

***MAP3K7* and *GSTZ1* are associated with human longevity: a two-stage case–control study using a multilocus genotyping**

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Abstract The pathways that regulate energy homeostasis, the mechanisms of damage repair, and the signaling response to internal environmental changes or external signals have been shown to be critical in modulating lifespan of model organisms and humans. In order to investigate whether genetic variation of genes involved in these pathways contribute to longevity, a two-stage case–control study in two independent sets of long-lived individuals from Calabria (Italy) was performed. In stage 1, 317 SNPs in 104 genes were analyzed in 78 cases (median age 98 years) and 71 controls (median age 67 years). In stage 2, 31 candidate SNPs identified in stage 1 ($\pi_{\text{markers}} = 0.1$)

were analyzed in an independent sample composed by 288 cases (median age 92 years) and 554 controls (median age 67 years). Two SNPs, rs282070 located in intron 1 of the *MAP3K7* gene, and rs2111699 located in intron 1 of the *GSTZ1* gene, were significantly associated (after adjustment for multiple testing) with longevity in stage 2 ($p=1.1 \times 10^{-3}$ and $p=1.4 \times 10^{-3}$, respectively). Interestingly, both genes are implicated in the cellular response to internal and external environmental changes, playing a crucial role in the inflammation processes that accompany aging. Our data confirm that long-lived subjects are endowed with genetic variants that allow them to optimize these cellular responses and to better deal with environmental and internal stresses.

Fausta Di Cianni and Daniele Campa, and Stefano Landi and Giuseppina Rose have equal contribution to the study.

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Introduction

Over the past decades, developed societies have experienced a gradual increase in life expectancy which sharply increased the percentage of elderly people in the population and, at the same time, increased the number of subjects who are not autonomous and are affected by invalidating pathologies (Vaupel 2010). Since this has important implications, especially from a social point of view, there is at present a considerable

interest in understanding how individuals achieve a long and healthy life.

Family-based studies demonstrated the existence of an important familial and genetic component of longevity. Indeed, parents, siblings, and offspring of long-lived subjects have a significant survival advantage and a higher probability to have been or to become long-lived (Gudmundsson et al. 2000; Kerber et al. 2001; Cournil and Kirkwood 2001; Perls et al. 2000, 2002; Schoenmaker et al. 2006; Willcox et al. 2006; Montesanto et al. 2011). Furthermore, the offspring of centenarians show a lower incidence of age-related diseases and a higher degree of physical functioning and autonomy, when compared to appropriately selected controls (Terry et al. 2003, 2004; Atzmon et al. 2006). Twin studies have found that approximately 25 % of the overall variation in human lifespan can be attributed to genetic factors (Herskind et al. 1996; Ljungquist et al. 1998; Skytthe et al. 2003), which becomes more relevant at advanced ages (Hjelmborg et al. 2006).

In the past years, there has been a surge of activity aimed at unraveling the effects of genetic variability on aging and lifespan. Specific mutations in single genes (age-1, daf2, sir2, methuselah, and p66) that extend or decrease lifespan in nematode worms (*Caenorhabditis elegans*), fruit flies (*Drosophila*), and mice have revealed evolutionary, often interconnected, conserved pathways for aging which are related to nutritional sensing, stress response signaling, and DNA repair/maintenance (Kenyon 2010; Fontana et al. 2010). The insulin/insulin-like growth factor-I (IGF-1) signaling pathway and downstream effectors such as the forkhead box O group (FoxO) family, the sirtuin family, the target of rapamycin (TOR) kinase, are perhaps the best studied pathways involved in aging and longevity.

Research in human longevity mainly focused on the orthologues of such candidate *loci*. Studies of centenarians or long-lived subjects have been successful in identifying specific genes and genotypes that influence human lifespan (for reviews see Christensen et al. 2006; Bonafè and Olivieri 2009; Chung et al. 2010; Slagboom et al. 2011). Particular striking examples include GH/IGF-1/insulin signaling (*GHR*, *IGF1R*, and *FOXO3A*), antioxidant (*SOD1*, *SOD2*, *PON1*, and *FOXO3A*), and inflammatory (*IL6*, *CETP*, and *Klotho*) pathways, silencing genes (*SIRT1* and *SIRT3*), and elements of lipid metabolism (*APOE*, *APOB*, *ACE*, and *APOC3*).

