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A novel VNTR enhancer within the *SIRT3* gene, a human homologue of *SIR2*, is associated with survival at oldest ages

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Abstract

SIR2 genes control life span in model organisms, playing a central role in evolutionarily conserved pathways of aging and longevity. We wanted to verify whether similar effects may act in humans too. First, we searched for variability in the human sirtuin 3 gene (*SIRT3*) and discovered a VNTR polymorphism (72-bp repeat core) in intron 5. The alleles differed both for the number of repeats and for presence/absence of potential regulatory sites. Second, by transient transfection experiments, we demonstrated that the VNTR region has an allele-specific enhancer activity. Third, by analyzing allele frequencies as a function of age in a sample of 945 individuals (20–106 years), we found that the allele completely lacking enhancer activity is virtually absent in males older than 90 years. Thus the underexpression of a human sirtuin gene seems to be detrimental for longevity as it occurs in model organisms.

Keywords: SIRT3; Human longevity; VNTR enhancer

Aging can be viewed as a lethal by-product of activities, such as reproduction and food intake, that are controlled by genes [1]. Since most of these genes are evolutionarily conserved, distant species may share common pathways of aging [2]. The insulin/insulin-like growth factor 1 (IGF1) signaling pathway could be one such common pathway, as it modulates aging in many species, including *Caenorhabditis elegans*, *Drosophila*, mice [3], and possibly humans [4]. An elegant study carried out in *C. elegans* by applying microarray techniques showed that a member of the SIR2-like protein family is regulated downstream of *DAF-16*, a FOXO-family transcription factor that affects the rate of aging in response to the insulin/IGF1 pathway [5]. SIR2 proteins constitute an evolutionarily conserved family of NAD-dependent deacetylases called sirtuins [6–8]. In model organisms the expression levels of *SIR2* modulate life span [9–11]. Since sirtuins are NAD^+ dependent these proteins through different routes may link energy metabolism, genome maintenance, and aging [11,12]. Thus *SIR2* genes may play a crucial role in conserved pathways of aging and longevity.

A human homologue of the *SIR2* genes, *SIRT3*, lies at the telomeric terminal on chromosome 11p15.5 (www.ncbi.nlm.nih.gov/omim; MIM 604481; contig NT_035113). The gene is ubiquitously expressed, particularly in metabolically active tissues, and the SIRT3 protein is targeted to mitochondria through an N-terminus signal necessary for

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mitochondrial localization [13,14]. By analyzing a large sample of individuals including people older than 100 years of age, we found an association between longevity and the silent marker G477T of *SIRT3* [15]. Owing to the silent nature of the SNP, we hypothesized a linkage disequilibrium between the G477T marker and an unknown functional variant of *SIRT3*. The aim of the present study was to identify the functional variant that could account for the association we observed between the silent marker of *SIRT3* and survival. Thus, first we searched for variability in the *SIRT3* gene in a large population sample; then we characterized the functional activity, and its association with longevity, of a VNTR polymorphism we identified in intron 5.

Results

To identify variability in the functional domains of *SIRT3*, we conducted DNA sequence analyses of PCR fragments around the FGE conserved motif (exon 5) in a panel of 50 unrelated subjects (25 males and 25 females randomly chosen from sample 1). We observed a VNTR polymorphism having a 72-bp core in the fifth intron of the gene (12,343 nt initial position referring to www.ncbi.nlm.nih.gov/omim; MIM 604481; contig NT_035113). After verification of Mendelian inheritance in 30 parent/offspring pairs collected in another study, we analyzed the VNTR polymorphism in all of sample 1. Six length-alleles were identified spanning one to six repeats (see Table 1); four alleles were common (alleles 1–4), while two were rare (alleles 5 and 6). Since the sample under study had been already typed for the silent variant G477T of *SIRT3* [15], we analyzed linkage disequilibrium between the markers G477T and VNTR. The disequilibrium was highly significant ($p < 0.001$), with 10 observed haplotypes of the 12 expected (estimated by MLE from both homozygous and heterozygous genotypes).

