Monochrome multiplex qPCR of 18S and 5.8S human rDNA

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Abstract

The ribosomal DNA (rDNA) encodes the structural RNAs of the ribosomes. Ribosomal DNA instability is a major contributor to aging in yeast, but the role of rDNA instability in human aging and longevity is largely unknown. Human 45S rDNA, which encodes the 18S, 5.8S, and 28S RNAs, occurs as tandem repeats on the short arms of the five acrocentric chromosomes (p13, p14, p15, p21, and p22). The 45S rDNA copy number has been reported to range from 60 to > 800 copies per cell, and to be prone to frequent homologous and non-homologous recombination. Here we present two monochrome multiplex quantitative PCR (MMqPCR) assays, one for 18S rDNA normalized to the single copy gene beta-globin (HBB), and the other for 5.8S rDNA normalized to the single copy gene albumin (ALB), with both measurements expressed relative to those obtained from a reference DNA sample, using the relative qPCR method. Longitudinally collected pairs of DNA samples from bloods drawn approximately 16 years apart from 40 females and 39 males, aged < 1 to 77 years at first blood draw, were assayed. Our results are consistent with essentially all subjects having the expected 1:1 ratio of 18S to 5.8S copies at each blood draw; however, we observed up to +/- 40% gains and losses in rDNA copy number between the first and second draws, while repeated assays of single DNA samples varied approximately +/- 10%. There was no apparent trend toward either increasing or decreasing rDNA copy number with age; and no apparent difference between the sexes in the magnitude of rDNA instability. To our knowledge this longitudinal study is the first to show that rDNA copy number can change across the lifetime of an individual. These assays will facilitate investigations of the biology of the ribosomal RNA genes and the roles they play in the molecular pathophysiology of diseases and aging.

Keywords: quantitative PCR, ribosomal DNA, genomic instability

Introduction

The role of rDNA instability in human aging and longevity is largely unknown. The copy number of rDNA (the genes encoding ribosomal RNA) is unstable in eukaryotes [1]. Stabilizing the rDNA copy number in yeast extends lifespan [2]. In humans the rDNA copy number can vary more than 10-fold between individuals [3], and has been reported to change significantly between parents and offspring [4], to be altered in various disease conditions (e.g. cancer [5] and neurodegeneration[6]), and to be lower in the adipose tissue of older individuals vs. young individuals [7]. Here we present two novel monochrome multiplex qPCR assays to make copy number assessments of the 18S and 5.8S rDNA amenable to low cost, high-throughput screening, and we report the first longitudinal study of human rDNA copy number stability, in pairs of DNA samples extracted from blood draws collected 12.5 to 20.3 years apart from the same individuals.

Human subjects and methods

Human DNA samples

DNA was extracted by standard phenol-chloroform methods from peripheral blood draws collected in the early 1980's from the three-generation Utah CEPH (Centre d'Etude du Polymorphisme Humain) families, which are of northern European ancestry [8]. Forty females and 39 males from this original cohort (25 from the youngest generation, 22 from the middle generation, and 32 from the grandparent generation) also had second bloods drawn and DNAs extracted in the mid- to late 1990's, for the Utah Genetic Reference Project (UGRP) using the Gentra Puregene Blood Kit [9]. All extracted DNAs were dissolved in 10 mM Tris-Cl, 0.1 mM EDTA, pH 7.5 at 25°C, at a concentration of 100-200 ng/μl, confirmed by agarose gel electrophoresis to consist of high molecular weight DNA with negligible degradation, and stored long term at 4°C. The 79 subjects were selected so that within each generation, all individuals of the same sex were unrelated (from different families). The University of Utah's Institutional Review Board has approved this research project.

Quantitative PCR

We used the monochrome multiplex polymerase chain reaction [10] with SYBR Green I as the detecting dye, to measure the rDNA copy number to single copy gene copy number ratio, relative to that in a reference DNA sample. The reaction mix composition used here is the same as in ref. 10, except for the primers used and the inclusion here of a restriction enzyme. The 18S rDNA to beta-globin gene (HBB) relative ratios were measured in one set of reactions, and the 5.8S rDNA to albumin gene (ALB) relative ratios were measured in another set of reactions. TaqI restriction endonuclease (New England Biolabs) was included in the master mix to a final concentration of 4 units of TaqI per 10 microliter final PCR reaction volume. We found that restriction enzyme digestion of the genomic DNA prior to PCR is essential in order to obtain reproducible, reliable results in rDNA quantification. All primer sequences are written 5' -> 3'.