Most of gene-longevity association studies have so far focused on a single or a few candidate genes. However, given the complexity of the longevity trait, a single-locus approach may not be suitable, as each locus is likely to exert a small, if any, effect on the trait. Thus, approaches have been developed that take into account that complex traits can be caused by an intricate pattern of genetic variants, where the epistatic effects may be not negligible. Recent technological improvements in genotyping technology have made whole genome genotyping (>100,000 SNPs) more accessible. Indeed, genome-wide association studies (GWAS) are at present widely used to find genetic variants contributing to variation in human lifespan (Puca et al. 2001; Lunetta et al. 2007; Newman et al. 2010; Walter et al. 2011; Deelen et al. 2011; Nebel et al. 2011; Malovini et al. 2011). Along with GWAS, studies that consider candidate biochemical pathways consisting of many biologically related genes, and a minimal number of “tagging” SNPs that efficiently capture all the common genetic variation in the assayed genomic region, are also being routinely used (Passarino et al. 2006; Nebel et al. 2009; Pawlikowska et al. 2009; Flachsbart et al. 2010).

Here, for testing the association of a number of polymorphic genes with longevity, we used a two-stage design, an approach that has proven to be useful for the identification of a number of genetic variants associated with pathological phenotypes such as cancer, Crohn’s disease, and migraine (Milne et al. 2006; Beckly et al. 2008; Corominas et al. 2009). We analyzed 317 SNPs in 104 selected genes involved in longevity pathways (DNA repair, apoptosis and cell cycles regulation, metabolism of xenobiotics, insulin/IGF-1, and neuromodulation). A first sample (stage 1) was used to test all selected SNPs, and only those showing some hint of association were subsequently retested in a second sample (stage 2), thus reducing the number of association tests (Zuo et al. 2006; Satagopan and Elston 2003; Satagopan et al. 2004; Thomas et al. 2004; Skol et al. 2007; Zheng et al. 2008).

Materials and methods

Study population

Stage 1 sample was recruited between 1994 and 1997 in Calabria (southern Italy) and included 149 unrelated

subjects of which 78 were considered as cases (≥ 90 years; median age, 98 years; 36 males and 42 females) and 71 as controls (< 90 years; median age, 67 years; 39 males and 32 females). Stage 2 sample was recruited subsequently between 2005 and 2008, also in Calabria, and included 842 unrelated subjects of which 288 are cases (≥ 90 years; median age, 92 years; 125 males and 163 females) and 554 are controls (< 90 years; median age, 67 years; 277 males and 277 females). Study participants, their parents, and grandparents were all born in Calabria, as ascertained from population registers.

Younger subjects were contacted through general physicians. Subjects older than 90 years were identified by screening of population registers in different municipalities distributed across the entire Calabria region. Subjects who were eligible for the study were contacted and invited to participate in the study. A written informed consent was obtained from all participants before enrolling in the study. The healthy status was ascertained by medical visit carried out by a geriatrician who also administered a structured questionnaire validated within European recruitment projects (available at the following web site: <http://biologia.unical.it/echa/results.htm>). The questionnaire was aimed to the collection of sociodemographic information, evaluation of physical and cognitive status, medications, and information on common diseases that occurred in the past. Subjects with dementia and/or neurologic disorders were not included. 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.

Candidate genes and SNP selection

Candidate genes were chosen from genetic pathways related to DNA repair, apoptosis and cell cycle regulation, metabolism of xenobiotics, insulin/IGF-1, and neuromodulation. The selection was based on their biological relevance and on published reports indicating their involvement in aging and longevity in either model organisms or humans. Using keywords as “aging AND longevity”, “human aging”, “human longevity”, “aging AND model organism”, a range of online databases were employed including Pubmed (<http://www.ncbi.nlm.nih.gov/pubmed/>) and GenAge (<http://genomics.senescence.info/genes/>). Additionally, pathway analysis tools, such as “Biocarta” ([\[cgap.nci.nih.gov/Pathways/BioCarta_Pathways\]\(http://cgap.nci.nih.gov/Pathways/BioCarta_Pathways\)\) and “Kegg” \(<http://www.genome.jp/kegg/>\) were used for identification of pathway-specific genes.](http://</p>
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The chromosomal region encompassing the candidate genes were identified through the databases <http://www.ncbi.nlm.nih.gov/> (gene and mapview) and <http://www.ensembl.org/index.html>. The gene region was defined as the region from up to 5 kbp upstream of the transcription start site to up to 5 kbp downstream of the transcription stop site, allowing no overlap with the neighboring genes. In the choice of candidate SNPs, we used different online databases (eg., <http://www.ncbi.nlm.nih.gov/projects/SNP>; <http://www.ensembl.org/index.html>; and <http://www.hapmap.org/cgi-perl/gbrowse/hapmap>).

