recruitments for the TCS has been previously reported (Gondo et al.

2006). The TCS cohort included 304 centenarians (65 men, 239 women) aged 100–108 years.

The SSC-J is a nationwide longitudinal survey mainly consisting of individuals aged 105 years or older, which started in 2002. In 2002, we recruited 135 SSC in whole Japan. After 2002, our recruitment strategy has relied on responses to local governments and nursing homes in the whole country and direct inquiries to our research team. Consequently, a total of 450 centenarians (58 men, 392 women) were enrolled into SSC-J by the end of November 2011.

The phenotype and disease characteristics of the Japanese centenarians are described elsewhere (Takayama et al. 2007), and the prevalence of hypertension, CAD, and dementia was of 63.6, 28.8, and 59.4 %, respectively.

Healthy controls (n= 920) Inclusion criteria for this group were man or woman aged <60 years and free of any diagnosed stroke, cardiac disease, and chronic renal failure. During the years 2008–2012, genomic DNA was extracted from venous blood samples in 143 men and 356 women (National Institute of Health and Nutrition, Japan; see later discussion). We also used data from blood samples previously obtained in 409 men and 511 women (during year 2008) in the Department of Molecular Pathogenesis (Medical Research Institute, Tokyo Medical and Dental University, Japan), most of which have been included in another study (Hinohara et al. 2008).

Controls with CAD (n= 395) Inclusion criteria for this group were (1) man or woman aged ≤65 years and (2) with diagnosed CAD. We used data from blood samples of 350 men and 45 women who were also previously genotyped (during the year 2008) in the Department of Molecular Pathogenesis (Medical Research Institute, Tokyo Medical and Dental University, Japan), most of which have been included in a published report on the association of rs1333049 and CAD risk (Hinohara et al. 2008).

Genotype assessment

In line with previous research (Emanuele et al. 2010), we focused only on the rs1333049 marker because the SNPs rs1333049, rs10757274, and rs10757278 on chromosome 9p21.3 identified as risk loci for cardiovascular disease in previous studies are in tight

linkage disequilibrium, and their associations with a selected phenotype are generally identical (Farzaneh-Far et al. 2009). Genotyping was performed specifically for research purposes.

Spanish cohort Genotyping was performed in the genomics laboratory of Vall d'Hebron Hospital (Barcelona, Spain) during the summer and autumn of 2012 (centenarians and healthy controls) and summer of 2013 (CAD controls). The two experienced researchers in charge of genotyping were totally blinded to the participants' identities, i.e., saliva samples were tracked solely with bar coding and personal identities were only made available to the main study researcher who was not involved in actual genotyping. We used the classical phenol-chloroform DNA extraction protocol with alcoholic precipitation. Genomic DNA was resuspended in 50 µl milli-Q H_2O and stored at −20 °C. We used custom-designed Taqman® SNP genotyping assays (assay ID C_1754666_10) (Applied Biosystems, Foster City, CA, USA). All samples were run in a 7.500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The analysis had a pre-read and post-read step of the plate consisting of 1 min at 60 °C before and after the PCR cycle. The cycle conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 92 °C, and 1 min at 60 °C. To ensure proper internal control, for each genotype analysis, we used positive and negative controls from different DNA aliquots which were previously genotyped by the same method.

Japanese cohort For all centenarians, as well as for the healthy controls whose samples were genotyped in the genomics laboratory of the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan), total DNA was isolated from venous blood by the use of QIAamp DNA Blood Maxi Kit (QIAGEN, Hilden, Germany). Genotyping was performed during Winter 2013 using custom-designed Taqman® SNP genotyping assays (assay ID C_1754666_10) (Applied Biosystems, Foster City, CA, USA). All samples were run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A total of 5 µl genotyping mixture contained 2.5 µl GTXpress™ master mix, 0.125 µl assay mix (40×), and 1.375 µl distilled water to mix with 1 µl genomic DNA (10 ng/µl) in each reaction. One or two negative controls were included on each plate.

TaqMan® assays for genotype calls were analyzed using StepOne™ Software v2.1 (Applied Biosystems, Foster City, CA, USA).

For healthy and CAD controls gathered from the Department of Molecular Pathogenesis (Medical Research Institute, Tokyo Medical and Dental University, Japan), venous blood samples and Taqman® SNP genotyping assay (Applied Biosystems, Foster City, CA, USA) were also used for rs1333049 genotyping in PCR products with premier pairs rs1333049-F (5′-CCTTCATGCTATT TTGAGGAG) and rs1333049-R (5′-GGAGATAAGT TGAGAATGTCA) (Hinohara et al. 2008).

