FNDC5 (irisin) gene and exceptional longevity: a functional replication study with rs16835198 and rs726344 SNPs

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Abstract Irisin might play an important role in reducing the risk of obesity, insulin resistance, or several related diseases, and high irisin levels may contribute to successful aging. Thus, the irisin precursor (FNDC5) gene is a candidate to influence exceptional longevity (EL), i.e., being a centenarian. It has been recently shown that two single-nucleotide polymorphisms (SNPs) in the FNDC5 gene, rs16835198 and rs726344, are associated with in vivo insulin sensitivity in adults. We determined luciferase gene reporter

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activity in the two above-mentioned SNPs and studied genotype distributions among centenarians ($n=175$, 144 women) and healthy controls ($n=347$, 142 women) from Spain. We also studied an Italian [79 healthy centenarians (40 women) and 316 healthy controls (156 women)] and a Japanese cohort [742 centenarians (623 women) and 499 healthy controls (356 women)]. The rs726344 SNP had functional significance, as shown by differences in luciferase activity between the constructs of this SNP (all $P\leq0.05$), with the variant *A*-allele having higher luciferase activity compared with the *G*-allele ($P=0.04$). For the rs16835198 SNP, the variant *T*-allele tended to show higher luciferase activity compared with the *G*-allele ($P=0.07$). However, we found no differences between genotype/allele frequencies of the two SNPs in centenarians versus controls in any cohort, and no significant association (using logistic regression adjusted by sex) between the two SNPs and EL. Further research is needed with different cohorts as well as with additional variants in the FNDC5 gene or in other genes involved in irisin signaling.

Keywords Myokines · Cardiometabolic · Disorders · Allele · Longevity · Centenarians

Introduction

Myokines are substances that are produced by skeletal muscles, especially induced by exercise, and modulate different metabolic processes locally or in other target tissues (Febbraio and Pedersen 2005; Duzova 2012). The peptidic myokine "irisin," secreted in response to activation of the peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1α), has been recently identified (Bostrom et al. 2012). The precursor of irisin is a type I transmembrane protein of skeletal muscle, the fibronectin type III domain-containing protein 5 (FNDC5) (Bostrom et al. 2012). Irisin acts in the white adipose tissue, promoting the acquisition of a brown adipocyte phenotype prone to energy expenditure (Polyzos et al. 2013). Accordingly, irisin might play an important role in reducing the risk of obesity (Spiegelman 2013), insulin resistance (Bostrom et al. 2012) [included in aged populations (Sanchis-Gomar et al. 2014b)], diabetes (Spiegelman 2013), or several related diseases (Sanchis-Gomar et al. 2012; Hojlund

and Bostrom 2013; Sanchis-Gomar 2013; Sanchis-Gomar and Perez-Quilis 2014). It can also help to preserve vascular function and skeletal muscle mass (Spiegelman 2013; Bostrom and Fernandez-Real 2014). On the other hand, circulating irisin levels can predict telomere length in healthy adults (Rana et al. 2014). Great expectations have been created based on the above-mentioned findings (Elbelt et al. 2013; Villarroya 2013) with a new provocative concept, "irisinemia," been proposed to monitor metabolic disorders such as diabetes, obesity, or the metabolic syndrome (Sanchis-Gomar and Perez-Quilis 2013). We recently hypothesized that high irisinemia may contribute to successful aging (Emanuele et al. 2014). Serum irisin levels were in fact higher in healthy centenarians compared to young healthy controls and, especially, to young adults with precocious myocardial infarction (Emanuele et al. 2014).

For the above-mentioned reasons, the irisin precursor (FNDC5) gene is a candidate to influence the risk of cardiometabolic diseases as well as the aging process. Staiger et al. recently showed that two single-nucleotide polymorphisms (SNPs) in the FNDC5 gene, rs16835198 and rs726344, were associated with in vivo insulin sensitivity (Staiger et al. 2013). In this regard, human exceptional longevity (EL), that is, being a centenarian, is associated with insulin sensitivity/signaling (Barbieri et al. 2008; Brunet 2012), e.g., with genetic variation in the insulin-human forkhead box O3A (FOXO3A) pathway influencing EL (Willcox et al. 2008; Flachsbart et al. 2009). Nonetheless, to the best of our knowledge, no previous research has studied the potential functional consequences of FNDC5 SNPs and their influence on EL. Centenarians are the paradigm of healthy aging, as they have postponed if not avoided major age-related diseases, including cardiometabolic diseases, as well as the onset of disability (Garatachea et al. 2014).