SNPs selection was based on the following criteria: minor allele frequency (MAF) $> 10\%$ in Caucasians, coding SNPs and SNPs having potential functional impact (non-synonymous SNPs, SNPs located in potential regulatory region), and SNPs previously identified in relevant association studies. Tagging SNPs were selected with the use of the Tagger program within Haploview (<http://www.broad.mit.edu/mpg/haploview/>; <http://www.broad.mit.edu/mpg/tagger/>; de Bakker et al. 2005), using pair wise tagging with a minimum r^2 of 0.8.

In total we identified 317 SNPs in 104 genes which are involved in the above-mentioned pathways. The complete list of genes, SNPs, and other relevant information is provided in Supplementary Table S1.

Genotyping

Genotyping of the samples in stage 1 was carried out using a microarray technique based on the arrayed primer extension (Kurg et al. 2000). Genotyping of samples in stage 2 was carried out using an allele-specific PCR-based KASPar SNP genotyping system (KBiosciences, Hoddesdon, UK) (Cuppen 2007). Genotyping quality control procedures consisted of inclusion of duplicates and inclusion of both positive and negative (water blanks) controls in each assay reaction.

Quality control

After genotype calling, in order to check errors at genotypic level, the datasets were subjected to a battery of quality control (QC) tests. In particular, SNPs were excluded if they had (1) a significant deviation

from Hardy–Weinberg equilibrium (HWE, $p < 0.001$) in the control group; (2) a Missing Frequency (MiF) higher than 20 %; and (3) a frequency of rare allele (MAF) < 1 %.

Statistical analyses

Single SNP analysis

In both stages, the chi-square test was used to assess the association between the variability of the analyzed polymorphisms and human longevity. Before to apply the test, each genotypic variable was first coded with respect to dominant, recessive, and heterozygote disadvantage/advantage model. For each of these models, the corresponding chi-square value was then obtained. The maximum of these three chi-square values (χ^2_{MAX}) was used as a measure of association between the polymorphism and the analyzed phenotype:

$$\chi^2_{\text{MAX}} = \max(\chi^2_{\text{DOM}}, \chi^2_{\text{REC}}, \chi^2_{\text{HET}})$$

where χ^2_{DOM} , χ^2_{REC} and χ^2_{HET} represent the three chi-square statistics assuming a dominant, recessive and an heterozygote disadvantage/advantage model of inheritance of the analyzed polymorphism. The p value of the proposed test statistics was obtained by the Monte-Carlo procedure described in Ziegler and König (2010). The procedure of ranking the SNPs not according to a single p value but to the minimum of different test statistics allows to obtain more robust results, as reported in Zheng et al. (2007).

In the present study, the χ^2_{MAX} test (maximum of three different chi-square tests) was used to assess the associations between the selected markers ($M=317$) and the analyzed phenotype. In the stage 1 the χ^2_{MAX} values were ranked, and a proportion π_{markers} equal to 0.1 (top 10 %) of the initial set of SNPs corresponding approximately to a p value < 0.15 was selected for stage 2 analysis. By following the standard strategy to consider the stage 2 data as a replication study, the same test was used to assess the association between the selected polymorphism and longevity, adopting the Bonferroni-corrected significance level $\alpha/(\pi_{\text{markers}} \cdot M)$.

Interaction analysis

In order to capture second-order SNP–SNP interactions, we used the model-based multifactor dimensionality

reduction (MB-MDR) method recently developed by Calle et al. (2010). It is an extension of the popular multifactor dimensionality reduction (MDR) method in which risk categories are defined using a regression model that also allows adjustments for main effects and covariates. By this approach, first, logistic regressions analyses are performed to define the nine possible genotypic combinations as high (H), low (L), or no risk (0). Then, these multilocus genotypes of the same risk category are merged and two Wald statistics (WH and WL, one for each risk) with the relevant p values (PH and PL) are obtained. The significance for the epistatic effect is based on the minimum between PH and PL (MIN.P). Finally, the significance of a specified model is assessed through a permutation test on the maximum Wald statistic, implemented in the function `mbmdr.PermTest` of MB-MDR package.