Statistical analysis

All statistical analyses were performed using PASW (v. 18.0 for WINDOWS, Chicago, IL, USA) except for statistical power, which we calculated with StatMate software, version 2.0 (GraphPad, San Diego, CA, USA) (Emanuele et al. 2010; Minoretti et al. 2006). We tested Hardy–Weinberg equilibrium and compared the genotype/allele frequencies of cases vs. healthy/CAD controls within each cohort (Spanish and Japanese) using the χ^2 test with α set at 0.05. Within each cohort of cases, we also compared allele/genotype distributions depending on disease status, i.e., centenarians with CAD and/or dementia vs. those who were free of these two conditions. We used logistic regression analysis to analyze the association between alleles and longevity within each of the two cohorts after adjusting for sex and age.

Results

Spanish cohort Failure rate of genotyping (due in all cases to insufficient amount of DNA) was 13.1 % in cases and 5.2 % and 1.01 % in healthy and CAD controls, respectively. Thus, the final number of cases and healthy and CAD controls entered in the analyses was $n=152$ (24 men, 128 women), $n=343$ (131 men, 212 women), and $n=98$ (66 men, 32 women), respectively. Genotype distributions of rs1333049 met Hardy–Weinberg equilibrium in the two control groups ($P>0.05$), but not in the cases' group ($P<0.05$). The frequency of the "risk" C-allele we found in healthy controls (52.9 %) was similar to that previously reported in other control cohorts of Southern European

Caucasians, e.g., 52 % in Italians from the Chianti (Toscana) region (Cluett et al. 2009), 53.6 % in another Italian cohort (Emanuele et al. 2011), or 53 % in Portuguese (Mendonca et al. 2011).

Allele/genotype distributions in the three Spanish groups are shown in Table 1. The main finding was that the frequency of the C-allele tended to be lower in cases than in healthy ($P=0.088$) or CAD controls ($P=0.078$), and significant differences were found in genotype distributions ($P=0.034$ and $P=0.045$), with a higher frequency of the GG genotype in cases than in both healthy and CAD controls and a lower proportion of the CG genotype in cases compared with healthy controls. On the other hand, allele and genotype distributions did not differ between centenarians with CAD and/or dementia vs. those who were free of these two diseases (Table 2). Results of logistic regression are shown in Table 3.

Japanese cohort Failure rate of genotyping was 1.6 % in cases and 0 % and 1.8 % in healthy and CAD controls, respectively. Thus, the final number of cases and healthy and CAD controls entered in the analyses was $n=742$ (119 men, 623 women), $n=920$ (409 men, 511 women), and $n=395$ (350 men, 45 women), respectively. Genotype distributions met Hardy–Weinberg equilibrium in the three groups ($P>0.05$). The frequency of the risk C-allele in healthy controls (47.3 %) was comparable to that reported in another control cohort of Japanese people, i.e., 49.1 % (Hiura et al. 2008).

Allele/genotype distributions in the three Japanese groups are shown in Table 1. The main finding was that allele ($P=0.602$) and genotype frequencies were similar in cases and healthy controls ($P=0.554$). In contrast, significant differences were found in allele and genotype distributions between cases and CAD controls (both $P<0.001$), with the "high-risk" C-allele and CC genotype being less frequent and the "low-risk" GG genotype more frequent in the former. On the other hand, allele and genotype distributions did not differ between centenarians with CAD and/or dementia vs. those free of these two conditions (Table 2). Likewise, allele ($P=0.251$) and genotype distributions ($P=0.188$) did not differ between TCS and SSC-J centenarians' subgroups (TCS: CC= 25.9 %, CG=47.2 %, GG=26.9 %; SSC-J: CC= 20.0 %, CG=52.5 %, GG=27.5 %). Results of logistic regression are shown in Table 3.

Table 1 Allele and genotype distributions of the rs1333049 (G/C) SNP in the two study cohorts