MMQPCR 1: 18S rDNA primers: TGTAGTCGCCGTGCCT (final concentration, 1μM) and CGTCACCCGTGGTCACCAT (f.c. 1.5μM); HBB primers (with GC-clamps): CGGCGGCGGGCGGCGCGGGCTGGGCGGCTTCATCCACGTTCACCTTG (f.c. 500nM) and GCCCGGCCCGCCGCGCCCGTCCCGCCGGAGGAGAAGTCTGCCGTT (f.c. 500nM)

MMQPCR 2: 5.8S rDNA primers: CCTCGTACGACTCTTAGCGGT (f.c. 1μM) and GCACGAGCCGAGTGATCC (f.c. 1.5μM); ALB primers (with GC-clamps): CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG (f.c. 400nM) and GCCCGGCCCGCCGCGCCCGTCCCGCCG GCATGGTCGCCTGTTCAC (f.c. 400nM)

Thermal profile for the 18S rDNA + HBB reaction: 65°C x 60 min (TaqI digestion); 95°C x 15 min (DNA polymerase activation and DNA denaturation); and 40 cycles of 96°C x 15 sec, 58°C x 30 sec, 72°C x 15 sec with signal acquisition, 86°C x 15 sec with signal acquisition. Thermal profile for the 5.8S rDNA + ALB reaction: same as above, except that the second signal acquisition is at 85°C instead of 86°C. All QPCR reactions were performed on a Bio-Rad CFX384 Real-Time PCR Detection System. Relative quantification was analyzed by the Standard Curve method using Bio-Rad's accompanying software.

