Original Article

Polymorphisms Falling Within Putative miRNA Target Sites in the 3′UTR Region of SIRT2 and DRD2 Genes Are Correlated With Human Longevity

Paolina Crocco,* Alberto Montesanto,* Giuseppe Passarino, and Giuseppina Rose

Department of Biology, Ecology and Earth Science, University of Calabria, Rende, Italy.

Address correspondence to Giuseppina Rose, PhD, Department of Biology, Ecology and Earth Science, University of Calabria, 87036 Rende, Italy.

Email: pinarose@unical.it

*These authors contributed equally to this work.

Received November 24, 2014; Accepted April 3, 2015

Decision Editor: Rafael de Cabo, PhD

Abstract

Many studies have suggested that individual differences in aging phenotypes may be associated to polymorphisms affecting gene regulation. As single-nucleotide polymorphisms (SNPs) in the 3′-untranslated regions (3′UTR) targeted by microRNAs (miRNAs) can alter the strength of miRNA binding (and, consequently, the regulation of target genes), we wondered whether these SNPs (known as miRSNPs) affect the individual chance to become long-lived. Thus, we estimated the effect of miRSNPs falling in the 3′-untranslated regions of 140 aging-related genes on the DNA/miRNA bond. The 24 miRSNPs with the highest difference of binding energy between the two alleles were then investigated for their association with longevity by case–control analysis. Two SNPs, SIRT2-rs45592833 G/T and DRD2-rs6276 A/G, provided a significant association with human longevity, also after correcting for multiple comparisons. For both SNPs, the minor allele was associated with a significantly decreased chance to become long-lived in an allele dose-dependent manner (p = 1.090 × 10−6 and 1.964 × 10−4 for SIRT2 and DRD2, respectively). The results indicate that the individual aging phenotype may be affected by the variability of specific miRNA targeted regions, as shown for SIRT2 and DRD2, and may suggest further studies to analyze the variability of gene expression regulation as a modulator of aging phenotypes.

Key Words: miRNA—Longevity—SIRT2—DRD2—SNP

Single-nucleotide polymorphisms (SNPs) are the most common source of genome sequence diversity and the main responsible for phenotypic variation in quantitative traits, and for the etiology of common diseases (1). While coding SNPs have an obvious impact on the encoded protein’s activity, less predictable are the variants located within non-coding regions of the genome. On the other hand, it has been estimated that more than 80% of the SNPs identified as associated with a trait or disease are located in noncoding regions such as promoters, enhancers, silencers, and other regulatory regions (2). These regulatory SNPs (rSNPs) may modulate gene expression in an allele-specific manner hence representing an important class of genetic variation that likely plays a significant role in the development of complex traits.

Aging is a very complex phenotype characterized by a progressive decrease in the ability to control metabolic processes and to maintain cellular homeostasis in response to physiological and environmental changes, as well as an increase in morbidity and mortality (3). There are indications from recent studies that many of the phenotypic changes that occur as an organism ages may be traced to widespread changes in gene expression (4). It has also emerged over recent years that posttranscriptional regulation by microRNAs (miRNAs) may contribute to the phenotypic alterations seen during the aging process by epigenetically modulating the expression of key regulatory proteins (5,6).

miRNAs are small (approximately 21 nucleotides in length) noncoding RNAs which can decrease the efficiency of the translation or induce the degradation of miRNAs via base pairing of their seed region (roughly nucleotides 2–7 from the 5′ end of the mature miRNA) with complementary sequences usually located to the
3′-untranslated region (UTR) of target genes (7). Accumulating evidence indicates that gene regulation by miRNA is essential to almost all biological processes and underlies many human diseases (8,9). In aging, data obtained from model systems such as Caenorhabditis elegans, Drosophila, and mouse, demonstrated that specific miRNAs regulate lifespan by controlling the activity of genes belonging to signaling pathways widely recognized as critical regulators of aging and longevity, including insulin/IGF-1, p53-, and SIRT1-mediated pathways (10,11), and also demonstrated that numerous miRNAs are significantly up- or down-regulated with aging (12–15). Extensive changes in miRNAs expression also occur in human aging, and recent studies suggested that circulating miRNAs and their predicted targets may be potential biomarkers of aging and age-related diseases (16–18).