Table 1
Genotypic and allelic frequencies of the VNTR polymorphism in sample 1 (20- to 80-year-old subjects)

Genotype	Count	Allele	Count
1.1	124 (17.6 ± 1.4)	1	587 (41.7 ± 1.3)
1.2	73 (10.4 ± 1.2)	2	166 (11.8 ± 0.9)
1.3	112 (15.9 ± 1.4)	3	291 (20.7 ± 1.1)
1.4	149 (21.2 ± 1.5)	4	351 (25.0 ± 1.2)
1.5	1 (0.1 ± 0.1)	5	6 (0.4 ± 0.2)
1.6	4 (0.6 ± 0.3)	6	5 (0.4 ± 0.2)
2.2	13 (1.9 ± 0.5)	Total	1406 (100.0)
2 3	27 (3.8 ± 0.7)		
2.4	38 (5.5 ± 0.9)		
2.5	2 (0.3 ± 0.2)		
3.3	40 (5.7 ± 0.9)		
3.4	69 (9.8 ± 1.1)		
3.5	2 (0.3 ± 0.2)		
3.6	1 (0.1 ± 0.1)		
4.4	47 (6.7 ± 0.9)		
4.5	1 (0.1 ± 0.1)		
Total	703 (100.0)		

Relative frequencies ± standard errors (×100) are given in parentheses. Allele nomenclature refers to the repeat number. Hardy–Weinberg equilibrium check (5000 permutations): $p = 0.185$.

Then we checked whether nucleotide variability did occur within the VNTR alleles. By sequencing the PCR-DNA fragments of all the subjects homozygous for the alleles having 1 to 4 repeats (see Table 1) we identified a T/C variation located in the second repeat, 63 nt from its starting point. MatInspector V2.2 analysis carried out on the 72-bp core fragment revealed that the T/C variation transformed a GATA3 site into a DeltaEF1 site, potentially having different regulatory activities [17,18]. On the occurrence of the GATA3/DeltaEF1 site in the second repeat, the length-alleles having one to four repeats were resolved into seven alleles. In fact, with the exception of allele 1 (which lacked a second repeat and thus had the GATA3 core sequence in all cases), each of the remaining common length-alleles was resolved into two categories, one having the GATA3 site (alleles 2a, 3a, and 4a) and the other the DeltaEF1 site (alleles 2b, 3b, 4b). Fig. 1 shows the position of the VNTR with respect to the G477T silent marker we previously identified in exon 3 [15], the seven length-alleles spanning one to four repeats, and the GATA3/DeltaF1 variable sequence of the second allelic repeat.

We analyzed possible enhancer effects of specific VNTR alleles. By using constructs including the alleles lacking the DeltaEF1 site (alleles 1a, 2a, 3a, 4a) we found that these alleles act as an enhancer whose activity appears to be related to the number of VNTR repeats (Fig. 2A). One-way ANOVA showed a statistically significant difference among the luciferase activities observed in the whole experiment ($p \leq 0.001$). Then LSD post hoc tests showed that: (i) the luciferase activity sustained by each of the four alleles is significantly higher than that present in the control ($p < 0.05$ for every allele) and (ii) the luciferase activity is significantly different between alleles that differ for two repeats at least ($p = 0.04$ between alleles 1a and 3a, $p = 0.01$ between alleles 1a and 4a, $p = 0.02$ between alleles 2a and 4a). But, when the alleles harboring the DeltaEF1 site in the second repeat were assayed (alleles 2b, 3b, 4b) allele 2b showed a dramatic reduction in the enhancer activity with respect to allele 2a (Fig. 2B), although the enhancer activity of alleles 3b and 4b did not differ from that of 3a and 4a, respectively (results not shown).

By the above approach we demonstrated that the VNTR acts in vitro as an enhancer on a reporter gene. To explore a possible role of this enhancer in human longevity we analyzed the VNTR polymorphism in sample 2 (90- to 106-year-old subjects) and compared the genotypic and allelic frequencies with those of sample 1 (20- to 80-year-old subjects). The analyses were carried out according to sex because of gender effects in mortality of the oldest people and sex specificity in gene/longevity associations. On the basis of data in Table 2, we rejected the hypothesis of homogeneity between the genotypic pools of samples 1 and