Results

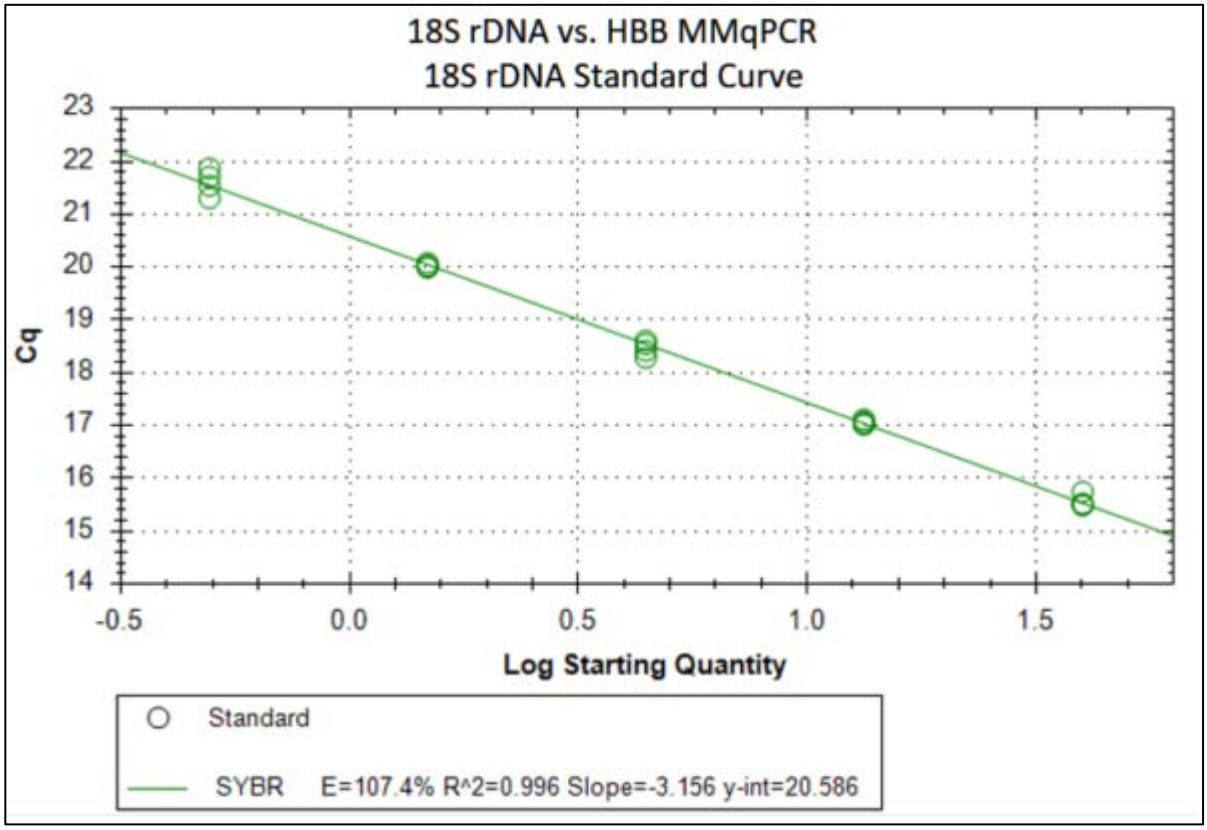

Figure 1.

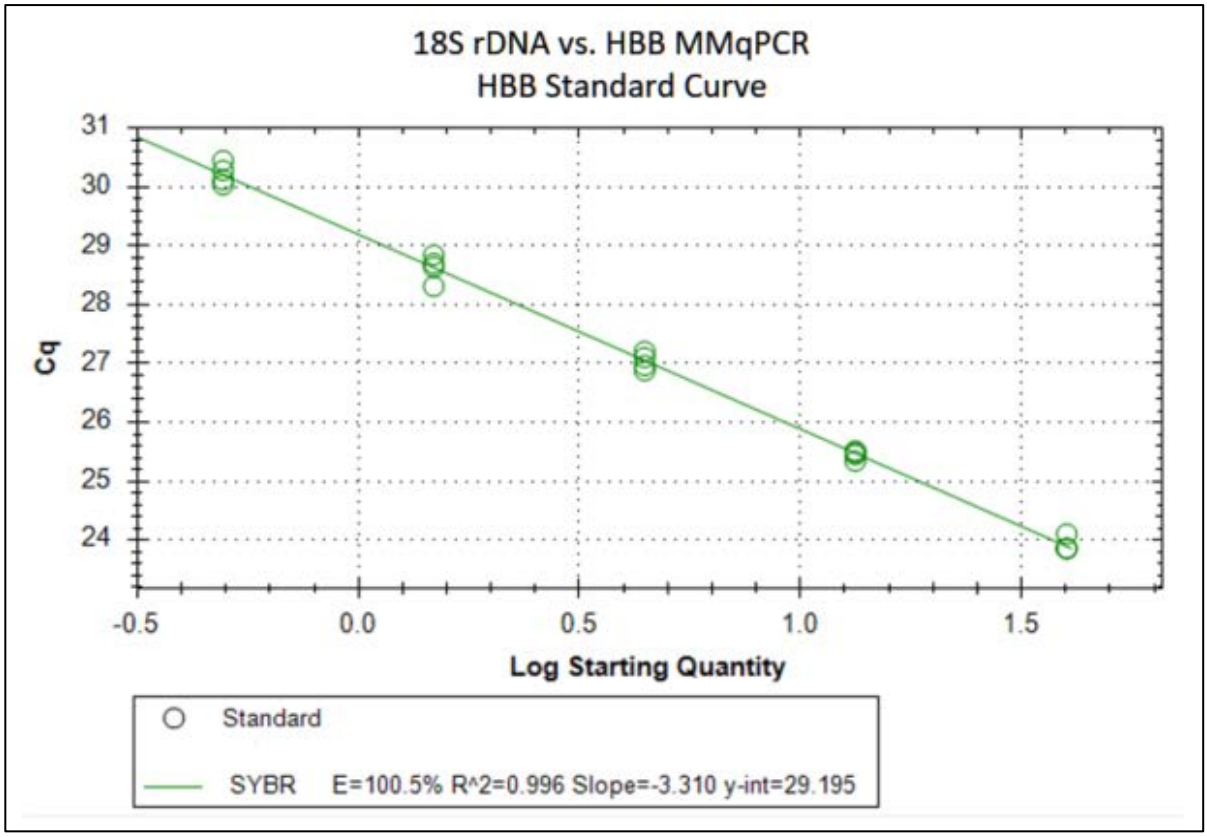

Figure 2.