The search for the gene variants that might influence the likelihood of reaching EL is of medical interest, particularly to help identifying potential targets of highly specific "antiaging" drugs. To gain insight into their functionality, here we determined luciferase gene reporter activity in the two above-mentioned FNDC5 SNPs, rs16835198 and rs726344. We then compared allele/genotype frequencies of rs16835198 and rs726344 among centenarians (cases) and disease-free controls of the same ethnic and geographic origin (Spanish cohort) as well as in two other

geographically and ethnically independent replication cohorts (from Italy and Japan).

Materials and methods

Functional analysis: luciferase reporter gene

The fragment, including the allele, was directly inserted into the pGL3-promoter at the restriction recognition sites MluI in the 5′ and XhoI in the 3′ (see below—in bold) of the sequences obtained from the genomes of the following:

1. One individual homozygous for the rs726344 C-allele and one homozygous for the rs726344 T-allele (see below—underlined)
2. One individual homozygous for the rs16835198 G-allele and one homozygous for the rs16835198 T-allele (see below—underlined)

rs726344-CC

ACGCGTTGTGAGGGCGGGGACAGTGTCTGG
TTCACCTGGGAGCCCCGCCTGGCCTGGTAG
CCGCTCGCAGGGCCTGTGGATGAGGCCGGG
CAGCAATGCCACTGCTCTCCTCATATCACCC
ATTTCACCCTCACAGACACCCGTGAGGTGG
AAGTTAATGTCCACAGCTTATAGGGAGCCA
AACTGAGCCTCAGAAAGGCTGACTTGCTCG
AG.

rs726344-TT

ACGCGTTGTGAGGGCGGGGACAGTGTCTGGTT
CACCTGGGAGCCCCGCCTGGCCTGGTAGCCGC
TCGCAGGGCCTGTGGATGAGGCCGGGCAGC
AATGCCACTGCTTTCCTCATATCACCCATTTCA
CCCTCACAGACACCCGTGAGGTGGAAGTTAAT
GTCCACAGCTTATAGGGAGCCAAACTGAGCCT
CAGAAAGGCTGACTTGCTCGAG.

rs16835198-GG

ACGCGTGGAGGATGCACCTTGGCTCCTATTGT
AGGAAGTAGGTAGAGTCTGGGAATCTTCTAAA
TTCTGTTTTTGGCAGATAAGGACACCACGACC
TAACCTCAGAGAATTAACTTGCCTGAGGTGT

TAGGTGATTACAAGGCAGGGTCTCCTGTCTCT
CAGACCAGCATTTCTCCCACAGGCCTGTGGTA
GTCTCCAAGTGAGGTCCTCGAG.

rs16835198-TT

ACGCGTGGAGGATGCACCTTGGCTCCTATTGT
AGGAAGTAGGTAGAGTCTGGGAATCTTCTAAA
TTCTGTTTTTGGCAGATAAGGACACCACGACC
TAACCTCAGATAATTAACTTGCCTGAGGTGTTA
GGTGATTACAAGGCAGGGTCTCCTGTCTCTCA
GACCAGCATTTCTCCCACAGGCCTGTGGTAGT
CTCCAAGTGAGGTCCTCGAG

Mice skeletal muscle C2C12 cell lines were used to represent muscle-specific expression. Cell cultures, transfections, and dual-luciferase reporter assays were performed as previously described (He et al. 2011). We used the pRL-SV40 vector as an internal control for variations in transfection efficiency. The pGL3-promoter vector without an insert was used as a negative control. The transfected cells were harvested after 48 h and assayed for firefly luciferase activity and renilla luciferase activity using the dual-luciferase reporter assay system (Promega Biotech, Beijing, China), as suggested by the manufacturer using a luminometer (TecanGenios Pro, Männedorf, Switzerland). From each measurement, relative luciferase activity was calculated by dividing the firefly luciferase activity reading by the renilla luciferase activity reading. Experiments were performed in triplicate. Relative luciferase activity values are expressed as the means±SD of the three different experiments.

Participants

Written consent was obtained from each participant. The study protocol was approved by the corresponding institutional ethics committees [European University of Madrid (Spanish cohort), University of Pavia (Italian cohort), and National Institute of Health and Nutrition, Medical Research Institute and Keio University (Japanese cohort)] and was in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2008).