	Cases (centenarians)	Healthy controls	CAD controls
Spanish cohort			
Sample size per group (n)	152	343	98
χ^2 and P-value for allele frequency comparison (cases vs. controls)		$\chi^2=2.911$ $P=0.088$	$\chi^2=3.099$ $P=0.078$
Allele frequency, n (%)	C=143 (47.0 %) G=161 (53.0 %)	C=363 (52.9 %) G=323 (47.1 %)	C=108 (55.1 %) G=88 (44.9 %)
χ^2 and P-value for genotype frequency comparisons (cases vs. controls)		$\chi^2=6.766$ $P=0.034$	$\chi^2=6.220$ $P=0.045$
Genotype frequency, n (%)	CC=45(29.6 %) CG=53(34.9 %) GG=54(35.5 %)	CC=105(30.6 %) CG=153(44.6 %)* GG=85(24.8 %)*	CC=31(31.6 %) CG=46(46.9 %) GG=21(21.4 %)*
Japanese cohort			
Sample size per group (n)	742	920	395
χ^2 and P-value for allele frequency comparison (cases vs. controls)		$\chi^2=0.272$ $P=0.602$	$\chi^2=23.99$ $P<0.001$
Allele frequency, n (%)	C=689 (46.4 %) G=795 (53.6 %)	C=871 (47.3 %) G=969 (52.7 %)	C=452 (57.2 %) G=338 (42.8 %)
χ^2 and P-value for genotype frequency comparisons (cases vs. controls)		$\chi^2=1.181$ $P=0.554$	$\chi^2=24.60$ $P<0.001$
Genotype frequency, n (%)	CC=158 (21.3 %) CG=373 (50.3 %) GG=211 (28.4 %)	CC=193 (21.0 %) CG=485 (52.7 %) GG=242 (26.3 %)	CC=133(33.7 %)* CG=186 (47.1 %) GG=76 (19.2 %)*

Significant P-values are in italic

CAD coronary artery disease, *SNP* single nucleotide polymorphism

*$P<0.05$ for the comparison of controls vs. cases for the specific genotype

Statistical power Based on the observed prevalence of the minor G-allele of the rs1333049 SNP in the CAD control group, the sample size of the Spanish cohort ensured a power of 0.80 (with $\alpha=0.05$) to detect an OR of 1.48 for being a centenarian between G-allele carriers and non-carriers. Based on the observed prevalence of the minor G-allele in the CAD control group, the sample size of the Japanese cohort ensured a power of 0.80 (with $\alpha=0.05$) to detect an OR of 1.20 for being a centenarian between G-allele carriers and non-carriers.

Discussion

Our main finding was that the "risk" C-allele of the rs1333049 (G/C) SNP located on chromosome 9p21.3 was associated with exceptional longevity in the Spanish cohort that we studied, with a trends towards a lower proportion of this allele in the group of centenarians compared with their healthy and CAD controls. We also found a higher frequency of the GG genotype in the Spanish cases than in their healthy and CAD controls as well as a lower proportion of the CG genotype compared with healthy controls. Further, the frequency of the C-allele (47.0 %) in our cohort of centenarians, most of whom were not disease-free, was similar to that recently reported (46.3 %) in 80 "super-healthy" Italian centenarians (men and women, 100–105 years) by Emanuele et al. (2010). Although our findings overall corroborate and extend pioneer data by Emanuele et al. in another Southern European (Mediterranean) cohort, it must be emphasized that (1) the putative association between rs1333049 and extreme longevity was not so strong in our cohort and (2) the findings were not replicated in a cohort of a totally different ethnic and geographic (Japanese) origin. In the latter, the "risk" C-allele was in fact the minor allele in both centenarians and healthy controls

Table 2 Allele and genotype distributions of the rs1333049 (G/C) SNP in cases (centenarians) depending on disease status

	Centenarians free of CAD and dementia	Centenarians with CAD and/or dementia
Spanish cohort		
Sample size per group (n)	54	98
χ^2 and P-value for allele frequency comparison		$\chi^2 = 0.005$ $P = 0.942$
Allele frequency, n (%)	C=50 (46.3 %) G=58 (53.7 %)	C=58 (51.0 %) G=66 (49.0 %)
χ^2 and P-value for genotype frequency comparison		$\chi^2 = 0.543$ $P = 0.762$
Genotype frequency, n (%)	CC=15 (27.8 %) CG=20 (37.0 %) GG=19 (35.2 %)	CC=31 (31.6 %) CG=38 (38.8 %) GG=29 (29.6 %)
Japanese cohort		
Sample size per group (n)	259	483
χ^2 and P-value for allele frequency comparison		$\chi^2 = 0.361$ $P = 0.548$
Allele frequency, n (%)	C=246 (47.5 %) G=272 (52.5 %)	C=443 (45.9 %) G=523 (54.1 %)
χ^2 and P-value for genotype frequency comparison		$\chi^2 = 2.138$ $P = 0.343$
Genotype frequency, n (%)	CC=62 (23.9 %) CG=122 (47.1 %) GG=75 (29.0 %)	CC=96 (19.9 %) CG=251 (52.0 %) GG=136 (28.1 %)

CAD coronary artery disease, *SNP* single nucleotide polymorphism

(~46–47 %, whereas its frequency is typically above 50 % in Caucasians), and the allele/genotype distribution of centenarians only differed from that of CAD controls.

