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Preferential loss of 5S and 28S rDNA genes in human adipose tissue during ageing

A. Zafiropoulos[a], E. Tsentelierou[a], M. Linardakis[b], A. Kafatos[b], D.A. Spandidos[a,*]

[a] *Laboratory of Virology, Medical School, University of Crete, Heraklion 71100, Crete, Greece*
[b] *Department of Social Medicine, Preventive Medicine and Nutrition Clinic, Medical School, University of Crete, Heraklion, Greece*

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Abstract

Loss of genomic rDNA has been associated with cellular and organismal ageing. The rDNA locus in humans comprises multiple copies of the 5.8S, 28S and 18S genes. Aim of the present study was to test the effect of aging on the copy number of the three rDNA genes individually in post-mitotic human tissue. We utilized real time polymerase chain reaction relative quantification to measure the copy number of 5.8S, 28S and 18S rDNA genes individually. We obtained adipose tissue from 120 male individuals aged from 9 to 94 years. The available data of each subject corresponding to the time of tissue sampling included: age, height, weight and calculated body mass index. Each rDNA gene was directly tested with Pearson correlation against age and body mass index. We found a significant negative correlation of the gene copy of 5.8S ($P < 0.001$) and 28S ($P < 0.003$) with age. Interestingly 18S gene copy displayed a different pattern with no statistically significant correlation with age. Conversely, we observed a significant negative correlation of the 18S gene copy with body mass index ($P = 0.004$) and a marginally non-significant negative correlation of the 5.8S ($P = 0.097$) gene copy with body mass index. In summary our results indicate that the rDNA recombination events in humans can be differentially targeted and regulated in response to ageing and/or fat accumulation. The proposed model generates possible implications regarding the effects of each rDNA gene loss in cell function as well as the mechanism of recombination targeting.

Keywords: Ageing; rDNA; Calorie restriction; Body mass index

1. Introduction

Ribosomal DNA (rDNA) genes encode the rRNA species that form the ribosomes and mediate proteinosynthesis in eukaryotic cells. The rDNA genes in the human genome exist in multiple copies, ranging from 150 to 300 per haploid genome, located in clusters

* Corresponding author. Tel.: +302810 394633; fax: +302810 394759.
E-mail address: Spandidos@spandidos.gr (D.A. Spandidos).

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at chromosomes 13, 14, 15, 21 and 22 (Worton et al., 1988). The rDNA repeating unit contains three genes, 18S, 5.8S, 28S, separated by intergenic sequences and other components of the ribosome assembly making a total of 42.9 kb. The close proximity of a large number of sequences with high homology such as the rDNA in the genome renders it a potential target for gene rearrangement through homologous recombination. Previously the rDNA gene copy number was found to be associated with ageing, and a selective loss of rDNA repeating units during the course of ageing was demonstrated. Age-dependent loss of rDNA has been demonstrated in beagle dog brain tissues (Johnson & Strehler, 1972), in human myocardium and cerebral cortex (Strehler & Chang, 1979; Strehler, Chang, & Johnson, 1979) and in mouse brain spleen and kidney tissues (Gaubatz & Cutler, 1978). The initial hypothesis stated that loss or inactivation of genes that have an impact on all protein synthesis should impair the function of post-mitotic cells in response to maximal stress (Strehler and Chang, 1979; Strehler, Chang, & Johnson, 1979). Nevertheless, a causal or effector relationship of rDNA loss with ageing has not been established until today.

The mechanism of rDNA gene loss has been studied extensively in *Saccharomyces cerevisiae* where the formation of extra-chromosomal circular rDNAs (ERCs) was demonstrated (Guarente, 1997). The ERCs appeared in the mother cell after the budding of the daughter cell and increased in number reaching 70% of the genomic DNA content. Ageing was found to be accelerated when exogenous ERCs were introduced (Sinclair & Guarente, 1997). Mutations affecting the recombination rate at the rDNA locus were found to greatly affect the life-span of the yeast cell (Defossez et al., 1999). The most important was the loss-of-function mutation of the SIR2 gene which normally is responsible for the silencing of the rDNA locus and the reduction of recombination (Guarente & Kenyon, 2000). In the same model an association was discovered between the modulation of recombination activity in the rDNA locus and the caloric restriction through the modulation of NAD metabolism (Lin, Defossez, & Guarente, 2000). Caloric restriction has been shown to extend life-span in several biological models (rodents, yeast, and worms) but the exact mechanism has not been elucidated yet.