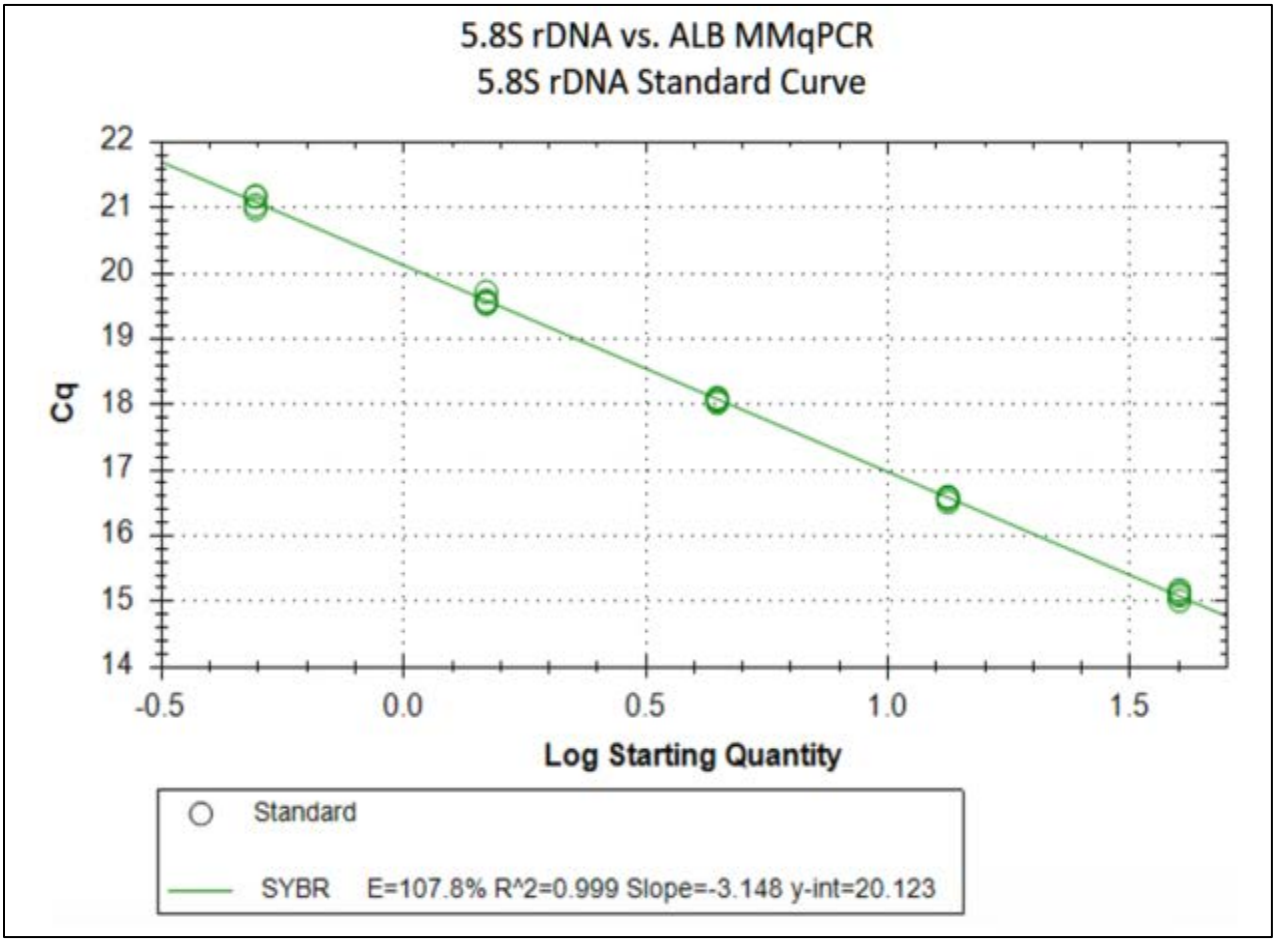

Figure 3.