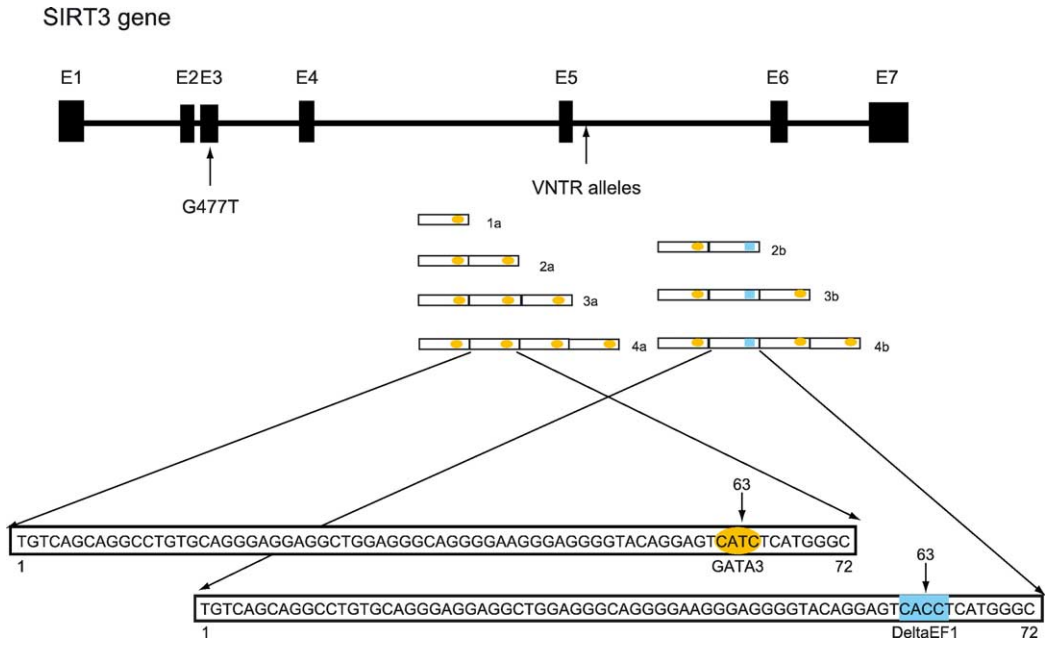

Fig. 1. A schematic representation of the *SIRT3* gene showing the positions of the G477T marker previously identified [15] and the VNTR discovered in the present study. The alleles including the GATA3 site in the second repeat (in yellow) are named 1a–4a, while the alleles including the DeltaEF1 site (in blue) are named 2b–4b. The variable sequence occurring in the second allele repeat is also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2 in males ($p = 0.026$) but not in females ($p = 0.674$). Likewise, allele distribution frequencies were different between samples 1 and 2 in males ($p < 0.001$) but not in females ($p = 0.923$). In particular, in males, the frequency of allele 2 decreased from sample 1 to sample 2 (Table 2B). Since length-allele 2 included the sole allele lacking enhancer activity, we checked possible age-related variations of the gene pool in terms of alleles 2a and 2b by sequencing allele 2 in all the males. Of the 89 alleles found in the young group (sample 1, Table 2B) 49 were 2a and 40 were 2b, while all 5 alleles found in the oldest group (sample 2, Table 2B) were 2a. Therefore, in the entire gene pool, allele 2a decreased from 7.8 ± 1.1% in sample 1 to 2.9 ± 1.3% in sample 2 ($p = 0.025$ by Fisher exact test), while allele 2b decreased from 6.4 ± 1.0% in sample 1 to 0 in sample 2 ($p = 8 \times 10^{-5}$ by Fisher exact test).

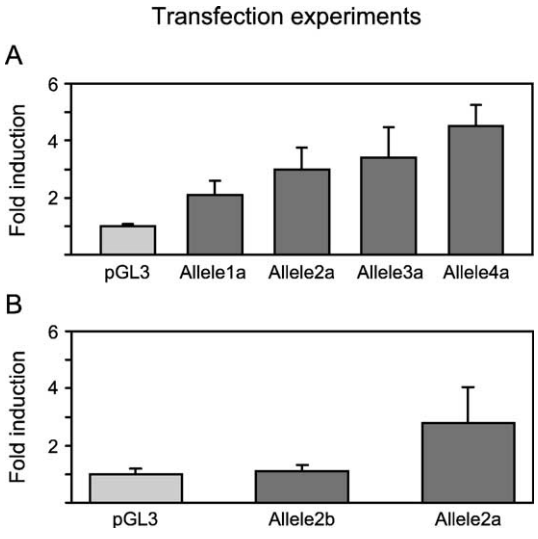

Fig. 2. (A) Luciferase expression of constructs including the 1a–4a alleles reported as fold induction (pGL3 negative control). (B) Luciferase expression of constructs including the allele 2b or the allele 2a reported as fold induction (pGL3 negative control). In both A and B the values reported for transfection experiments are the means ± SD of triplicate experiments.