Spanish cohort

Two groups of Spanish subjects (most born and living in the central area of Spain, *Meseta Castellana*) were

investigated: (i) 175 cases (centenarians, age range 100–111 years, 144 women, 31 men) and (ii) 347 healthy controls (20–50 years, 142 women, 205 men). All the Spanish participants were of the same Caucasian (Spanish) descent for ≥3 generations and all spoke Spanish as mother language. Centenarians were living mainly in nursing residencies of the Spanish central area, where they were recruited during years 2009–2012 after we had access to the ages' lists of the people living in the residencies. The participants' ages were ascertained by the dates of birth as stated on identity cards. This group included the oldest European individual (111 years) alive in June 2012 (http://www.grg.org/Adams/E.HTM), and ~7 % of the cohort was aged ≥105 years. The most prevalent diseases were osteoarthritis (66 %), hypertension (57 %), dementia (51 %), and cardiovascular disease (29 %). Only two centenarians were free of any diagnosed disease.

The DNA of 347 younger controls was collected from saliva samples during 2008–20012 in the European University (Madrid, Spain). This was a convenient sample composed of students and staff from this institution; all of them were free of any major cardiometabolic disease (including cardiovascular disease (CVD) or Alzheimer) and had no known family history of high longevity (90+ years), as reported in a questionnaire.

Italian cohort

Two groups of Italian subjects born and living in Northern Italy were studied: (i) 79 cases (healthy centenarians, 100–104 years, 40 women, 39 men) and (ii) 316 healthy controls (27–81 years, 156 women, 160 men). The participants' ages were defined by the dates of birth as stated on identity cards. All patients and controls were Caucasian whites ascertained to be of Italian descent. The criterion of "Italian descent" was met when all the parents and grandparents of an individual originated from Italy. The Italian centenarians were ascertained mainly via general practitioners in the community. These centenarians represent a convenience sample that has been previously described (Emanuele et al. 2010). The history of past and current diseases was accurately collected, checking the centenarians' medical documentation and the current drug therapy. Accordingly, all the Italian centenarians were free of major age-related diseases, i.e., severe cognitive impairment, clinically evident cancer, CVD, renal insufficiency, or severe physical impairment (only, part of this group had decreased visual or auditory acuity). Thus, all of the Italian centenarians were in apparently good health relative to their very advanced age.

Controls were in apparent good physical health, with exclusion criteria being the following: presence of major CVD or cerebrovascular disease, cancer, dementia, chronic autoimmune or inflammatory disorders, renal or hepatic failure, and major psychiatric disorders.

Japanese cohort

Two groups of Japanese subjects [of the same Asian (Japanese) descent] were studied: (i) 742 cases (centenarians, 100–116 years, 623 women, 119 men) and (ii) 499 healthy controls (23–59 years, 356 women, 143 men). The group of cases was gathered from to 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 consisting mainly of individuals aged 105 years or older, which started in 2002 (with $n=135$ SSC). After 2002, the recruitment strategy has relied on responses to local governments and nursing homes in the whole country, and direct inquires to our research team. Consequently, a total of 450 centenarians (58 men, 392 women) were enrolled in the SSC-J by the end of November 2011. The phenotype and disease characteristics of the Japanese centenarians are described elsewhere (Takayama et al. 2007), with a prevalence of hypertension, CVD, and dementia of 63.6, 28.8, and 59.4 %, respectively. Inclusion criteria for the control group, which was recruited during years 2008–2012 by advertising, were being man or woman aged <60 years, and free of diagnosed stroke, cardiac disease, and chronic renal failure (as reported in a questionnaire).

Genotype assessment

Spanish cohort

Genomic DNA was extracted from buccal cells according to standard phenol/chloroform procedures followed by alcohol precipitation. Allelic discrimination analysis was performed in the genetics laboratory of the

European University of Madrid with predesigned Life Technologies TaqMan® SNP Genotyping Assays on demand for rs16835198 (ID C_34204885_10) and rs726344 (ID C_927694_10). PCR amplification was performed using a StepOne™ Plus Real-Time PCR System (Life Technologies, Foster City, CA) with a denaturation stage at 95 °C for 10 min, 50 cycles of denaturation at 92 °C for 15 s, annealing/extension at 60 °C for 1 min, and a final extension stage of 30 s at 60 °C. Positive and negative controls were used in each run, and a random 20 % of the samples were repeated (where all the results matched with the initial genotyping). Two investigators independently reviewed all the results.

Italian cohort

Genomic DNA was purified from peripheral blood samples using the QiaAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genotyping was performed at the Cellular Pathophysiology and Clinical immunology Laboratory (University of Pavia using the TaqMan® rs726344 and rs16835198 SNP genotyping assays (Applied Biosystems, Foster City, CA, USA)). For quality control, genotyping analyses were done blind with respect to the age of the study participants, and a random 20 % of the samples were repeated. All genotyping repeated for quality control did not differ from the initial genotyping. Two investigators independently reviewed all results.