In the present study we applied the MB-MDR algorithm to the stage 2 dataset. The permutation procedure (10,000 permutations) was applied to the interaction models with a MIN.P < 0.05 .

Results

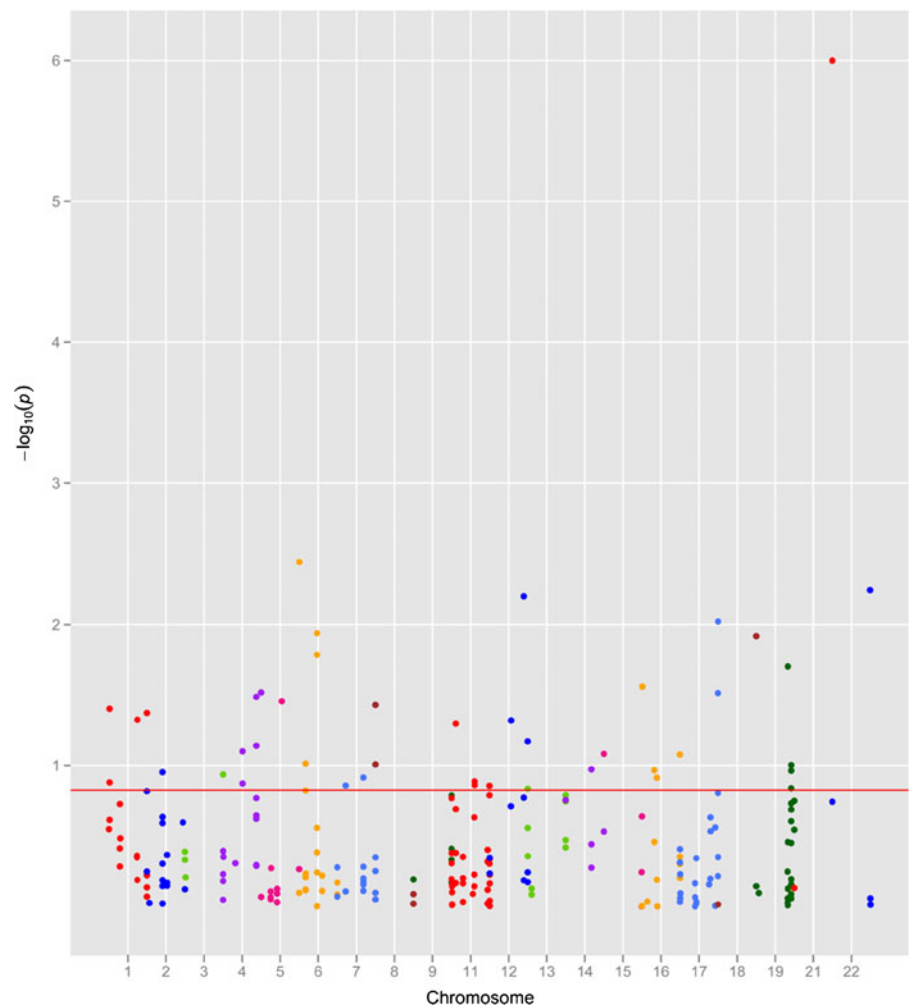
Dataset stage 1

Initially, 317 SNPs from 104 candidate genes (see Supplementary Table S1) were genotyped in 78 cases and 71 controls. After the QC phase, the final number of analyzed polymorphisms in stage 1 was 235. In particular, 49 SNPs were excluded from the analysis because of a significant deviation from HWE. Thirty SNPs were excluded from the analysis because of a MiF value higher than 20 %. Finally, three SNPs were excluded on the basis of MAF criterion (< 1 %). The cleaned dataset was then analyzed for association with longevity by using the χ^2_{MAX} test.

Single-locus analysis

The plot reported in Fig. 1 displays the p values computed on the basis of the χ^2_{MAX} test with respect to the analyzed polymorphisms. Supplementary Table S2 reports the list of the top-ranked SNPs ($\pi_{\text{markers}}=0.1$) obtained according to the association results and corresponding approximately to a p value < 0.15 .