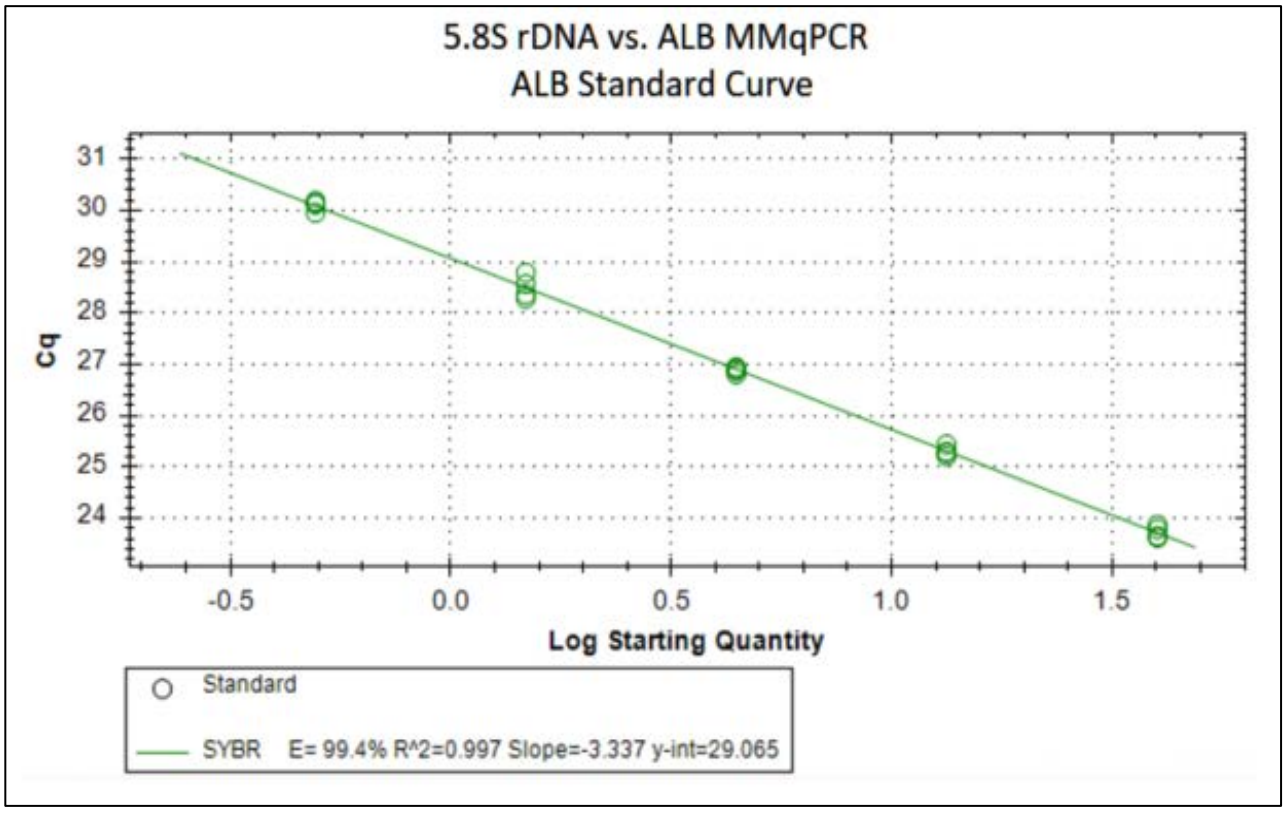

Figure 4.