Discussion

The aim of this study was to identify a putative functional variant occurring in *SIRT3* that could account for the association previously observed between longevity and a silent marker of this gene [15]. Transfection experiments demonstrated that the VNTR we identified in intron 5 of *SIRT3* is a functional polymorphism and that different VNTR alleles are able to modulate the expression of a reporter gene in an allele-specific way, according to number of repeats and occurrence of GATA3/DeltaEF1 site in the second repeat of the length-allele 2.

Is this polymorphism the functional variant we were searching for? Some hints indicate that this may be the case. First, a strong linkage disequilibrium occurs between the G477T silent marker and VNTR alleles; second, both genotypic and allelic frequency distributions differ between the group selected for longevity (male sample 2) and the younger control group (male sample 1).

It is intriguing that the sole allele lacking an enhancer effect in vitro, allele 2b, is completely absent in the oldest

Table 2
(A) Genotypic and (B) allelic counts of VNTR polymorphism in samples of males and females of different ages

	Males		Females	
(A) Genotype	Sample 1 (20–80 years)	Sample 2 (90–106 years)	Sample 1 (20–80 years)	Sample 2 (90–105 years)
1.1	46 (14.7 ± 2.0)	14 (16.3 ± 4.0)	78 (19.9 ± 2.0)	27 (17.3 ± 3.0)
1.2	36 (11.5 ± 1.8)	3 (3.5 ± 2.0)	37 (9.5 ± 1.5)	13 (8.3 ± 2.2)
1.3	42 (13.5 ± 1.9)	15 (17.4 ± 4.1)	70 (17.9 ± 1.9)	31 (19.9 ± 3.2)
1.4	69 (22.1 ± 2.3)	23 (26.7 ± 4.8)	80 (20.5 ± 2.0)	33 (21.2 ± 3.3)
1.5	—	—	1 (0.3 ± 0.3)	—
1.6	—	—	4 (1.0 ± 0.5)	—
2.2	10 (3.2 ± 1.0)	—	3 (0.8 ± 0.4)	4 (2.6± 1.3)
2.3	12 (3.9 ± 1.1)	—	15 (3.8 ± 1.0)	6 (3.8 ± 1.5)
2.4	20 (6.4 ± 1.4)	2 (2.3 ± 1.6)	18 (4.6 ± 1.1)	5 (3.2 ± 1.4)
2.5	1 (0.3 ± 0.3)	—	1 (0.3 ± 0.3)	—
3.3	18 (5.8 ± 1.3)	4 (4.7 ± 2.3)	22 (5.6 ± 1.2)	10 (6.4 ± 2.0)
3.4	37 (11.9 ± 1.8)	11 (12.8 ± 3.6)	32 (8.2 ± 1.4)	13 (8.3 ± 2.2)
3.5	1 (0.3 ± 0.3)	2 (2.3 ± 1.6)	1 (0.3 ± 0.3)	—
3.6	—	—	1 (0.3 ± 0.3)	—
4.4	19 (6.1 ± 1.4)	10 (11.6 ± 3.5)	28 (7.1 ± 1.3)	11 (7.1 ± 2.0)
4.5	1 (0.3 ± 0.3)	1 (1.2 ± 1.2)	—	2 (1.3 ± 0.9)
4.6	—	1 (1.2 ± 1.2)	—	1 (0.6 ± 0.6)
Total	312 (100.0)	86 (100.0)	391 (100.1)	156 (100.0)
(B) Allele				
1	239 (38.3 ± 1.9)	69 (40.1 ± 3.7)	348 (44.5 ± 1.8)	131 (42.0 ± 2.8)
2	89 (14.3 ± 1.4)	5 (2.9 ± 1.3)	77 (9.9 ± 1.1)	32 (10.3 ± 1.7)
3	128 (20.5 ± 1.6)	36 (20.9 ± 3.1)	163 (20.8 ± 1.5)	70 (22.4 ± 2.4)
4	165 (26.4 ± 1.8)	58 (33.7 ± 3.6)	186 (23.8 ± 1.5)	76 (24.4 ± 2.4)
5	3 (0.5 ± 0.3)	3 (1.8 ± 1.0)	3 (0.4 ± 0.2)	2 (0.6 ± 0.5)
6	—	1 (0.6 ± 0.6)	5 (0.6 ± 0.3)	1 (0.3 ± 0.3)
Total	624 (100.0)	172 (100.0)	782 (100.0)	312 (100.0)