Japanese cohort

Total DNA was isolated from venous blood by use of QIAamp DNA Blood Maxi Kit (QIAGEN, Hilden, Germany). The rs16865198 was genotyped using TaqMan® SNP Genotyping Assays (Assay ID C__32747086_10) and StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) in the genomics laboratory of the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). 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).

The rs726344 polymorphism was not genotyped in the totality of the Japanese cohort because this polymorphism has not been detected in the Japanese population according to HapMap data and our preliminary analysis in 96 samples showed no presence of the variant A-allele.

Determination of serum irisin levels in the Italian cohort

Serum irisin levels were measured in Italian cases and controls in duplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) (Aviscera Biosciences, Santa Clara, CA, USA) in accordance with the manufacturer's instructions. The sensitivity of the assay was 0.2 ng/ml, the linear range of the standard was 5 to 500 ng/ml, and the intra- and inter-assay coefficients of variation were 4.2 and 6.8 %, respectively. The results of the cases' group have been recently published by our group (Emanuele et al. 2014).

Statistical methods

Allele frequencies of rs726344 and rs16835198 were calculated from genotypes using the gene-counting method. We tested Hardy–Weinberg equilibrium (HWE) and compared the genotype/allele frequencies of cases versus controls within each cohort (Spanish, Italian, and Japanese) using the χ^2 test with α set at 0.05. We used logistic regression analysis to analyze the association between genotypes/alleles and EL under the five genetic models (codominant, dominant, recessive, overdominant, and log-additive) within each of the three cohorts after adjustment for sex. In the Italian cohort, we compared mean serum irisin levels among the three genotypes of each SNP using a one-way ANOVA test.

All statistical analyses were performed using the PASW (v. 18.0 for WINDOWS, Chicago), except for statistical power, which we calculated with the StatMate software, version 2.0 (GraphPad, San Diego, CA, USA) and analysis of haplotypes, which was performed using the free Web-based application SNPStats (Sole et al. 2006). Haplotype frequencies were estimated using the implementations of the EM algorithm coded into the *haplo.stats* package.

Results

Functional analysis

The results of luciferase report analyses are presented in Fig. 1. The rs726344 SNP had functional significance in vitro, as shown by differences in luciferase activity between the constructs of this SNP (all $P \leq 0.05$), with the variant A-allele having higher luciferase activity compared with the G-allele ($P=0.04$). Statistical significance was almost reached for the rs16835198 SNP (the variant T-allele tended to have higher luciferase activity compared with the G-allele, $P=0.07$).
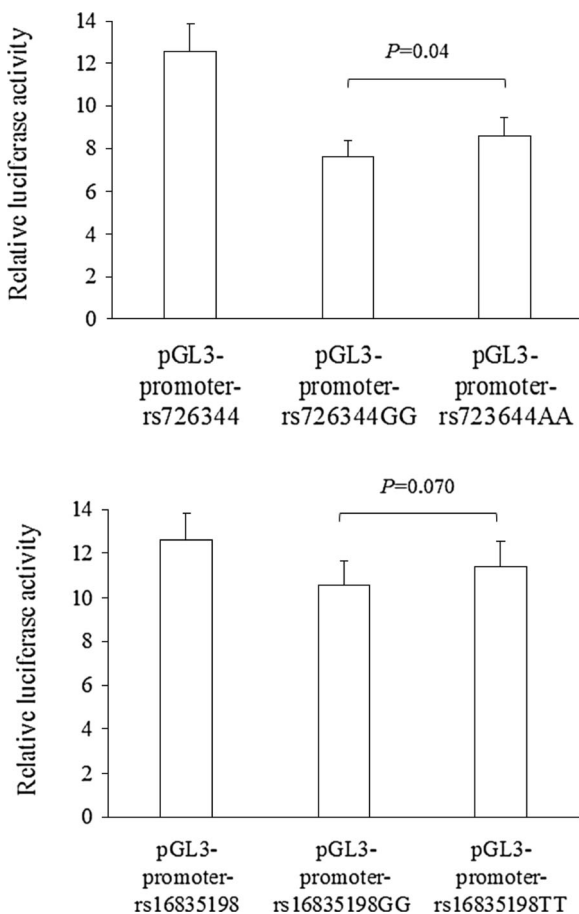

Fig. 1 Comparison of relative luciferase activity (i.e., firefly luciferase activity divided by renilla luciferase activity) between plasmids for rs726344 (*upper figure*) and rs16835198 (*lower figure*). Values are mean±SD of three different experiments, each performed in triplicate. *Upper figure* significant differences were found within the rs726344 variant for all comparisons between plasmids (all $P<0.01$). *Lower figure* statistical significance was reached for all the comparisons between plasmids (all $P<0.01$), except but for *GG* vs *TT* ($P=0.070$)