Fig. 1 Plot of the stage 1 association results using the χ^2_{MAX} test (maximum of three different chi-square tests). Red line represents the p value corresponding approximately to the π_{markers} value (0.1) for the definition of the top-ranked SNPs ($p < 0.15$)



Dataset stage 2

The 31 selected SNPs were genotyped in the additional 288 cases and 554 controls in the subsequent stage 2. Two SNPs were excluded from the following association analysis because of a MiF value higher than 20 %. The cleaned dataset was then analyzed for association with longevity by using the χ^2_{MAX} test. Table 1 reports the SNPs which we found to have a significant effect on the phenotype in the χ^2_{MAX} test with the most likely best model. The complete list of the results obtained for the 29 SNPs of stage 2 is reported in Supplementary Table S3. After the Bonferroni's correction (p value threshold $< 0.05/29$), two of the observed associations remained statistically significant: rs282070 (p value equal to 1.1×10^{-3}) and rs2111699 (p value equal to 1.4×10^{-3}). In particular, for the SNP rs282070, located in *MAP3K7* gene, the subjects carrying the C allele

showed an increased chance to attain longevity; for the SNP rs2111699 located in *GSTZ1* gene, the subjects carrying two copies of the G allele showed an increased chance to survive at very old age.

Interaction analysis

Supplementary Table S4 reports the significant second-order interactions results ($p < 0.05$) in the stage 2 dataset from the MB-MDR analysis. In this table for each interaction model, a permutation testing was performed for each of the top-ranked models (MIN.P < 0.05) by permuting the outcome variable calling the *mbmdr.PermTest* function of the *mbmdr* package (10,000 permutations). By this approach, we found that all these interactions resulted to be significant ($p \leq 0.05$). However, after a Bonferroni's correction for multiple testing no significant differences remained.

Table 1 Significant SNPs obtained according to χ^2_{MAX} test in the stage 2 sample

SNP ID	dbSNP RS	Variation	Position	Gene	Best model	χ^2_{MAX}	<i>p</i> value best model	<i>p</i> value ^a
6	rs282070	G/C	Intron 1	<i>MAP3K7</i>	Dominant	13,939	5×10^{-4}	0.0011
145	rs2111699	A/G	Intron 1	<i>GSTZ1</i>	Recessive	12,511	3×10^{-4}	0.0014
306	rs11066301	A/G	Intron 1	<i>SHP2</i>	Dominant	8,444	0.005	0.0111
189	rs1042571	C/T	3' UTR	<i>POMC</i>	Recessive	4,413	0.046	0.0874
103	rs3212948	C/G	Intron 3	<i>ERCC1</i>	Recessive	4,024	0.048	0.1062

χ^2_{MAX} represents the maximum of the three chi-square values

^a*p* value was adjusted for multiple testing of different genetic models obtained by the proposed permutational approach

Discussion

In the present study, we investigated possible associations between longevity and 317 SNPs in 104 candidate genes involved in the regulation of aging-related biological pathways by using a two-stage approach. To improve the efficiency of this study design, we used a robust test that is powerful when the underlying genetic model is unknown and that was successfully applied to genome-wide association studies (Zheng et al. 2007; Sladek et al. 2007). We found that two SNPs, rs282070 on intron 1 of *MAP3K7* gene and rs2111699 on intron 1 of *GSTZ1* gene, were significantly associated with survival at advanced age after Bonferroni's correction.

MAP3K7 (also known as transforming growth factor- β activated kinase-1) is a serine/threonine kinase, member of the mitogen-activated protein kinase kinase family, which plays a crucial role in interleukin 1, tumor necrosis factor, and Toll-like receptor signaling pathways. Various stressors and inflammatory cytokines trigger the activation of MAP3K7 which in turn activates key intracellular kinases such as the p38 MAPK, c-jun N-terminal kinase, and I-kappa B kinase complex. These pathways ultimately activate transcription factors nuclear factor-kappa B and activator protein-1 which are critical regulators of genes of the immune-inflammatory responses (Ninomiya-Tsuji et al. 1999; Sato et al. 2005; Chen et al. 2006; Landström 2010). Studies have also demonstrated that the oxidative stress caused by reactive oxygen species (ROS) may induce or mediate the activation of the MAPK pathways, including the one mediated by MAP3K (Son et al. 2011 for a review). In particular, Omori et al. (2008) reported that MAP3K7 regulates the levels of ROS and prevent inflammation. Thus, MAP3K7 may be

considered as a crucial factor in the cascade of intracellular events initiated by environmental and/or physiological stressors that induce cells to survive or to die and therefore may be important for regulating aging. However, although several components of MAPK signaling pathways play important roles in the regulation of cellular senescence (Maruyama et al. 2009; Debacq-Chainiaux et al. 2010) and have been also implicated in the pathogenesis of many human diseases (Kim and Choi 2010), no data have been documented so far about the influence of MAP3K7 on human aging and longevity. The results of our study provide the first evidence of such an influence with the rs282070-C allele positively affecting longevity in a dominant manner.