Relative frequencies ± SE (×100) are given in parentheses.
(A) Genotypic homogeneity tests between samples 1 and 2: $p = 0.026$ in males, $p = 0.674$ in females.
(B) Allelic homogeneity tests between samples 1 and 2: $p < 0.001$ in males, $p = 0.923$ in females.

men while it is present in the younger group ($p = 8 \times 10^{-5}$). This finding suggests that allele 2b is detrimental for male longevity. On the other hand as the sample's age increases the frequency of allele 2a decreases as well ($p = 0.025$), thus suggesting that this allele is also unfavorable to male longevity. It follows that the GATA3/DeltaEF1 variability is not the only factor affecting the age-related variation of the gene pool and that other regulatory sites present in the 72-bp core do not act independent of the number of the VNTR repeats. We are now carrying out appropriate molecular studies to verify what is the exact portion of the VNTR repeat that links nuclear proteins, what are the proteins interacting with the repeat core, and, most importantly, how do these proteins interact according to the number of repeats.

Although the effect of the VNTR alleles as enhancer sequences has been demonstrated on a reporter gene, on the whole our findings are in line with the possibility that the VNTR acts in vivo on *SIRT3* and that variations in the expression of a *SIR2*-like gene may modulate life span in mammals [19]. Since the SIRT3 protein could deacetylate mitochondrial proteins involved directly in apoptosis [14], possibly by mimicking SIRT1 in inhibiting apoptosis by p53 deacetylation [20,21], the level of expression of *SIRT3* may affect longevity by acting on apoptotic mitochondrial patterns [22].

The association between VNTR alleles and longevity was restricted to males, because no difference was observed, either in the genotypic or in the allelic pool, between female samples 1 and 2 (Table 2). This finding confirms that longevity factors are sex-specific [23–25] and that males and females probably follow different trajectories to attain longevity [16].

Although cross-sectional data have been used for estimating the association between VNTR alleles and longevity, the present study should be reliable and not affected by bias caused by population stratification. In fact the population under study is highly homogeneous because of historical and geographical factors; furthermore, we did not find any stratification in this sample (unpublished results) by analyzing a panel of neutral markers spanning the genome [26].

In conclusion, our study has discovered a VNTR polymorphism in intron 5 of the *SIRT3* gene that has an allele-specific enhancer activity on a reporter gene. The frequency distribution of VNTR genotypes (and alleles) is significantly different between a sample of older men and a sample of younger men from the same population. Of course we need to confirm by appropriate longitudinal studies the effect of the VNTR on male longevity, and these studies are going on.

Materials and methods

Population samples

A population sample of 945 subjects was analyzed. All the subjects lived in Calabria (southern Italy) and their origin in the area was ascertained up to the grandparents' generation (interview). The sample consisted of two subsamples: sample 1 was made up of 20- to 80-year-old subjects (median age 59 years); sample 2 was made up of 90- to 106-year-old subjects (median age 102 years). Sample 1 comprised 703 subjects (312 males and 391 females); sample 2 comprised 242 subjects (86 males and 156 females). For collecting sample 1, an appropriate campaign was addressed to Calabria University students and staff, as well as to people who attended the University for the Elderly or used local thermal baths. For collecting sample 2, subjects older than 90 years were identified by consulting the population registers of the Municipalities of Calabria, contacted by phone, and then visited.

After a detailed explanation of the aims of the research, the subjects who agreed to participate donated a blood sample for routine laboratory analyses and DNA preparation; furthermore, people older than 60 years underwent a complete clinical and geriatric assessment. Subjects free of clinically overt pathologies and having blood and biochemical parameters in the normal age- and sex-specific range were enrolled in the study. In particular, the subjects enclosed in sample 2 belonged to health categories A and B previously described [16].

All the subjects analyzed in this study have given written informed consent for genetic studies on aging carried out by the present research group.

DNA samples

Six milliliters of blood was drawn from each subject. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from blood buffy coats following standard procedures.