Spanish cohort

Failure rate of genotyping was 21.71 % in cases for both studied genotypes and 0.57 and 0.86 % in controls for rs726344 and rs16835198, respectively. The distribution of rs726344 and rs16835198 genotypes was consistent with the HWE in both groups ($P>0.05$). The results of genotype/allele frequency distributions as well as of binary logistic regression are shown in Table 1 and summarized below. The allele frequency distribution of the 2 SNPs did not differ between cases and controls (rs726344 $\chi^2=0.671$, $P=0.41$; rs16835198 $\chi^2=1.671$, $P=0.20$). Likewise, genotype frequencies did not differ between centenarians and controls (rs726344 $\chi^2=2.821$, $P=0.244$; rs16835198 $\chi^2=1.540$, $P=0.463$). Using logistic regression analysis, no significant associations were found between the two SNPs and EL after adjusting for sex. No significant associations were found either when analyzing both sexes separately (data not shown). When the two SNPs were considered together, four haplotypes were estimated, i.e., $G_{rs726344}$-$G_{rs16835198}$ (55.1 %), $G_{rs726344}$-$T_{rs16835198}$ (34.1 %), $A_{rs726344}$-$G_{rs16835198}$ (10.8 %), and $A_{rs726344}$-$T_{rs16835198}$ (<1.0 %). We found no association between haplotypes and EL ($P>0.1$).

Based on the observed prevalence of the G-allele of the rs726344 and rs16835198 SNPs in the control group, the Spanish cohort's sample size had an 80 % power to detect a relative likelihood of 1.07 and 1.20, respectively, for being a centenarian between G-allele carriers and noncarriers with a significance level (α) of 0.05 (two-tailed).

Italian cohort

Rate failure of genotyping was 0 %. The distribution of rs726344 and rs16835198 genotypes was consistent with the HWE in both groups ($P>0.05$). The results of genotype/allele frequency distributions as well as data of binary logistic regression are shown in Table 2 and summarized below. Allele (rs726344 $\chi^2=0.096$, $P=0.756$; rs16835198 $\chi^2=0.810$, $P=0.368$) and genotype frequencies did not differ between groups (rs726344 $\chi^2=0.122$, $P=0.941$; rs16835198 $\chi^2=1.128$, $P=0.569$), and no significant association was found between the two SNPS and EL using logistic regression adjusted by sex. No significant associations were found either when analyzing both sexes separately (data not shown). When the two SNPs were considered

Table 1 Genotype/allele frequencies of rs726344 and rs16835198 and binary logistic regression adjusted by sex in the Spanish cohort

		rs726344							rs16835198						
		Controls		Cases					Controls		Cases				
		N	%	N	%	OR	95%CI	P		N	%	N	%	OR	95%CI	P
Codom	GG	274	79.4	111	81.0	1.00		–	GG	163	47.4	58	42.3	1.00		0.48
	GA	64	19.0	26	19.0	1.19	0.69–2.06		GT	136	39.5	56	40.9	0.81	0.51–1.29	
	AA	7	2.0	0	0.0	–	–		TT	45	13.1	23	16.8	0.71	0.38–1.33	
Dom	GG	274	79.2	111	81.0	1.00		0.23	GG	163	47.4	58	42.3	1.00		0.25
	GA-AA	71	20.6	26	19.0	1.38	0.81–2.36		GT+TT	181	52.6	79	57.7	0.78	0.51–1.20	
Reces	GG-GA	338	98.0	137	100.0	1.00		–	GG+GT	299	86.9	114	83.2	1.00		0.42
	AA	7	2.0	0	0	–	–		TT	45	13.1	23	16.8	0.78	0.43–1.41	
Overdom	GG-AA	281	81.5	111	81.0	1.00		0.63	GG-TT	208	60.5	81	59.1	1.00		0.56
	GA	64	18.6	26	19.0	1.14	0.66–1.96		GT	136	39.5	56	40.9	0.88	0.57–1.36	
Log-additive	–	–	–	–	–	1.52	0.94–2.48	0.08		–	–	–	–	0.83	0.62–1.12	0.23
Allele	G	612	88.7	248	90.5	1.00			G	462	67.2	172	62.8	1.00		
	A	78	11.3	26	9.5	0.65	0.39–1.06	0.84	T	226	32.8	102	37.2	0.81	0.59–1.12	0.20

Codom codominant, *Dom* dominant, *OR* odds ratio, *Overdom* overdominant, *Reces* recessive; *95 % CI* 95 % confidence interval

together, four haplotypes were estimated, i.e., $G_{rs726344}$-$G_{rs16835198}$ (44.3 %), $G_{rs726344}$-$T_{rs16835198}$ (36.7 %), $A_{rs726344}$-$G_{rs16835198}$ (18.2 %), and $A_{rs726344}$-$T_{rs16835198}$ (<1.0 %). No association between haplotypes and EL was observed (P>0.1).