Given the crucial role of miRNAs, it follows that any interference in miRNA–mRNA interactions, for instance, due to alterations in miRNA expression or function, might affect the physiological control of gene expression (19). In recent years it has become apparent that SNPs in the 3′UTR of genes are able to affect miRNA binding, and consequently the expression of specific target genes, by either modulating existing binding sites or by creating novel binding sites (20,21). These SNPs, known as miRNA-binding SNPs or miRSNPs, are now being recognized as a new class of rSNPs in the human genome and their biological relevance has been investigated in various diseases including cancer (22), neurodegeneration (23), hypertension (24), and cardiovascular diseases (25).

The aim of this study was to investigate the role of miRSNPs in human aging and longevity. Starting from a list of 140 genes belonging to pathways relevant to aging and longevity and using a stringent in silico approach, thanks to the availability of publicly available online databases, which provide predictions of SNP effects on miRNA targeting:MirSNP (http://compbio.uthsc.edu/miRSNP) (28), mirDSNP (http://mirdsnp.ccr.buffalo.edu/) (28), Patrocles (http://www.patrocles.org/) (29), Polymirts (http://compbio.uthsc.edu/miRSNP) (30), miRNASNP (http://www.bioguo.org/miRNASNP) (31), and mirnspscore (www.bigh.medisin.ntnu.no/mirnspscore/) (32). The rational of this choice is that in the context of experimental strategies for miRNA target identification, it is advisable to use multiple databases to predict miRNA binding for genotyping studies because they often predict distinct miRNA-binding sites. 3′UTR SNP with a putative effect in at least two of the mentioned databases were selected for the assessment of the binding free energy (expressed as ΔG, Gibbs free energy) for both the “wild-type” and the “variant” alleles. Although some of these databases provide measurements of the variation of ∆Gs between the variant and the common alleles within each target site, in order to obtain comparable measurements of the variation of ∆Gs we used the algorithm implemented in miRanda (http://www.microrna.org) (33), which is the most specialized and widely used for the calculation of ∆Gs.

Schematically, the protocol for the in silico identification of putative miRSNPs was as follows:

1. Selection of the 3′UTR regions.
2. Identification of SNPs in the 3′UTR regions with minor allele frequency (MAF) greater than or equal to 5%. This choice was in agreement with simulation studies which clearly showed that with the analyzed sample size (about 300 cases and 300 controls), considering a phenotype prevalence of 10% and assuming an additive effect, our study design had only a 45% power to detect a disease predisposing variant with an MAF equals to 0.05 and a genotype relative risk of 1.5 at a 5% significance (26).
3. Identification of putative miRNA-binding sites within the 3′UTR SNP.
4. Assessment of the binding free energy (expressed as ΔG, Gibbs free energy) for both the “wild-type” and the “variant” alleles identified as in point 2.
5. Prioritizing the identified SNP on the basis of the variation of ΔG.

For each gene, we selected the 3′UTR region according to the UCSC genome browser (http://genome.ucsc.edu). Three public databases were used to collect information about SNPs in the 3′UTR regions: NCBI (http://www.ncbi.nlm.nih.gov), Ensembl (http://www.ensembl.org), and HapMap (http://www.hapmap.org). SNPs were considered if they had MAF greater than or equal to 5%. In order to assess the effects of these SNPs on miRNA binding, we queried the following publicly available online databases, which provide predictions of SNP effects on miRNA targeting:MirSNP (http://combi.bjmu.edu.cn/mirsnp) (27), mirDSNP (http://mirdsnp.ccr.buffalo.edu/) (28), Patrocles (http://www.patrocles.org/) (29), Polymirts (http://compbio.uthsc.edu/miRSNP) (30), miRNASNP (http://www.bioguo.org/miRNASNP) (31), and mirnspscore (www.bigh.medisin.ntnu.no/mirnspscore/) (32). The rational of this choice is that in the context of experimental strategies for miRNA target identification, it is advisable to use multiple databases to predict miRNA binding for genotyping studies because they often predict distinct miRNA-binding sites. 3′UTR SNP with a putative effect in at least two of the mentioned databases were selected for the assessment of the binding free energy (expressed as ΔG, Gibbs free energy) for both the “wild-type” and the “variant” alleles. Although some of these databases provide measurements of the variation of ∆Gs between the variant and the common alleles within each target site, in order to obtain comparable measurements of the variation of ∆Gs we used the algorithm implemented in miRanda (http://www.microrna.org) (33), which is the most specialized and widely used for the calculation of ∆Gs.