Also *GSTZ1* is implicated in the cellular response to environmental insults. *GSTZ1* is a member of the glutathione S-transferase (GSTs) superfamily of multifunctional enzymes which are involved in the cellular detoxification, via conjugation with glutathione (GSH) of a wide range of endogenous and exogenous toxic compounds, and various reactive products generated under oxidative stress. Diverse cellular functions, ranging from apoptosis to cell survival, are also modulated by GSTs (Nebert and Vasiliou 2004; Hayes et al. 2005; Laborde 2010). The GSTs comprise three families of genes designed as cytosolic, mitochondrial, and microsomal transferase. The cytosolic GSTZ class of GSTs is expressed in many eukaryotic species, including fungi, plants, insects, and mammals (Board et al. 1997). Human *GSTZ1*, which is identical to maleylacetoacetate isomerase, catalyses two distinct reactions: the biotransformation of a range of α -haloacids, including dichloroacetic acid, a drinking water contaminant, and the GSH-dependent isomerization of maleylacetoacetate to fumarylacetoacetate, the penultimate step in the phenylalanine-tyrosine

catabolic pathway (Board and Anders 2005; Board and Anders 2011). Analysis of *Gstz1*-knockout mice provided more insight about the physiological role of *Gstz1*. Besides a range of pathological changes, *Gstz1*^{-/-} mice display the induction of some of the cytosolic GST isoenzymes, and the constitutive expression of genes that are regulated by antioxidant-response elements (AREs) and that respond to oxidative stress via the Keap1/Nrf2 signaling pathway, which regulates the expression of numerous detoxifying and antioxidant genes (Lim et al. 2004; Lee and Johnson 2004). All of these findings support the view that the lack of *GSTZ1* may induce oxidative stress (Blackburn et al. 2006). The antioxidant role of GSTs is well known and derives essentially from their ability to regulate the concentration of GSH, the most powerful cellular antioxidant, in different cellular compartments including mitochondria, the principal source and target of ROS. For instance, specific mitochondrial GST isoforms are able to protect mitochondria from oxidative insults either indirectly by maintaining appropriate levels of GSH or directly by reducing cellular peroxides and reactive aldehydes, such as 4-hydroxynonenal, generated from lipid peroxidation (Raza 2011). The recent discovery that *GSTZ1* also localizes in mitochondria (Li et al. 2011) leads to assume that *GSTZ1* may also contribute to the cellular redox status and then may be relevant to aging and longevity. Interestingly, Starr et al. (2008) showed that the *GSTZ1* R42G variant affects both telomere length, a biomarker of stress and physical aging, and suggest that this may occur through oxidative stress. Moreover it has been shown that the *GSTZ1*-1002 G>A variant influences cognitive ability in the elderly, probably because of its effect on dopamine production and dopamine by-product disposal (Starr et al. 2008). In our study, the rs2111699 resulted to be significantly associated to the longevity phenotype. It seems to act in a recessive manner, with the rs2111699-G allele favoring survival at very old age. To our knowledge, this is the first study reporting an association between *GSTZ1* genetic variability and human survival.

Overall, based on our data, it can be concluded that *MAP3K7* and *GSTZ1* are longevity genes whose variability actually affects the organism's chances of reaching advanced age. As the rs282070 in *MAP3K7* and the rs2111699 in *GSTZ1* are intronic, their functional significance is unclear. It is possible that these SNPs are located in regions containing *cis*-regulatory

elements essential for gene expression, and that the allelic variants differently affect the transcriptional rate, but linkage disequilibrium of these SNPs with other functional variants is also possible. This is an interesting issue that needs to be investigated in future research. Nevertheless, we believe that our results reinforce the view (synthesized in the oxidation-inflammatory theory of aging, see De la Fuente and Miquel 2009) that the immune, inflammatory, and stress responses, which are essential components of the body's network of defense against external and internal damaging agents, play an essential role in life span and in age-related phenotypes.

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