Based on the observed prevalence of the *G*-allele of the rs726344 and rs16835198 SNPs in the control group, the Italian cohort's sample size had an 80 % power to detect a relative likelihood of 1.20 and 1.31, respectively, for being a centenarian between *G*-allele carriers and noncarriers (two-tailed α=0.05).

We failed to identify a significant association between serum irisin levels and rs726344 and rs16835198 SNPs. Indeed, in the entire Italian cohort, the distribution of serum irisin levels according to the rs726344 genotypes was as follows

Table 2 Genotype/allele frequenciesof rs726344 and rs16835198 and binary logistic regression adjusted by sex in the Italian cohort

		rs726344							Irs16835198						
		Controls		Cases					Controls		Cases				
		N	%	N	%	OR	95%CI	P		N	%	N	%	OR	95%CI	P
Codom	GG	234	74.1	57	72.1	1.00		0.941	GG	104	32.9	31	39.2	1.00		0.569
	GA	78	24.7	21	26.6	0.88	0.50–1.55		GT	151	47.8	34	43.0	1.31	0.77–2.24	
	AA	4	1.2	1	1.3	0.98	0.12–8.47		TT	61	19.3	14	17.8	1.27	0.66–2.64	
Dom	GG	234	74.1	57	72.2	1.00		0.732	GG	104	32.9	31	39.2	1.00		0.288
	GA-AA	82	25.9	22	27.8	0.86	0.54–1.60		GT+TT	212	67.1	48	60.8	1.33	0.81–2.20	
Reces	GG-GA	312	98.7	78	98.7	1.00		1	GG+GT	255	80.7	65	82.3	1.00		0.748
	AA	4	1.3	1	1.3	1.01	0.13–9.11		TT	61	19.3	14	17.7	1.12	0.61–2.11	
Overdom	GG-AA	238	74.3	58	73.4	1.00		0.727	GG+TT	165	52.2	45	57.0	1.00		0.449
	GA	78	24.7	21	26.6	0.91	0.49–1.57		GT	151	47.8	34	43.0	1.22	0.71–2.01	
Log-additive	–	–	–	–	–	0.84	0.61–1.78	0.814		–	–	–	–	1.16	0.87–1.98	0.378
Allele	G	546	86.4	135	85.4	1.00			G	359	56.8	96	60.8	1.00		
	A	86	13.6	23	14.6	0.89	0.50–1.59	0.756	T	273	43.3	62	39.2	1.15	0.83–1.71	0.368

Codom codominant, *Dom* dominant, *OR* odd ratio, *Overdom* overdominant, *Reces* recessive, *95 % CI* 95 % confidence interval

(mean±SD): GG, 22.4±6.4 ng/ml; GA, 20.9± 7.1 ng/ml; and AA, 24.7±8.2 ng/ml ($P=0.67$ for the genotype effect). The concentrations of serum irisin for rs16835198 genotypes were as follows: GG, 20.1±5.7 ng/ml; GT, 23.3±7.4 ng/ml; and TT, 22.9±9.1 ng/ml ($P=0.81$). Similarly, no statistical differences were found between genotypes when analyzing cases and controls separately (data not shown).

Japanese cohort

Rate failure of genotyping was 1.21 % in cases and 0.02 % in controls. The distribution of rs16835198 genotypes was consistent with the HWE in both groups ($P>0.05$). The results of rs16835198 genotype/allele frequency distributions as well as data of binary logistic regression are shown in Table 3 and summarized below. Allele ($\chi^2=1.001$, $P<0.317$) and genotype frequencies did not different between groups ($\chi^2=5.337$, $P<0.069$), and no significant association was found between rs16835198 and EL using logistic regression adjusted by sex. No significant associations were found when analyzing both sexes separately (data not shown). Haplotype analysis of the two SNPs could not be performed owing to the absence of the variant A-allele of rs726344 in this cohort.

Based on the observed prevalence of the *G*-allele of the rs16835198 SNP in the control group, the Japanese cohort's sample size had an 80 % power to detect a relative likelihood of 1.19 for being a centenarian between *G*-allele carriers and noncarriers (two-tailed $\alpha=0.05$).