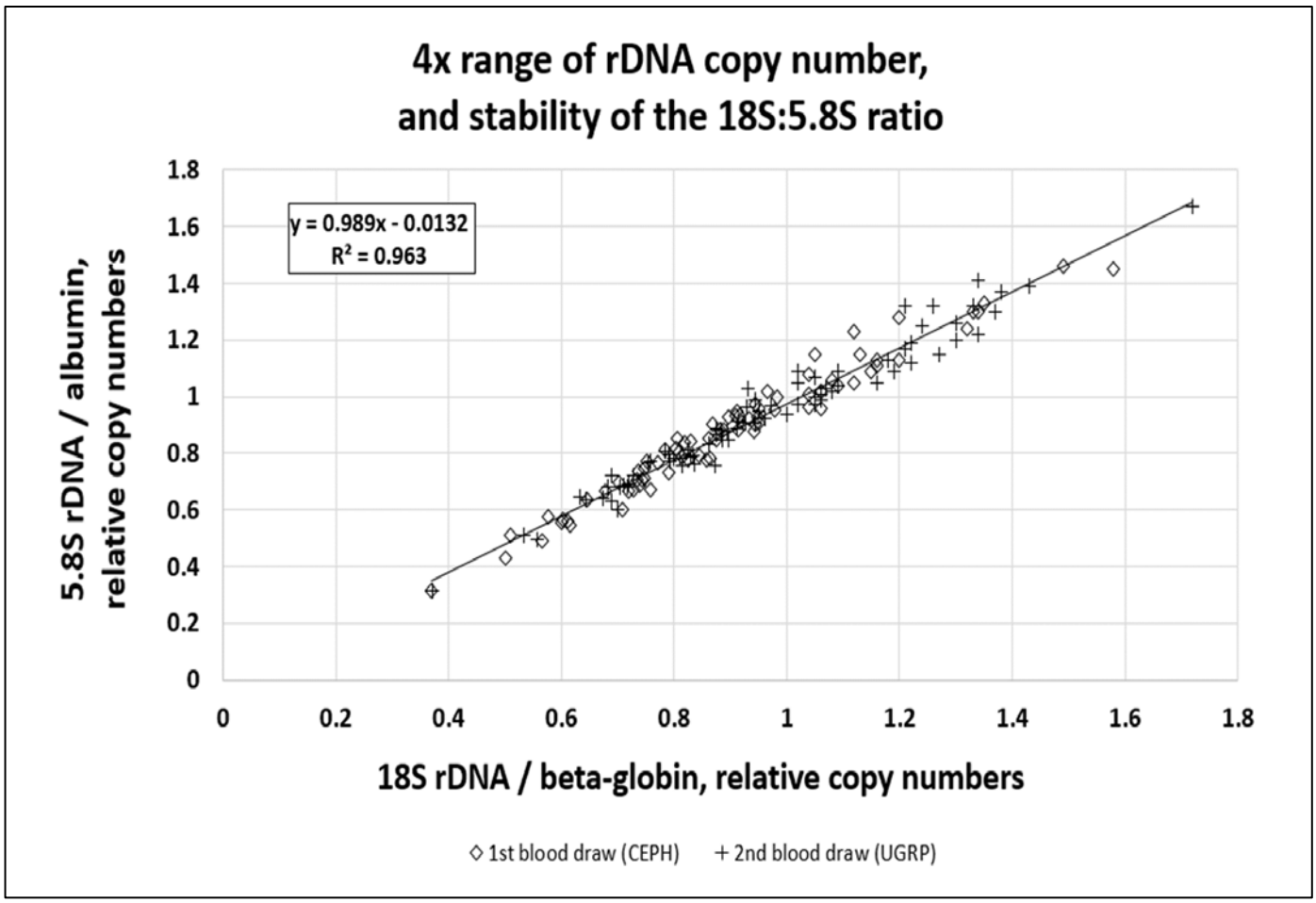

Figure 5. Quantitative PCR determination of the average rDNA copy number per cell in 79 individuals, relative to that in a reference DNA sample. (The single copy genes are a proxy for cell count.) The 18S-5.8S-28S rDNA unit is tandemly repeated along the acrocentric p-arms of chromosomes 13, 14, 15, 21, and 22. Our results are consistent with there being the expected 1:1 ratio of 18S to 5.8S rDNA sequences in all DNA samples. In absolute numbers, we estimate that the average rDNA copy number per cell ranges from ~170 – 740 in these subjects.