The difference of the free energies between the two alleles was computed as “variation of ΔG” (ie, ΔΔG). The ΔΔG was calculated by deducting the variant ΔG from the wild-type ΔG. A negative ΔΔG indicates decreased binding in the variant compared with the wild-type allele, whereas a positive ΔΔG indicates increased binding in the variant.

Because in some genes the same sequence is predicted to bind several miRNAs, and thus, SNPs in these sequences could affect the binding site of more than one miRNA, we used the sum of all the ΔΔGs for each SNP (∑ΔΔG) as parameter for predicting the biological impact of the polymorphism. As proposed by Landi et al. (34), although each target can bind only one miRNA at a time, the basic idea behind this approach is that the more miRNAs are predicted to target the same mRNA, the more likely it is that at least one of them actually binds to it.

Materials and Methods

Candidate Genes and SNP Selection

Candidate genes were selected by using the following two-step procedure:

1. Identification of biological processes and pathways influencing aging and longevity in either model organisms or humans. To this purpose, Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) and GenAge (http://genomics.senescence.info/genes/) were employed using key words as “aging AND longevity,” “human aging,” “human longevity,” “aging AND model organism,” “pathway AND aging”. By this approach the following signaling pathways were identified: DNA repair, apoptosis and cell cycle regulation, metabolism of xenobiotics, insulin/IGF-1, mitochondrial biology, response to oxidative stress, and neuromodulation.
2. Pathway analysis tools, such as “Biocarta” (http://cgap.nci.nih.gov/Pathways/BioCarta_Pathways) and “Kegg” (http://www.genome.jp/kegg/) were used for identification of genes belonging to the pathways identified at point 1.

Based on this search strategy, we finally selected 140 genes that are listed in Supplementary Table 1.
The input list of miRNAs in miRanda was taken from miRBase (http://www.mirbase.org) (35), which is the most updated and comprehensive database of miRNAs. Finally, SNPs were scored on the basis of the $\Delta\Delta G$ values and only those that caused a change in the $\Delta\Delta G_{\text{unz}}$ 8.44 kcal/mol (upper tertile) were considered biologically relevant and included in the case–control study. Figure 1 provides a schematic overview of the SNP selection process.

Study Population
A total of 622 subjects (286 men and 336 women) whose ages ranged from 65 to 107 years participated in this study. Subjects older than 90 years were defined as cases ($n = 320$; mean $\pm$ SD age, 96.0 $\pm$ 3.45 years; 127 males and 193 females), while the remaining ones were classified as controls ($n = 302$; mean $\pm$ SD age, 75.1 $\pm$ 6.84 years; 159 males and 143 females). All subjects were of Calabrian origin (South Italy) up to their grandparents. The samples were collected between 1999 and 2004, within the framework of appropriate recruitment campaigns carried out for monitoring the quality of aging in Calabria. Subjects younger than 90 years of age were contacted through family physicians, while subjects older than 90 years of age were identified through the birth registers of the 409 municipalities of Calabria and then contacted by specialized personnel and invited to join the study. The different campaigns were approved by the ethical committee of the University of Calabria, and all subjects consented to their phenotypic and genetic data to be used anonymously for genetic studies on aging and longevity (informed consent). The healthy status was ascertained by medical visit carried out by a geriatrician who also administered a structured questionnaire, validated within European recruitment projects, for the collection of sociodemographic information, evaluation of physical, cognitive, depressive status, sensory deficits, medications, and self-reported health status. At the time of the visit, peripheral venous blood samples were also obtained. White blood cells from blood buffy coats were used as source of DNA.