Discussion

The main findings of our study are two-fold. First, the rs726344 SNP had functional significance in vitro, with the variant *A*-allele upregulating luciferase activity compared to the common *G*-allele. On the other hand, we found no major association between *FNDC5* rs16835198 or rs726344 and EL. Regarding the results of the functionality analyses (luciferase constructs), to the best of our knowledge, this is the first attempt to gain insights (using in vitro analyses) into the potential functional consequences of these two *FNDC5* variants.

The rs16835198 and rs726344 SNPs are not likely to influence the amino acid sequence of the protein product owing to the fact that the rs16835198 and rs726344 SNPs are located in the 3′-flanking region and in intron 5, respectively, of the *FNDC5* gene. Alterations in DNA sequence in the 3′-UTR region of a gene (e.g., rs16835198) have the potential to alter the level, location, or timing of gene expression whereas intronic genomic variants (e.g., rs726344) can influence gene expression and thus phenotype, by altering mRNA stability, alternative mRNA splicing, or the binding of transcription factors (Tabor et al. 2002; Knight 2005;

Table 3 Genotype/allele frequencies of rs16835198 and binary logistic regression adjusted by sex in the Japanese cohort

		Controls		Cases				
		N	%	N	%	OR	95%CI	P
Codom	TT	148	29.7	200	27.3	1.00		0.58
	GT	239	47.9	356	48.6	1.10	0.84–1.44	
	GG	111	22.4	177	24.1	1.18	0.86–1.62	0.35
Dom	TT	148	29.7	200	27.3	1.00		
	GT-GG	350	70.3	533	72.7	1.13	0.88–1.45	0.45
Reces	TT-GT	387	77.6	556	75.8	1.00		
	GG	111	22.3	177	24.1	1.11	0.85–1.45	
Overdom	TT-GG	259	52.1	377	51.4	1.00		0.84
	GT	239	47.9	356	48.6	1.02	0.93–1.27	
Log-additive	—	—	—	—	—	1.09	0.93–1.27	0.30
Allele	T	535	54	756	52	1.00		0.29
	G	461	46	710	48	1.09	0.93–1.28	

Codom codominant, *Dom* dominant, *OR* odd ratio, *Overdom* overdominant, *Reces* recessive, *95 % CI* 95 % confidence interval

Mercado et al. 2005; Sasabe et al. 2007). However, Staiger et al. (2013) found no association between the A-allele of the rs726344 SNP and FNDC5 mRNA expression in human myotubes ($P=0.19$). Although the minor A-allele of rs726344 is characterized by higher luciferase activity compared with the wild-type G-allele and has been recently associated with decreased insulin sensitivity in vivo (Staiger et al. 2013), our results indicated that this SNP was neither associated with EL nor with serum irisin levels. We can therefore hypothesize that the previously observed association between serum irisin levels and EL (Emanuele et al. 2014) is not mediated by this specific SNP. This result is only partly surprising because it is known that serum irisin concentrations are regulated by a number of factors (including food intake and exercise) (Lopez-Legarrea et al. 2014) that are not necessarily limited to genetic variants in the irisin gene. On the other hand, although the association between the A-allele of rs726344 and increased luciferase activity shown here in vitro is counterintuitive given the insulin-desensitizing effect of this allele in middle-aged adults at increased risk for type II diabetes recently shown by Staiger and coworkers (Staiger et al. 2013), here we did not measure insulin resistance. Thus, we cannot exclude that the previously demonstrated insulin-desensitizing effect of the A-allele of rs726344 could be either modified or abolished by other factors, including age, drug use, or comorbidities.