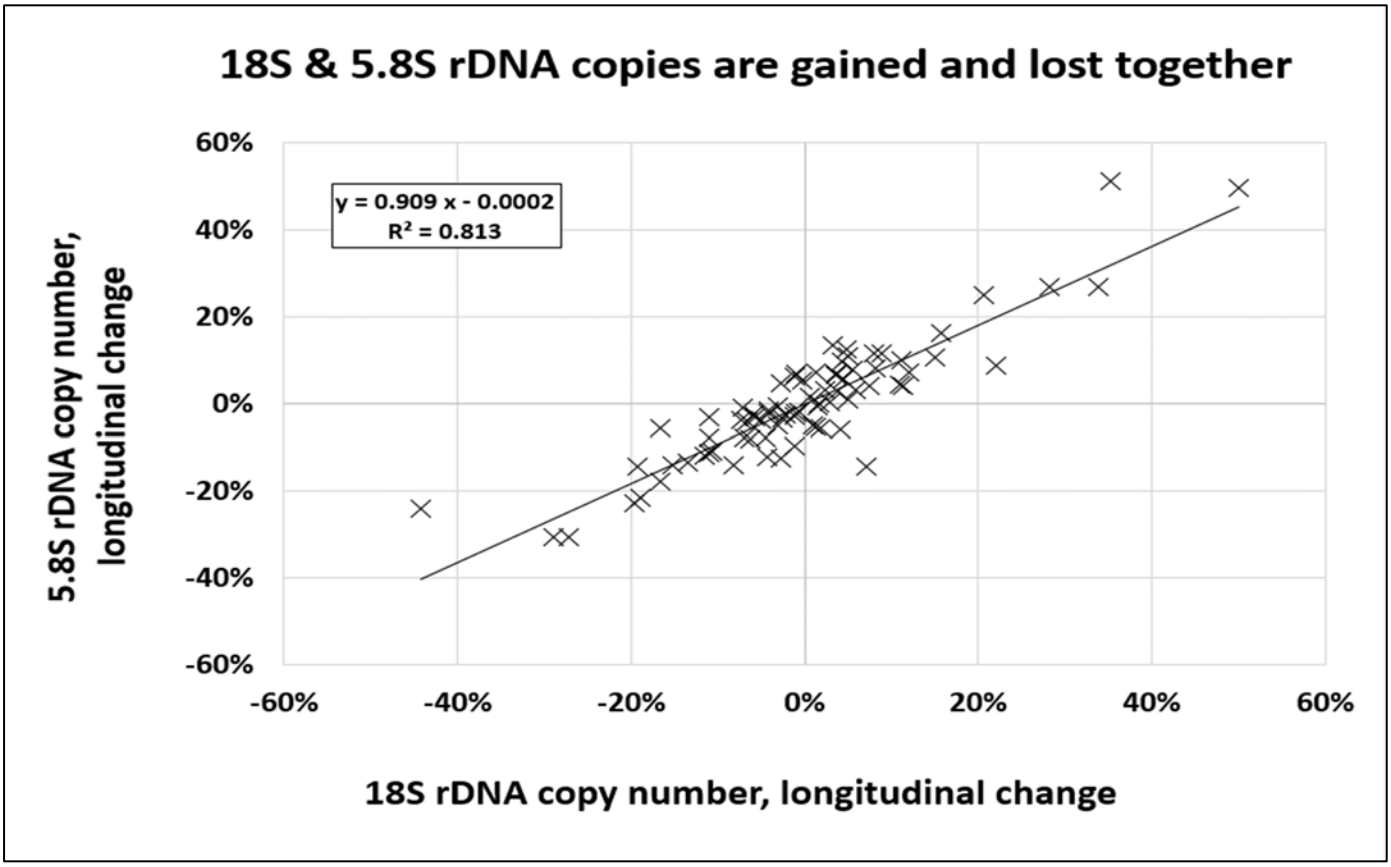

Figure 6. Instability of the rDNA copy number within the 79 individuals. Pairs of blood draws were taken years apart (range: 12.5-20.3 years, average: 16.1 years) from children and young to elderly adults (age at first draw: < 1 year-77.2 years). Gains and losses of rDNA copy number up to approximately +/- 40% between the two blood draws were observed. The strong correlation between the two independent measures (18S/beta-globin gene and 5.8S/albumin gene) of change in rDNA copy number provides confidence that the changes are not artifactual. In contrast, replicate measurements on any single DNA sample by either assay display only approximately +/- 10% variation.