Genotyping Using the Sequenom iPLEX™ Assay
Multiplex SNP genotyping was performed using polymerase chain reaction followed by primer extension and Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry using iPLEX Gold technology from Sequenom (Sequenom Inc, San Diego, CA). Sequenom MassARRAY Assay Designer software (version 3) was used to design primers for polymerase chain reaction, and unincorporated nucleotides were deactivated with the shrimp alkaline phosphatase. A primer extension reaction was subsequently implemented using the mass extension primer and the terminator. The primer extension products were then desalted on resin, and spotted onto the 384-element SpectroCHIP (Sequenom) for MALDI-TOF analysis using SpectroACQUIRE v3.3.1.3 (Sequenom). Spectra were analyzed using MassARRAY Typer v3.4 Software (Sequenom).

Statistical Analysis
For each SNP, allele and genotype frequencies were estimated by gene counting from the observed genotypes. Hardy–Weinberg equilibrium was tested by Fisher’s exact test.

The association between the analyzed genetic variants and the longevity phenotype was assessed by fitting for each analyzed SNP the following logistic regression model:

$$\ln \frac{p}{1-p} = \beta_0 + \beta_1 \text{Sex} + \beta_2 \text{SNP}$$

where $p$ represents the probability of belonging to the long-lived group (90+ years), $\beta_0$, $\beta_1$, and $\beta_2$ are the parameters to be estimated, Sex is the sex of the subject (1 for males and 2 for females), and SNP represents...
the analyzed polymorphism coded in an additive fashion (number of copies of the minor allele). In such model, in order to test if $\beta_1$ was significantly different from zero the Wald’s test was used, while the strength of the association was measured in terms of odd ratio (OR).

To capture the sex-dependent effects of the analyzed genetic variants, model 1 can be reformulated as follows:

$$g(\mu) = \beta_0 + \beta_1 Sex + \beta_2 SNP + \beta_3 Sex \times SNP$$ (2)

where $\beta_3$ is the coefficient of the interaction effect; it indicates how the effect of the SNP on the phenotype of interest changes between males and females. Also in this case, to assess if $\beta_3$ was significantly different from zero the Wald’s test was used.

Adjustment for multiple comparisons has been carried out using the Bonferroni procedure ($\alpha = 0.05/\text{number of tests}$). All statistical analyses were carried out using Plink v1.07 (36).

Results

In Silico Analysis

To identify putative miRSNPs we analyzed SNPs located in the 3'UTR region of genes with reported relevance for aging and longevity. As schematized in Figure 1, of the 140 genes analyzed, 45 did not harbor any SNP or SNP with MAF greater than or equal to 5% in Caucasian population. The remaining 95 genes presented at least one SNP in their 3'UTR region with an MAF greater or equal to 5%, for a total of 432 SNPs. These SNPs were analyzed using different target prediction tools. From this analysis, 67 SNPs in 46 genes resulted to have a potential impact on miRNA binding in at least two different databases. A total of 182 different miRNAs were predicted to bind to the regions encompassing these SNPs. For the 67 selected SNPs, the algorithm implemented in miRanda was run to assess the Gibbs binding free energy for each identified miRNA ($\Delta G$, expressed in kcal/mol), both for the common and the variant alleles. Finally, the sum of the $\Delta G$s for all miRNAs targeting a given SNP ($|\Delta G_{tot}|$) was obtained. Supplementary Table 1 reports the list of the 67 SNPs classified according to the $|\Delta G_{tot}|$. The list of 24 top-ranked SNPs (upper tertile, $|\Delta G_{tot}| \geq 8.44$ kcal/mol) is reported in Table 1. In Supplementary Table 3 also the miRNAs predicted to bind the relevant SNPs are reported.