There is a strong rationale in postulating that the gene, FNDC5, encoding the precursor of irisin might be associated, at least partly, with healthy aging (Bostrom et al. 2012), based on the beneficial multisystemic effects of this myokine, i.e., browning of white adipocytes (Polyzos et al. 2013), reduced risk of obesity (Spiegelman 2013) and related diseases (Sanchis-Gomar et al. 2012; Hojlund and Bostrom 2013; Sanchis-Gomar 2013), potential preservation of vascular function and skeletal muscle mass ((Huh et al. 2012; Spiegelman 2013; Bostrom and Fernandez-Real 2014), higher aerobic fitness in cardiac patients (Lecker et al. 2012), improved neurogenesis in animal models (Hashemi et al. 2013), and also based on the association between irisin levels and higher telomere length in healthy adults (Rana et al. 2014). On the other hand, inhibition of another myokine, myostatin, can have irisin-like effects, e.g., "browning" of the white adipose tissue (though AMPK-PGC-1α-irisin pathway) and amelioration of muscle weakness (see Fiuza-Luces et al. 2013 for a review). Interestingly, the K153R polymorphism in the gene (MSTN) encoding myostatin could be associated with EL, as shown by previous research from our group (Garatachea et al. 2013). There is some controversy among the numerous studies that have assessed circulating blood levels of irisin in people with obesity, diabetes, and CVD (Huh et al. 2012; Choi et al. 2013; Liu et al. 2013; Moreno-Navarrete et al. 2013; Park et al. 2013; Stengel et al. 2013; Vamvini et al. 2013; Sanchis-Gomar et al. 2014a; Sanchis-Gomar et al. 2014b). Some authors have expressed their concerns about the notion that irisin is a real myokine (i.e., being released by contracting muscles) or that this molecule has actual beneficial effects on health (Timmons et al. 2012; Erickson 2013; Pekkala et al. 2013; Raschke et al. 2013). And yet, the bulk of evidence seems to indicate that irisin plays an overall protective role against cardiometabolic disorders (Sanchis-Gomar et al. 2012; Eckardt et al. 2014) and can be upregulated by muscle exercise (Bostrom et al. 2013). However, despite the overall metabolic-protective effect of irisin, no evidence is available on the potential benefits of irisin to prevent sarcopenia, with recent data showing no differences in irisin levels between subjects with sarcopenia and healthy controls, and also indicating no association between this molecule and skeletal muscle mass index (Choi et al. 2014). Arguably, the strongest support for a potential involvement of irisin on EL comes from a recent study from our group showing that irisin levels were ~70 and ~133 % higher in the present Italian cohort of healthy centenarians compared with younger controls who were healthy or had cardiovascular disease, respectively (Emanuele et al. 2014).

Studies on genetic polymorphisms in the irisin (FNDC5) gene are scarce and nonexistent in aged population. In this regard, EL is associated with insulin sensitivity and insulin signaling (Willcox et al. 2006; Barbieri et al. 2008; Guevara-Aguirre et al. 2011; Brunet 2012). Particularly, genetic variation in the insulin-FOXO3A gene pathway influences EL (Willcox et al. 2008; Flachsbart et al. 2009). Long-lived men exhibit several biological markers indicative of greater insulin sensitivity, which is associated with the FOXO3A GG genotype (Willcox et al. 2008). Also, a favorable glucose metabolism is associated with familial longevity (Rozing et al. 2010). Here, we failed to detect any significant association between the two FNDC5 SNPs we studied and EL, suggesting that other factors (e.g., maybe physical activity levels throughout lifespan,

nutritional habits or complex gene-environment associations that are yet to be determined) might have a stronger influence on irisinemia, despite the potential functional consequence of at least one of the two SNPs we studied (i.e., rs726344). In fact, we found no FNDC5 genotype effect on the irisin levels of the Italian cohort, which is in line with recent data on the rs16835198 SNP and serum irisin in Japanese men aged 21–79 years) (Tanisawa et al. 2014). On the other hand, the role of epigenetic factors in irisinemia remains to be determined. Indeed, epigenetic modifications might also influence longevity by affecting gene expression without changing the DNA sequence (Wolffe and Matzke 1999), i.e., through DNA methylation, histone modifications, and altered expression of RNAs or small, noncoding RNAs that regulate gene expression profiles associated with longevity (Mango 2011).

Besides the rationale for postulating the FNDC5 gene as a candidate to influence EL and the clear definition we used for the criterion of EL (with all cases being centenarians), a further strength from our study derives from the luciferase construct study we performed as well as from the fact that the analyses were conducted in three independent cohorts, with one of them, the Italian cohort, being composed of apparently healthy centenarians and another one, the Japanese cohort, including a very large sample of centenarians. On the other hand, there are some limitations in our study, such as the use of convenience samples, which increases the risk of bias due to population stratification or the relatively high rate of genotyping failure in the Spanish centenarians. The latter suggests that DNA extraction from saliva in frail individuals (which we were forced to adopt due to ethical reasons) might result in small amounts of DNA available for analyses. Interpretation of gene association studies with centenarians and younger controls as the one we conducted here may be also biased by differences in date of birth (i.e., early 1900s vs 1930 and onward, respectively).

In summary, we found no major association between FNDC5 rs16835198/rs726344 SNPs and EL despite the fact that the latter variation might have functional consequences and despite the rationale for postulating that irisin is a myokine with the potential to promote longevity owing to its overall cardiovascular and metabolic protective effect. Further research is needed with different cohorts as well as with different variants in the FNDC5 gene or in other genes involved in irisin signaling.

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