The validation of the above theories in higher mammals and humans is still pending. The early experiments on non-mitotic human tissue employed a small set of samples and examined the whole of the rDNA repeating unit and not the individual genes. The aim of our study was to quantify independently the 18S, 5.8S and 28S rDNA genes, linear or circular, in a cohort of 120 humans and examine the relation of gene copy number with age and obesity. We identified a significant negative correlation of the 5.8S and 28S rDNA with age, in agreement with the early findings, but no correlation of the 18S raising the possibility that the rDNA recombination event can be differentially targeted. We also identified a surprising negative correlation of the 18S rDNA with body mass index (BMI) linking obesity and rDNA recombination in humans.

2. Materials and methods

2.1. Donors and anthropometric measures

Hundred and twenty male donors, aged from 9 to 94 years were recruited from the Preventive Medicine and Nutrition Clinic of the University of Crete (Heraklion, Greece). The donors were selected from two previously described cohorts (seven country study (Kafatos et al., 1997), lawyers study (Mamalakis, Tornaritis, & Kafatos, 2002a)) and from new recruits in order to cover the above specified age range. The geriatric part of the population has a longitudinal follow-up for the last 40 years. We selected donors without hypertasis, diabetes type 2 and cardiovascular disease. All participants gave informed consent and the study was approved by the institute's ethics committee. Body weight was assayed by a digital scale (Seca) with an accuracy of 100 g. Subjects were weighed without shoes, in their underwear. Standing height was measured without shoes to the nearest 0.5 cm with the use of a commercial stadiometer with the shoulders in relaxed position and arms hanging freely. Body mass index was calculated by dividing weight (kg) by height squared (m^2).

2.2. Tissue specimens and extraction of total DNA

Buttock subcutaneous tissue samples were collected by aspiration, using the method described by Beynen and Katan (1985). The particular method has been reported to be rapid and safe, and to cause no more discomfort than a routine venipuncture (Beynen & Katan,

Table 1
Primer sequences used for the amplification of different ribosomal DNA regions

Primers	Position	Sequence	Optimal Mg (mM)	Product size (bp)
18S forward	3638–3659	cgccgcgctctaccttaccta	5	159
18S reverse	3756–3777	taggagaggagcgagcgacca		
5.8S forward	6514–6535	gaggcaaccccctctcctctt	6	136
5.8S reverse	6609–6630	gagccgagtgatccaccgcta		
28S forward	7927–7948	ctccgagacgcgacctcagat	5	173
28S reverse	8059–8080	cgggtcttccgtacgccacat		

1985), and such tissue samples can be safely stored for up to 1.5 years without changes in the component fatty acids (Beynen & Katan, 1985). Samples were taken from the left upper outer quadrant of the gluteal area, through the use of a 10 ml vaccutaneous tube. Aspiration sites were sprayed with local anesthetic (ethyl chloride) prior to aspiration. Adipose tissue samples were stored at −80 °C until DNA extraction. DNA was extracted as previously described (Saridaki et al., 2003) and stored at −20 °C until polymerase chain reaction (PCR) amplification. The quantity and purity of the genomic DNA preparation was assessed with 260/280 spectrophotometry.