VNTR polymorphism identification

PCR amplification of genomic DNA was carried out in 25-μl final volume containing 100 ng of DNA; 0.08 μM each forward primer, 5′-TTCCTGAAGCTGGGTACA-3′ (5′ position 12,030 nt referring to www.ncbi.nlm.nih.gov/omim; MIM 604481; contig NT_035113), and reverse primer, 5′-CATTCACCTTCCCAAAGTGG-3′; 200 μM dNTP; 0.3 U Finnzymes *Taq* DNA polymerase; 1.5 mM $MgCl_2$; and 1× thermophilic buffer. The following thermal cycling conditions were used: 1 cycle at 95°C for 45 s and 25 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C. An extra step was performed at 72°C for 5 min. PCR-amplified fragments were analyzed by automated sequencing in a 310 DNA sequencer with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

VNTR polymorphism typing

Both Gene Scan DNA fragment analysis (TAMRA 500 internal size standard) in a 310 DNA sequencer (PE Applied Biosystems) and electrophoresis in a 1.5% agarose gel (Marker Step Ladder 50 bp, Sigma, as molecular weight marker) were used on PCR-amplified products for VNTR genotyping.

Identification of alleles 2a and 2b

Allele 2 was analyzed by DNA sequencing in all the male carriers of the allele (10 homozygous plus 74 heterozygous subjects). After PCR amplification (see VNTR polymorphism identification) and electrophoresis, the DNA fragment containing two repeats was recovered from agarose gel in TAE buffer 1× (0.04 M Tris acetate, 0.001 M EDTA) by using the QIAquick gel extraction kit (Qiagen). The sequencing reactions were automatically carried out with the forward primer 5′-TCTTGCTGCATGTGGTTG-3′ (5′ position 12,275 nt).

Plasmid construction

The pGL3 promoter vector (Promega) containing an SV40 promoter was used in the construction of reporter plasmids for the analysis of allele-specific regulatory functions. Preliminarily, to discriminate between alleles having the same length but different nucleotide sequence (T/C at position 63 in the second repeat) the common alleles (one to four repeats) were amplified and automatically sequenced from subjects who were homozygous for the 1–4 length-alleles. The forward primer 5′-*CTCCCCGGG*TGGGAACTCCCTGGAGGTT-3′ (5′ position 12,324 nt referring to www.ncbi.nlm.nih.gov/omim; MIM 604481, contig NT_035113) and the reverse primer 5′-*CTCCCCGGG*TGGCACCAGCCCTGGAAG-3′ were used to have DNA fragments containing the VNTR sequence only (the italicized sequence was added to include the *Sma*I site for cloning). The PCR conditions were the same as described above. The PCR products of subjects having (CC) or (TT) homozygous genotype were purified by agarose gel in TAE buffer by using the QIAquick gel extraction kit (Qiagen) and digested with *Sma*I restriction enzyme. After purification by phenol extraction, the fragments were cloned into the *Sma*I-digested and dephosphorylated pGL3 vector, upstream of the *Luc*-promoter transcriptional unit. The sequence of each insert (and its orientation in the reporter vector) was confirmed by restriction mapping and automated DNA sequencing. Cloned vectors were purified by Qiagen Plasmid Maxi Kit and used for subsequent experiments.

Transfection assays

HeLa cells were cultured in DMEM (Sigma Aldrich) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin. Cells were transferred to 24-well plates with 500 μl of regular growth medium/well the day before transfection. Transfections were performed with the Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics) with a mixture containing 1 μg of each reporter plasmid and 2 ng of *Renilla* luciferase (Promega). Luciferase activity was measured after 24 h by using the Dual Luciferase Kit (Promega) in a Lumat LB9507 luminometer (EG&G Berthold). The luciferase activity of the reporter plasmids was normalized to the activity of *Renilla* luciferase. All transfections were performed in triplicate and repeated at least three times.

Statistical analyses

VNTR allele frequencies in samples 1 and 2 were estimated by counting genes from the observed genotypes, after verification of Hardy–Weinberg equilibrium by allele shuffling (5000 random permutations). The null hypothesis of homogeneity between the genotypic (or allelic) pools of samples 1 and 2 was checked by permutation tests (1000 permutations). The statistical significance of the differences among the enhancer activity of different VNTR alleles in transfection experiments was tested by ANOVA and LSD post hoc test. The MATLAB statistical textbook was used for all the statistical analyses, excepted for linkage disequilibrium analysis between the markers G477T [15] and VNTR, for which the Arlequin software was used; this software utilizes a maximum likelihood estimation procedure by using genotypic data from both homozygous and heterozygous subjects.

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