Table 1. Candidate SNPs With $|\Delta G_{tot}| \geq 8.44$ kcal/mol (Upper tertile of the distribution of $|\Delta G_{tot}|$) and MAF > 0.05, Evaluated in the Case–Control Association Study

| Gene  | dbsNP ID   | $|\Delta G_{tot}|$ | Gene  | dbsNP ID   | $|\Delta G_{tot}|$ |
|-------|------------|-----------------|-------|------------|-----------------|
| SIRT6 | rs350846   | 26.23           | PDK1  | rs1530865  | 9.82            |
| TORC  | rs10038    | 18.08           | SULT1A2| rs710410   | 9.79            |
| IL1B  | rs1071676  | 15.83           | PPARA | rs10154348 | 9.78            |
| POMC  | rs1042571  | 15.24           | PDK1  | rs11904366 | 9.66            |
| SOD3  | rs2855262  | 13.48           | LEP   | rs11761556 | 9.29            |
| IGF-1 | rs5742714  | 13.37           | PPARA | rs11704979 | 9.10            |
| IL1B  | rs1368439  | 12.71           | CCND1 | rs7177     | 9.00            |
| DRD2  | rs6278     | 12.5            | NBS1  | rs14448    | 8.79            |
| FKHRL1 (FOXO3) | rs4946936 | 11.61           | SIRT2 | rs45592833 | 8.71            |
| PPARDb| rs3734254  | 11.33           | SLCA6A| rs1042098  | 8.65            |
| DRD2  | rs6276     | 10.4            | INSR  | rs1366600  | 8.44            |
| PPARA | rs41388244 | 9.97            | NRF1  | rs13241028 | 8.44            |

*The $\Delta G$ was calculated by deducting the variant $\Delta G$ from the wild-type $\Delta G$. $|\Delta G_{tot}|$ is the total of the absolute values of $\Delta G$ ($|\Delta G|$).

Genetic Analysis

The above-selected SNPs were further investigated by a case–control study. Among them, three SNPs gave a very low call rate, while two showed significant departures from Hardy–Weinberg equilibrium. These SNPs were then excluded from the further analyses. Results from association analyses are reported in Table 2. We found six SNPs (SIRT2-rs45592833, DRD2-rs6278, DRD2-rs6276, SOD3-rs2855262, FOXO3-rs4946936, and SULT1A2-rs710410) nominally associated with the probability to attain longevity ($p < .05$). No SNP showed a significant sex-specific effect in association with human longevity. However, after adjustment for multiple comparisons (Bonferroni correction), only the variants rs45592833-GT of SIRT2 and rs6276-AG of DRD2 remained statistically significant (0.05/19 = 0.0026). In particular, carriers of rs45592833-T allele showed a reduced probability to attain longevity in a log-additive manner. In fact, a subject with a copy of minor allele T had a probability to become a long-lived people reduced of about 3.7 times, while for a subject with two copies this reduction amount to about 13 times with respect to a subject homozygous for the most frequent allele G ($OR = 0.2701, 95\%$ confidence intervals ($CI = 0.1596–0.4572, [p = 0.090 \times 10^{-4}$]). Likewise, a subject with a copy of minor allele G of the rs6276 variant of DRD2 had a probability to become a long-lived people reduced of about 1.84 times, while for a subject with two copies this reduction amount to about 3.4 times with respect to a subject homozygous for the most frequent allele A ($OR = 0.5424, 95\% CI = 0.3931–0.7485, [p = 1.964 \times 10^{-4}$].

Discussion

There are many indications that the presence of SNPs in miRNA-binding sites (miRSNPs) increases the risk of complex genetic diseases by perturbing the expression of target genes (20, 21). The growing interest in elucidating the role of these regulatory SNPs has led, in the last years, to the development of several databases and prediction tools that may facilitate the research in this field. In the present study, we took a stringent in silico approach to identify miRSNPs in the 3'UTR of 140 genes related to human aging and longevity. We identified 24 candidate miRSNPs that could affect miRNA–mRNA interactions. Their effect on human longevity was assessed.