2.3. Real time PCR

Specific oligonucleotide primer sequences were designed from Genbank sequence U13369, targeting the junctions of 18S, 5.8S and 28S genes (Table 1). The relative localization of the selected primers is shown in Fig. 1. The β2-microglobulin (β2m) gene was selected as a single copy gene to normalize the ribosomal gene content. The primers for β2m have been previously published (Zafiropoulos, Tsentelierou, Billiri, & Spandidos, 2003). For the real time PCR reaction we utilized the Dynamo SYBR Green qPCR kit (Finnzymes, Finland) and added 0.5 mM of each primer, appropriate amount of $MgCl_2$ and 100 ng of genomic DNA in a 50 μl reaction. Each PCR reaction was standardized with respect to Mg concentration (Table 1). We prepared a single master mix for all the expected measurements for each gene to reduce pippeting errors. The real time PCR protocol included: 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 40 s and 72 °C for 40 s, where measurements on SybrGreen channel (470 nm/585 hp) were acquired. Then a melting curve analysis was employed to verify the presence of correct product through comparison with the predicted melting point temperature. Finally we performed an extension step at 72 °C for 10 min and the PCR products were visualized by agarose electrophoresis and ethidium bromide staining to verify the correct size of each product.

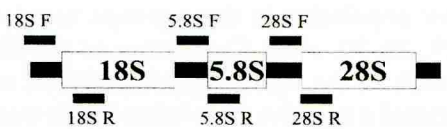

Fig. 1. Schematic representation of the rDNA repeating unit and the exact location of the oligonucleotide sets used for the quantification. F: forward primer, R: reverse primer.

All real time PCR measurements were performed on the Rotor-Gene 3000 (Corbett Research, Australia). For each sample four PCR reactions were run in parallel (18S, 5.8S, 28S and β2m) and the cycle threshold (Ct) value was calculated. The reproducibility of the experimental setup was tested by doing triplicate measurements in 10 samples in all markers (b2m, 18S, 5.8S and 28S). The standard deviations of the Ct value between replicates was in the range 0.03–0.08 (if we consider Ct = 1). For each ribosomal gene the dCt value (Ct β2m -Ct rDNA) was calculated to normalize for sample variation in genomic DNA quantity or quality (Fig. 2). The dCt value is directly related to the rDNA gene copy number.

2.4. Statistical analysis

Each rDNA gene dCt was correlated sequentially according to Pearson with age and BMI. Statistical significance was defined as $P < 0.05$. All analyses were performed using SPSSv10 (SPSS Inc., Chicago, IL, USA).

