












Original Research

Copy Number Variations of Human Ribosomal Genes in Health and Disease: Role and Causes

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Abstract

Background: A number of association studies have linked ribosomal DNA gene copy number (rDNA CN) to aging and pathology. Data from these studies are contradictory and depend on the quantitative method. **Methods:** The hybridization technique was used for rDNA quantification in human cells. We determined the rDNA CN from healthy controls (HCs) and patients with schizophrenia (SZ) or cystic fibrosis (CF) (total number of subjects N = 1124). For the first time, rDNA CN was quantified in 105 long livers (90–101 years old). In addition, we conducted a joint analysis of the data obtained in this work and previously published by our group (total, N = 3264). **Results:** We found increased rDNA CN in the SZ group (534 ± 108 , N = 1489) and CF group (567 ± 100 , N = 322) and reduced rDNA CN in patients with mild cognitive impairment (330 ± 60 , N = 93) compared with the HC group (422 ± 104 , N = 1360). For the SZ, CF, and HC groups, there was a decreased range of rDNA CN variation in older age subgroups compared to child subgroups. For 311 patients with SZ or CF, rDNA CN was determined two or three times, with an interval of months to several years. Only 1.2% of patients demonstrated a decrease in rDNA CN over time. We did not find significant rDNA CN variation in eight different organs of the same patient or in cells of the same fibroblast population. **Conclusions:** The results suggest that rDNA CN is a relatively stable quantitative genetic trait statistically associated with some diseases, which however, can change in rare cases under conditions of chronic oxidative stress. We believe that age- and disease-related differences between the groups in mean rDNA CN and its variance are caused by the biased elimination of carriers of marginal (predominantly low) rDNA CN values.

Keywords: rDNA; ribosomal gene; copy number variation; cystic fibrosis; schizophrenia; dosage effect

1. Introduction

Copy number variants (CNVs) are structural genomic repetitive elements (repeats) ranging in size from a thousand to several million base pairs per copy [1,2]. Repetitive elements comprise more than 70% of the human genome. Some of the repeats are clustered into tandem arrays. Tandem repeats are organized in a head-to-tail orientation and are characterized by a pronounced quantitative polymorphism. The tandem repeats play a principle role in the functioning of the nuclear chromatin through its regulation and spatial organization. CNVs of the tandem repeats have been implicated in many diseases [3–9].

Ribosomal repeats (ribosomal genes, ribosomal DNA [rDNA]) are a special type of tandem repeat. Ribosomal genes encode the ribosomal RNA (rRNA) species that form the backbone of the ribosome. The human genome includes hundreds of rDNA copies (Fig. 1A), which are arranged in tandem arrays on five acrocentric chromosome pairs 13, 14, 15, 21, and 22. Each rDNA unit codes for a 47S pre-rRNA

that is processed into the 18S rRNA (for the small ribosomal subunit) and the 5.8S and 28S rRNAs (for the large ribosomal subunit). During interphase, the ribosomal repeats form the structure of the nucleolus, where rDNA transcription and the initial stages of ribosome biogenesis occur. The biogenesis of ribosomes determines the ability of a cell to successfully proliferate, perform its functions, and respond to genotoxic stress. Dysregulation of rRNA biogenesis has been implicated in a series of human pathologies [10]. One of the factors that cause rRNA biogenesis is the copy number (CN) of the ribosomal genes [11,12].

The nucleolus consists of three rDNA fractions, which are distinguished by the transcription activity, methylation level, and the presence of nucleosomal organization. The fractions of active and poised (potentially active) repeats are euchromatic, hypomethylated at CpG sites, and marked with histone modifications typical for transcriptionally active genes. The rDNA CN of the active rDNA copies is estimated to be 30% to 50% of the total rDNA copies in the cell