As a result, two SNPs, SIRT2-rs45592833 G/T and DRD2-rs6276 AG, have provided a significant association with human longevity.
Table 2. Results of the Logistic Regression Analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>CHR</th>
<th>SNP</th>
<th>A1</th>
<th>MAF Controls</th>
<th>MAF Cases</th>
<th>OR</th>
<th>L95</th>
<th>U95</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK1</td>
<td>2</td>
<td>rs1530865</td>
<td>C</td>
<td>0.0386</td>
<td>0.04085</td>
<td>1.003</td>
<td>0.5526</td>
<td>1.821</td>
<td>.9918</td>
</tr>
<tr>
<td>PDK1</td>
<td>2</td>
<td>rs11904366</td>
<td>T</td>
<td>0.1407</td>
<td>0.1438</td>
<td>1.081</td>
<td>0.7704</td>
<td>1.517</td>
<td>.6516</td>
</tr>
<tr>
<td>SOD3</td>
<td>4</td>
<td>rs2835226</td>
<td>T</td>
<td>0.338</td>
<td>0.3974</td>
<td>1.267</td>
<td>0.9904</td>
<td>1.62</td>
<td>.05971</td>
</tr>
<tr>
<td>SLC6A3</td>
<td>5</td>
<td>rs1042098</td>
<td>C</td>
<td>0.3933</td>
<td>0.3495</td>
<td>0.8289</td>
<td>0.6495</td>
<td>1.058</td>
<td>.1313</td>
</tr>
<tr>
<td>IL12B</td>
<td>5</td>
<td>rs1368439</td>
<td>G</td>
<td>0.1962</td>
<td>0.1906</td>
<td>0.9922</td>
<td>0.7343</td>
<td>1.341</td>
<td>.9592</td>
</tr>
<tr>
<td>FHKRL1</td>
<td>6</td>
<td>rs4946936</td>
<td>T</td>
<td>0.3765</td>
<td>0.4444</td>
<td>1.415</td>
<td>1.084</td>
<td>1.847</td>
<td>.01058</td>
</tr>
<tr>
<td>(FOXO3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEP</td>
<td>7</td>
<td>rs11761556</td>
<td>C</td>
<td>0.4568</td>
<td>0.3966</td>
<td>0.7996</td>
<td>0.6314</td>
<td>1.013</td>
<td>.06353</td>
</tr>
<tr>
<td>NRF1</td>
<td>7</td>
<td>rs13241028</td>
<td>C</td>
<td>0.1172</td>
<td>0.1393</td>
<td>1.2</td>
<td>0.8477</td>
<td>1.698</td>
<td>.3039</td>
</tr>
<tr>
<td>NBS1</td>
<td>8</td>
<td>rs14448</td>
<td>C</td>
<td>0.04494</td>
<td>0.04902</td>
<td>1.03</td>
<td>0.5958</td>
<td>1.781</td>
<td>.9155</td>
</tr>
<tr>
<td>CCND1</td>
<td>11</td>
<td>rs7177</td>
<td>C</td>
<td>0.4291</td>
<td>0.4826</td>
<td>1.212</td>
<td>0.9573</td>
<td>1.534</td>
<td>.1102</td>
</tr>
<tr>
<td>DRD2</td>
<td>11</td>
<td>rs6278</td>
<td>T</td>
<td>0.1703</td>
<td>0.1113</td>
<td>0.611</td>
<td>0.4323</td>
<td>0.8636</td>
<td>5.267×10⁻³</td>
</tr>
<tr>
<td>DRD2</td>
<td>11</td>
<td>rs6276</td>
<td>G</td>
<td>0.4824</td>
<td>0.3906</td>
<td>0.5424</td>
<td>0.3931</td>
<td>0.7485</td>
<td>1.964×10⁻⁴</td>
</tr>
<tr>
<td>SULT1A2</td>
<td>16</td>
<td>rs701410</td>
<td>C</td>
<td>0.197</td>
<td>0.2588</td>
<td>1.342</td>
<td>1.015</td>
<td>1.774</td>
<td>.03922</td>
</tr>
<tr>
<td>SIRT6</td>
<td>19</td>
<td>rs350846</td>
<td>C</td>
<td>0.08727</td>
<td>0.08361</td>
<td>0.959</td>
<td>0.6436</td>
<td>1.429</td>
<td>.8372</td>
</tr>
<tr>
<td>INSR</td>
<td>19</td>
<td>rs1366600</td>
<td>C</td>
<td>0.01481</td>
<td>0.0178</td>
<td>1.294</td>
<td>0.5086</td>
<td>3.292</td>
<td>.5886</td>
</tr>
<tr>
<td>TORC</td>
<td>19</td>
<td>rs10038</td>
<td>A</td>
<td>0.2361</td>
<td>0.2365</td>
<td>0.9955</td>
<td>0.7595</td>
<td>1.305</td>
<td>.9741</td>
</tr>
<tr>
<td>SIRT2</td>
<td>19</td>
<td>rs45592833</td>
<td>T</td>
<td>0.1342</td>
<td>0.0369</td>
<td>0.2701</td>
<td>0.1596</td>
<td>0.4572</td>
<td>1.090×10⁻⁴</td>
</tr>
<tr>
<td>PPARA</td>
<td>22</td>
<td>rs11704979</td>
<td>A</td>
<td>0.08118</td>
<td>0.1127</td>
<td>1.351</td>
<td>0.9178</td>
<td>1.99</td>
<td>.1272</td>
</tr>
<tr>
<td>PPARA</td>
<td>22</td>
<td>rs41388244</td>
<td>T</td>
<td>0.01866</td>
<td>0.01629</td>
<td>0.6957</td>
<td>0.04312</td>
<td>11.22</td>
<td>.7981</td>
</tr>
</tbody>
</table>