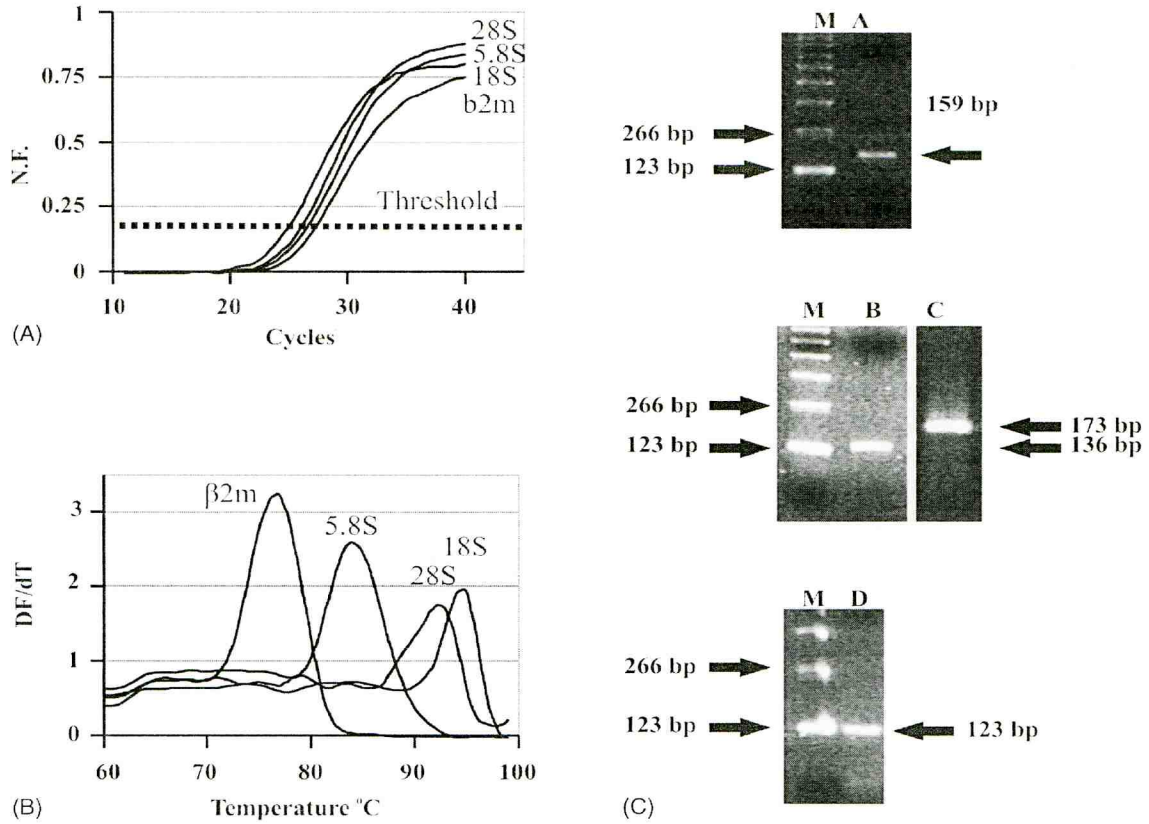

Fig. 2. rDNA gene copy quantification: (A) representative real time PCR curves for 18S, 5.8S, 28S and β2-microglobulin (β2m) gene analysis. N.F.: normal fluorescence. (B) Melting temperature analysis of the resulting real time PCR products. (C) Real time PCR products visualized with ethidium bromide staining in a 2% agarose gel electrophoresis. M: 123 bp ladder, A: 18S product, B: 5.8S, C: 28S, D: β2-microglobulin.

3. Results and discussion

We recruited 120 male individuals aged from 9 to 94 years. After measuring height and weight we obtained the gluteal adipose tissue sample. Visceral adipose tissue has been previously linked to insulin resistance and diabetes type 2 in geriatric populations but also in younger age groups and children (Mamalakis, Kafatos, Manios, Kalogeropoulos, & Andrikopoulos, 2002b). The interabdominal fat and the subcutaneous abdominal wall fat have different composition from the gluteal fat as has been shown in children.

Genomic DNA was extracted from the adipose tissue and the quantification of the 18S, 5.8S and 28S rDNA was performed as described in Section 2. The quantity of each gene was tested according to Pearson for correlation with age and body mass index (BMI). We observed a highly significant linear negative correlation of the age with 5.8S ($P < 0.001$) and 28S ($P = 0.003$) (Fig. 3B and C) while there was no correlation with 18S (Fig. 3A). Conversely we documented a linear negative correlation of BMI with 18S ($P = 0.004$) (Fig. 3D) and no correlation with 5.8S and 28S (Fig. 3E and F). Further statistical analysis was performed to evaluate the possible confounding effect of age on BMI in relation to 18S rDNA. We separated the donor population in three groups based on their age (<29, 29–70, >70). Correlation of the BMI with 18S in each of the age groups <29, 29–70 and >70, demonstrated a negative correlation which was getting gradually more pronounced in the older age groups ($b = -0.14$, P = ns; $b = -294$, $P = 0.074$; $b = -613$, $P = 0.012$, respectively).