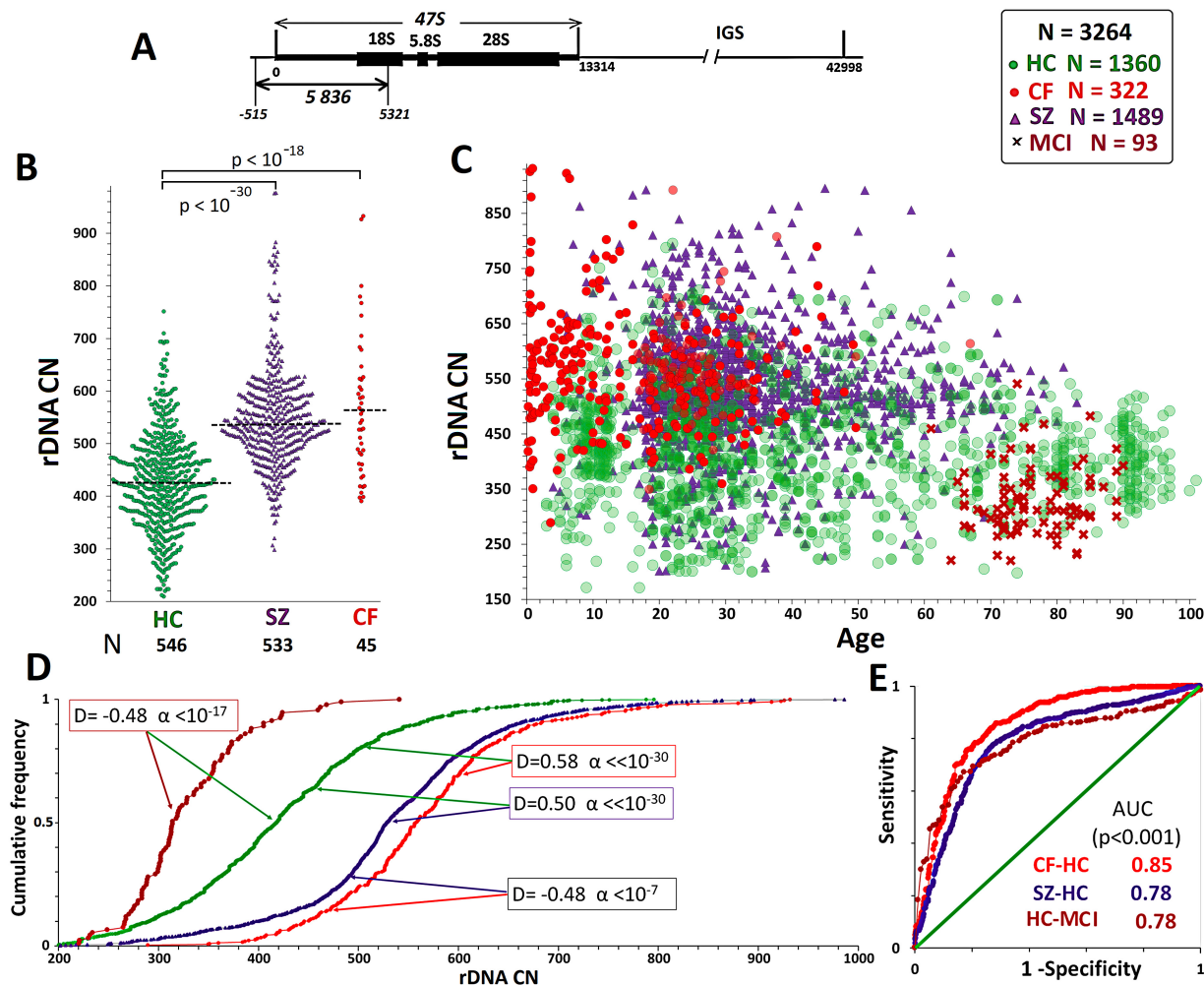


Fig. 1. Quantification of rDNA copy number in a general sample that includes all the groups studied. (A) Organization of ribosomal repeats. One copy of the repeat includes three rRNA genes and three transcribed spacers (transcribed region of rDNA) and a non-transcribed intergenic spacer. Probe p(ETS-18S) (fragment of rDNA from -515 to 5321 relative to the transcription initiation point, HSU 13369, GeneBank) was used for human rDNA detection. (B) rDNA CN in the HC, SZ, and CF groups, which were analyzed in this study. (C) Distribution of rDNA CN by age in the analyzed groups. The study findings (1124 DNA samples) are combined with the data previously reported by our group. (D) Comparison of rDNA CN distributions in various groups. The Kolmogorov–Smirnov (D and α) test statistics are presented in the boxes. (E) ROC curves for the groups. The AUC is a measure of how well a parameter can distinguish between two compared groups. rDNA CN, ribosomal DNA gene copy number; HC, healthy control; SZ, schizophrenia; CF, cystic fibrosis; ROC, receiver operating characteristic; AUC, area under the ROC curve; MCI, mild cognitive impairment.

[13–15]. A part of inactive rDNA copies in the nucleolus is assumed to be methylated at CpG sites within the rDNA promoter region and able to reactivate under certain conditions. About 10% of human blood cell DNA samples contain inactive hypermethylated rDNA copies. These copies are unable to reactivate and are located in the peripheral regions of the nucleolus. They constitute heterochromatin that encompasses the nucleolus. There is a positive correlation between the total rDNA CN in the genome and the number of active or methylated copies [15–27].

The rDNA function is not limited to ribosome biogenesis. The rRNA gene arrays constitute a center from which the stability of the whole genome is regulated and the

cell's lifespan is controlled [28–30]. The rDNA contributes to global chromatin regulation. The rDNA arrays have thousands of contacts in the folded genome, with rDNA-associated regions and genes dispersed across all chromosomes [31–36].