Notes: For each model odds ratios (OR), 95% confidence intervals (CI) and p values (Wald’s test). CHR = chromosome; MAF = minor allele frequency; SNP = single-nucleotide polymorphism.

after correcting for multiple comparisons. Additionally, we observed some suggestive associations that were significant at a nominal level of 0.05: rs6278 of DRD2, rs2855262 of SOD3, rs4946936 of FOXO3, and rs710410 of SULT1A2 genes.

The variant T allele of rs45592833 in SIRT2 was associated with decreased chance to survive at very old age in an allele dose-dependent manner (OR = 0.2701, 95% CI = 0.1596 to 0.4572).

SIRT2 is one of seven (SIRT1-7) mammalian sirtuins (Sir2-like proteins), a family of NAD+-dependent deacetylases, which play important roles in cellular functions such as metabolism and differentiation (37–39). More than a decade ago, the sir2 gene family came out as a key regulator of lifespan in different species ranging from yeast to human (40,41). SIRT2 is the ortholog of the yeast sir2 gene which mediates lifespan extension by caloric restriction (42,43). It resides primarily in the cytoplasm where it deacetylates α-tubulin (44), but shuttles to the nucleus during G2/M phase where it deacetylates histones (45), thereby modulating cell-cycle progression (46). SIRT2 also deacetylates several other substrates (PEPCK1, FOXO1, FOXO3a, p65, and p53) that are implicated in fundamental cellular processes related to the healthy state of the organisms such as maintenance of energy homeostasis, control of oxidative stress and inflammation, and regulation of cell growth and death (47).

Our in silico analysis revealed that the variant rs45592833 of SIRT2 lies within a binding site recognized by three different microRNAs (miR-3170, miR-92a-1-5p, and miR-615-5p), which were all predicted to bind more tightly to the T allele (Supplementary Table 3), likely resulting in a reduction of SIRT2 expression. Decreased levels of SIRT2 have been found in several human cancers (48,49); moreover, it has been shown that SIRT2-deficient mice develop tumors as they age (50). Interestingly, miR-615-5p, which shows the highest binding energy change caused by rs45592833, has been found to be deregulated in cancer cell lines (51,52), in patients with aging-associated conditions such as Huntington’s and cardiovascular diseases (53,54), and in muscles of old mice (55), thus suggesting that downstream targets of miR-615-5p could be involved in signaling pathways that play critical roles in the aging process.

It may be speculated that miR-615-5p mediated posttranscriptional control of SIRT2 could possibly affect biochemical pathways that may contribute to the development of age-related diseases (eg, cardiovascular diseases and cancer) that strongly influence survival.