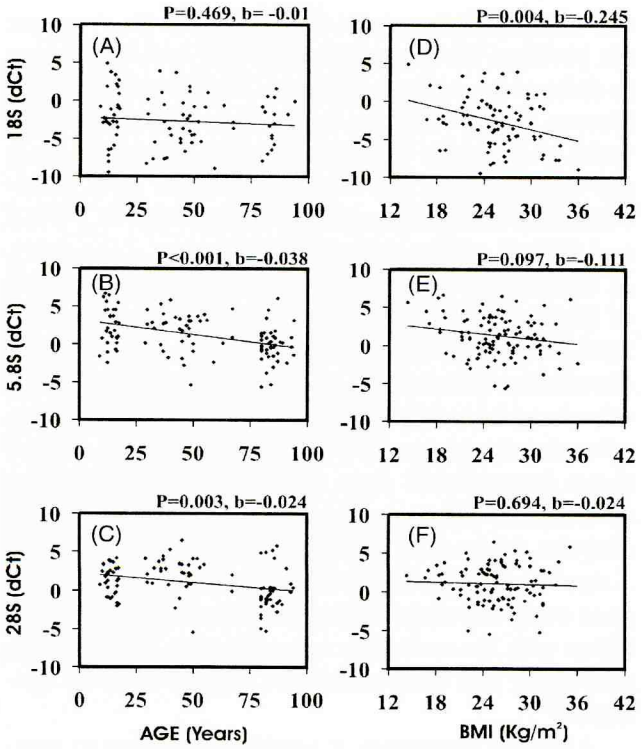

Fig. 3. Pearson correlation of the rDNA with age and BMI: (A) 18S vs. age, (B) 5.8S vs. age, (C) 28S vs. age, (D) 18S vs. BMI, (E) 5.8S vs. BMI, (F) 28S vs. BMI. *P*: probability, *b*: slope.

3.1. rDNA locus and ageing

Bernard Strehler in 1972 made the initial observation that rDNA is selectively lost in the non-dividing cells of human brain, heart and skeletal muscle (Burkle, 2002). Although the sample groups were rather small he calculated a rate of rDNA loss which was 0.9% per year for brain and 0.5% per year in myocardium. The methodology employed by Strehler was based on liquid hybridization and the reported rDNA loss refers to the sum of 18S and 28S rDNA gene copy. In the present study we quantified the 18S, 5.8S and 28S genes individually in 120 human adipose tissue samples and demonstrated that the rDNA loss described in Strehler's initial general hypothesis can be attributed to 28S reduction, since the 18S was not found to change in an age-dependent manner. Furthermore, we demonstrate for the first time the age-dependent loss of 5.8S rDNA gene. Subsequent experiments by others in mitotic tissues demonstrated an age-dependent loss or rDNA structures in polyclonally stimulated peripheral lymphocytes (Das, Rani, Mitra, & Luthra, 1986) but no rDNA loss in human fibroblast cultures (Machwe, Orren, & Bohr, 2000). The conflicting results on rDNA loss in mitotic cells could be explained by the counteraction of the ongoing natural selection process which is observed in cell culture models. In animal models, losses in the 18S and 28S rDNA genes were detected in dog brain with a loss rate higher than human brain (Johnson & Strehler, 1972), and in mice liver, brain and spleen (Gaubatz & Cutler, 1978).

The mechanism of rDNA loss has been studied extensively in an ageing model based on the dividing mother cell of *Saccharomyces cerevisiae* (Guarente & Kenyon, 2000). rDNA loss in yeast even though has been implied by several studies, it has not been directly demonstrated as yet. The yeast mother cell undergoes a series of events which lead finally to morphologic changes and sterility (Smeal, Claus, Kennedy, Cole, & Guarente, 1996). Other age related events included redistribution of the transcriptional silencing complex from heterochromatic regions to the rDNA locus and formation of extra-chromosomal ribosomal DNA circles (ERCs) (Guarente & Kenyon, 2000). In fact the formation of ERCs through homologous recombination has been proposed to be the mechanism of rDNA loss from the chromosomes. Nevertheless the formation of ERCs is not obligatory for yeast cell ageing (Ashrafi, Sinclair, Gordon, & Guarente, 1999). Instead, rDNA silencing through the SIR protein complex seems to be of greater importance for the recombination in the rDNA locus and ageing (Kaeberlein, McVey, & Guarente, 1999). The recombination event is believed to be due to the presence of multiple repeating units in close proximity. The differential dependence to ageing of 18S versus 5.8S and 28S rDNA copy number, reported in the present study, raises the following possibilities: (a) the recombination event is unequal leading to selective loss of specific rDNA genes and (b) the recombination point is located between 18S and 5.8S genes. Corroborating with our hypothesis, a recent study on human fibroblasts demonstrated the age-dependent accumulation of an rDNA RFLP fragment which can be explained by a genomic alteration event located between the 18S and 5.8S rDNA genes (Machwe, Orren, & Bohr, 2000). Finally, our observation that the rDNA genes are differentially related to ageing and BMI, raises the possibility that the initiation and the