Table 3 Results of the association between the rs1333049 (G/C) SNP and the status of being a centenarian in the two cohorts

	Spanish cohort		Japanese cohort	
	Centenarians vs. healthy controls	Centenarians vs. CAD controls	Centenarians vs. healthy controls	Centenarians vs. CAD controls
Dominant model				
CC+CG vs. GG	0.625 (95 % CI 0.409, 0.955; *P= 0.030*)	0.549 (95 % CI 0.269, 1.121; *P*=0.100)	0.932 (95 % CI 0.743, 1.170; *P*=0.544)	0.654 (95 % CI 0.436, 0.983; *P= 0.041*)
Recessive model				
CC vs. CG+GG	0.932 (95 % CI 0.607, 1.429; *P*=0.745)	1.106 (95 % CI 0.559, 2.191; *P*=0.772)	1.120 (95 % CI 0.872, 1.439; *P*=0.374)	0.696 (95 % CI 0.475, 1.022; *P*=0.064)
Co-dominant				
CG vs. GG	0.587 (95 % CI 0.362, 0.950; *P= 0.030*)	0.413 (95 % CI 0.191, 0.890; *P= 0.024*)	0.892 (95 % CI 0.701, 1.136; *P*=0.354)	0.708 (95 % CI 0.450, 1.112; *P*=0.134)
CG vs. CC	1.185 (95 % CI 0.735, 1.910; *P*=0.487)	1.766 (95 % CI 0.863, 3.615; *P*=0.120)	1.165 (95 % CI 0.0895, 1.517; *P*=0.256)	0.774 (95 % CI 0.515, 1.164; *P*=0.219)

The OR values were adjusted for sex and age. Significant P-values are in italic

CAD coronary artery disease, *CI* confidence intervals, *OR* odds ratio, *SNP* single nucleotide polymorphism

There is evidence indicating an association, especially in Caucasian cohorts, of the rs1333049 SNP we studied here and risk of CAD (Wellcome Trust Case Control Consortium 2007; Angelakopoulou et al. 2012; Bressler et al. 2010; Dandona et al. 2010; Ellis et al. 2010; Karvanen et al. 2009; McPherson 2010; Mendonca et al. 2011; Muendlein et al. 2009; O'Donnell et al. 2011; Palomaki et al. 2010; Plichart et al. 2012; Samani et al. 2009; Samani et al. 2007; Schunkert et al. 2008) and vascular dementia (besides Alzheimer's disease) (Emanuele et al. 2011), with the C-allele exerting an unfavorable effect. The rs1333049 SNP has been linked with CAD-related phenotypes in non-Caucasian cohorts, and especially with myocardial infarction in Japanese people, with a frequency of the risk C-allele of 52.8 % in 589 individuals who had suffered myocardial infarction (Hiura et al. 2008). Further, the C-allele was recently associated with excess mortality independent of CAD risk factors in old (≥71 years) individuals (Dutta et al. 2011).

The fact that we found significant differences in the genotype distribution of Spanish centenarians vs. their *healthy* (CAD-free) controls suggests that the potential influence of the rs1333049 SNP on exceptional longevity goes beyond its documented influence on CAD. With regards to this, although the functional significance of the rs1333049 SNP remains to be clearly elucidated, and the 9p21.3 locus does not contain any known expressed gene products, it lies adjacent to *CDKN2A* and *CDKN2B*, two important cell cycle regulators. Both are cyclin-dependent kinase inhibitor genes, which regulate the G1 phase of the cell cycle by inhibition of cyclin/cyclin-dependent kinases, which raises the possibility that rs1333049 may modulate the rate of cellular aging (Emanuele et al. 2010). In fact, recent genomewide scanning has suggested an association between *CDKN2A*/*CDKN2B* and extreme longevity, and CDKN2A performs a key step in the p53 pathway, the latter playing a crucial role in inducing cellular senescence (Sebastiani et al. 2012).

On the other hand, a limitation from our study stems from the relatively high rate of genotyping failure in the Spanish cohort, particularly in cases. The main reason might lie on the fact that DNA extraction from saliva in frail individuals such as centenarians might result in small amounts of DNA available for analyses (we did not collect blood samples based on ethical constraints). Thus, blood sampling is likely preferable

in this population segment. In contrast, the major strengths of our study were the clear definition that we used for the criterion of exceptional longevity (with all cases being centenarians or "semi-supercentenarians") and the fact that the analyses were performed in two totally independent cohorts from both ethnic and geographic perspectives.

In summary, our data overall replicate and extend the original data by Emanuele et al. (2010) in that they suggest that the rs1333049 (G/C) polymorphism in chromosome 9p21.3 polymorphism could be among the numerous variants exerting a certain contribution to exceptional longevity in Mediterranean (Southern European) cohorts. However, such association was not replicated in the healthy Japanese population and further corroboration is needed in other ethnic/geographic groups worldwide.

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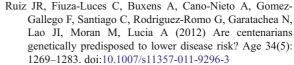
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