Discussion

To our knowledge this study is the first to demonstrate rDNA copy number changes longitudinally across the lifetime in humans. Measures of rDNA instability may reflect the level of genomic instability generally. Cells with high rDNA instability (or high genomic instability generally) may be prone to undergoing cellular senescence or apoptosis, processes believed to contribute to the dysfunction, atrophy, and degeneration of various tissues with age. Alternatively, depending on the cell type and other conditions present, the rDNA (or general genomic) instability may contribute to the development of various cancers. These two novel MMqPCR assays of the 18S and 5.8S rDNAs will facilitate further studies to explore these possibilities. Recent studies in mice and humans have shown that the copy number of the 5S rDNA tandem repeats on Chromosome 1 approximately co-vary with the copy number of the 45S rDNA tandem repeats on Chromosomes 13p, 14p, 15p, 21p, and 22p [11]. Ribosomal DNA copy numbers in humans also appear to be coordinately regulated with the copy numbers of the mitochondrial DNA (mtDNA) [12]. Therefore, there may be healthy, as well as pathologic, ranges for the absolute and relative copy numbers of 5S rDNA, 45S rDNA, and mtDNA. Further research will be needed to determine whether these quantitative traits will be clinically useful biomarkers of aging, accounting for a signficant fraction of the variation in age at death in late middle-aged and older adults, and whether medical interventions stabilizing them to healthy ranges will be effective in extending the human healthspan.

Disclosure statement

The authors have no competing interests to declare.

Data availability. Figures 1-6 have associated raw data collected during QPCR runs on Bio-Rad CFX384 Real-time Detection System, under the control of the Bio-Rad CFX Manager Software, and stored in a computer file. Copies of this file are available, without restriction, from Dr. Cawthon, upon request at rcawthon@genetics.utah.edu.

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References

1. Ganley AR, Kobayashi T. Ribosomal DNA and cellular senescence: new evidence supporting the connection between rDNA and aging. FEMS Yeast Res. 2014 Feb;14(1):49-59. doi: 10.1111/1567-1364.12133. Epub 2014 Jan 21. Review. PubMed PMID: 24373458.

2. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 2003 Sep 11;425(6954):191-6. Epub 2003 Aug 24. PubMed PMID: 12939617.

3. https://www.ncbi.nlm.nih.gov/gene/100861532

4. Stults DM, Killen MW, Pierce HH, Pierce AJ. Genomic architecture and inheritance of human ribosomal RNA gene clusters. Genome Res. 2008 Jan;18(1):13-8. Epub 2007 Nov 19. PubMed PMID: 18025267; PubMed Central PMCID: PMC2134781.

5. Lindström MS, Jurada D, Bursac S, Orsolic I, Bartek J, Volarevic S. Nucleolus as an emerging hub in maintenance of genome stability and cancer pathogenesis. Oncogene. 2018 May;37(18):2351-2366. doi: 10.1038/s41388-017-0121-z. Epub 2018 Feb 12. Review. PubMed PMID: 29429989; PubMed Central PMCID: PMC5931986.

6. Parlato R, Kreiner G. Nucleolar activity in neurodegenerative diseases: a missing piece of the puzzle? J Mol Med (Berl). 2013 May;91(5):541-7. doi: 10.1007/s00109-012-0981-1. Epub 2012 Nov 20. Review. PubMed PMID: 23179684; PubMed Central PMCID: PMC3644402.

7. Zafiropoulos A, Tsentelierou E, Linardakis M, Kafatos A, Spandidos DA. Preferential loss of 5S and 28S rDNA genes in human adipose tissue during ageing. Int J Biochem Cell Biol. 2005 Feb;37(2):409-15. PubMed PMID: 15474985.

8. Prescott SM, Lalouel JM, Leppert M. 2008. From linkage maps to quantitative trait loci: the history and science of the Utah genetic reference project. Annu Rev Genomics Hum Genet. https://doi.org/10.1146/annurev.genom.9.081307.164441.

9. https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/gentra-puregene-blood-kit/#orderinginformation

10. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res. 2009 Feb;37(3):e21. doi: 10.1093/nar/gkn1027. Epub 2009 Jan 7. PubMed PMID: 19129229; PubMed Central PMCID: PMC2647324.

11. Gibbons JG, Branco AT, Godinho SA, Yu S, Lemos B. Concerted copy number variation balances ribosomal DNA dosage in human and mouse genomes. Proc Natl Acad Sci U S A. 2015 Feb 24;112(8):2485-90. doi: 10.1073/pnas.1416878112. Epub 2015 Jan 12. PubMed PMID: 25583482; PubMed Central PMCID: PMC4345604.

12. Gibbons JG, Branco AT, Yu S, Lemos B. Ribosomal DNA copy number is coupled with gene expression variation and mitochondrial abundance in humans. Nat Commun. 2014 Sep 11;5:4850. doi: 10.1038/ncomms5850. PubMed PMID: 25209200.