A significant association with the longevity phenotype was also observed for a SNP, rs6276 A/G, in the DRD2 gene which encodes a G protein-coupled receptor located on postsynaptic dopaminergic neurons. Signaling through DRD2 is essential for proper regulation of diverse physiological functions, such as locomotion, behavior, and hormone production (56). Through in silico analysis, six different microRNAs were predicted to bind to the sequence containing the polymorphism rs6276, among them, miR-485-5p showed the highest energy binding level to 3′UTR with the minor G allele (Supplementary Table 3). Thus, G allele binding is expected to be associated with increased microRNA–mRNA binding, and this may, in theory, lead to a more stringent repression of DRD2 expression. It is of interest to notice on this point that a down-regulation of DRD2 expression has been reported in both striatal and extrastriatal regions of the brain of the elderly people (57), and that decreases in the density or activity of DRD2 receptor has been correlated with age-related decline in motor and cognitive functions (58), and with neurodegenerative diseases and behavioral disorders (59). In addition, a recent study indicates that astrocitic DRD2 activation suppresses neuroinflammation in the central nervous system (60), suggesting that DRD2 signaling may be involved in neuroinflammation that occurs in the nervous system during aging. Interestingly, Faghihi and colleagues (61) provided evidences that miR-485-5p is involved in BACE1 (beta-secretase-1) posttranscriptional regulation and is deregulated in RNA samples from Alzheimer's disease subjects. Very recently, a genetic variant within the DRD2 3′UTR (rs1130354), not considered in our study because it did not fulfill our selection criteria) has been found to interfere with the brain-expressed miR-326-mediated repression of DRD2 expression (62).

In our study we found that the minor G allele of rs6276, which is expected to increase the interaction of miR-485-5p with DRD2 3′UTR, was associated with decreased chance to become long-lived (OR = 0.5424, 95% CI = 0.3931 to 0.7485).
We are aware that there are some limitations to our study. First, it is worth mentioning that our selection procedure could be biased by our choices in the mining of literature data, and by the availability of data on genes involved in different pathways. As to the results, the potential effect of these SNPs on gene expression has not been validated by in vitro studies and, therefore, we cannot exclude the possibility that the associated SNPs are in linkage disequilibrium with causal variants either in the SIRT2 and DRD2 genes or genes located in nearby regions. We used Linkage Disequilibrium (LD) information from the 1000 Genomes Project to search SNPs in LD (cutoff of $r^2 < .9$) with rs45592833 and rs6276. LD patterns indicated that four SNPs were in LD with rs45592833 which are all located in intronic regions of SIRT2, while for rs6276 we identified 19 SNPs spanning a region of about 17 kb. We also explored the potential regulatory function of these variants. The more likely to be functionally relevant was the rs4938016 missense variant in the ANKK1 gene which is in LD with rs6276 in DRD2. It should be however considered that for this study we followed a stepwise and stringent bioinformatic analysis to identify miRSNPs in the 3′UTR of aging-implicated genes, and only good-confidence SNPs that may perturb the miRNA-mRNA interaction were considered for genotyping. In addition, it is worth to notice that the prioritizing approach we used to select the SNPs to test in the case-control study is not significantly influenced by the criterion we adopted (sum of all $|\Delta\Delta G|$ values). In fact, when we used the prioritizing approach based on the determination of the upper tertile of individual $|\Delta\Delta G|$ values, SIRT2 and DRD2 polymorphisms resulted again among the top-ranked SNPs. This result suggests that the “summation approach” we used is not slanted toward the group of miRNA recognizing a similar but not necessarily identical sequence.

To summarize, this study comprehensively evaluated the associations between SNPs in miRNA target binding sites of genes with a strong a priori biological relevance and probability to be involved in human longevity. The results support an association between two candidate miRSNPs, rs45592833 in SIRT2 and rs6276 in DRD2, and human longevity. Despite some limitations, we believe that our findings are of interest because they emphasize the importance of looking at miRSNPs and, in general, to gene expression regulation mechanisms, as potentially functional elements that may modulate human survival and longevity.

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

Funding
This work was partially supported by the European Union’s Seventh Framework Programme (FP7/2007–2011) [grant number 259679] and by funds from Programma Operativo Nazionale [01_00937]—MIUR “Modelli sperimentali biotecnologici integrati per lo sviluppo e la selezione di molecule di interesse per la salute dell’uomo.”

Conflict of Interest
The authors state that they have no conflict of interest to declare.

References