targeting of the recombination event in the rDNA locus have distinct controlling mechanisms.

3.2. rDNA and BMI

A calorie restricted diet by 40% compared to ad libitum feeding has been shown to extend life span in rodents worms and yeast (Finkel & Holbrook, 2000; Merry, 2002), however, the mechanism is still unclear. Several theories have been developed among which the most prominent implicated the slowing of metabolism, the reduction of the oxidative damage from metabolic by-products and thus slowing of the ageing process (Carrard, Bulteau, Petropoulos, & Friguet, 2002; Harman, 1981). Nevertheless the theory was challenged by the discovery of caloric restriction regimens that increased life span without decreasing the metabolic rate. The theory was further challenged by the discovery of mutations that extend the life-span without affecting the rate of metabolism. Recent discoveries in the yeast demonstrated a direct relation of caloric restriction with the recombination events at the rDNA locus. Specifically caloric restriction was shown to shift metabolism towards NAD production which itself activates SIR2 protein. SIR2 normally is responsible for gene silencing. Sir2 protein already at the rDNA locus becomes more active in response to increased NAD availability and directly reduces recombination events thus affecting the ageing process (Lin, Defossez, & Guarente, 2000). Another very recent finding connected caloric restriction with Sir2 activation through the action of PCN1 gene which modulates intracellular nicotinamide (Anderson, Bitterman, Wood, Medvedik, & Sinclair, 2003). None of the above pathways has been demonstrated in higher organisms or humans. Cretan populations had the highest life expectancy as compared to the 16 other populations of the "seven country study" (Kafatos et al., 1997). This finding was associated with the life style and diet of the cretan population. The fasting schedule of the Greek orthodox church which was strictly adapted by the cretan population included reduced calorie intake for 180–200 days per year.

Obesity in humans is characterized by increased calorie intake and excessive fat accumulation. BMI is the most widely used descriptor of obesity worldwide (Cole, Bellizzi, Flegal, & Dietz, 2000; WHO, 2004). The efficiency with which BMI describes obesity varies in different ethnic groups (WHO, 2004). To overcome the problem the research community has been trying to define new markers of obesity like the waist circumference (WC), waist to height ratio (WHR) and the waist to hip ratio (WHpR). Studies conducted from our laboratory in the Greek population have established that BMI is fully compatible with the WC, WHR and WHpR markers (Bertsias, Mammas, Linardakis, & Kafatos, 2003). Furthermore, our previous studies of BMI on human population have demonstrated that BMI increased with age (Kafatos, Kouroumalis, Vlachonikolis, Theodorou, & Labadarios, 1991). On the other hand it has been demonstrated that BMI is directly related to the composition of the fat content in adipose tissue (unpublished data) and can increase either due to increased calorie intake or due to lack of sufficient exercise. In the present population study we identified a significant negative relation of BMI with 18S rDNA gene copy, therefore linking in humans the fat accumulation with the molecular recombination events which previously had been associated exclusively with ageing.

Although premature, it would be tempting to speculate that lipid content of adipose tissue and ageing possess mutually exclusive regulatory capacity on the recombination events of the rDNA locus. Further mechanistic studies are necessary to investigate the points raised by the present study on the targeting and initiation of the rDNA recombination events in relation to ageing and caloric restriction.

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