The available literature suggests that the variability of rDNA is an important aspect of normal cell physiology and phenotypic variation [16,17,37–39]. Neurodegenerative disorders, psychiatric diseases, and cancer are associated with changes in rDNA CN. Mild cognitive impairment (MCI) and Alzheimer's disease are accompanied by an increase in rDNA CN in the brain [40,41]. On the other hand, a decrease in rDNA CN in the blood leukocytes of patients

with MCI has been observed [42]. Studies that had used experimental methods found an increase in rDNA CN in the blood leukocytes and brain of patients with schizophrenia (SZ) [43–46]. A very recent analysis of the blood transcripts of SZ patients vs. healthy controls showed that the genes most influential in clustering to distinguish between SZ and controls were enriched in pathways related to the ribosome biogenesis system [47], thus indirectly corroborating the upregulated ribosome biogenesis in SZ, which may be underpinned by the elevated rDNA CN in the patients. However, a study using a bioinformatics approach did not identify differences in rDNA CN in the genomes of healthy individuals and patients with SZ [18]. A large number of rDNA copies are contained in the blood leukocytes of patients at high risk of lung cancer [48]. It has been shown that rDNA CN is unstable in human cancers [49,50]. Recently, rDNA was shown to be positively correlated with neutrophil levels in the blood, a marker often linked to renal dysfunction, and negatively correlated with body weight [16,17].

A number of association studies have linked rDNA CN with aging. Johnson and Strehler [51] were the first to discover aging-related loss of rDNA in neurons. Age-dependent loss of rDNA was found in beagle dog brain tissues; mouse brain, spleen, and kidney tissues; human myocardium and cerebral cortex; and human adipose tissue [52–55]. Cytogenetic studies [56,57] showed a decrease in the number of silver-stained nucleolus organizer regions per cell, that is, a decrease in the number of transcriptionally active rDNA clusters, with aging, which may be explained by either a deletion of rDNA repeats, or a full repression of the transcription from an rDNA array or several arrays. By contrast, a several-fold increase in rDNA CN has been found in the liver [58] and bone marrow cells [59] of old mice compared to young ones. However, some studies have not shown a correlation of changes in genomic rDNA content with aging. Rather, several reports have documented that rDNA CN is largely stable during normal aging [41,60–62].

The conflicting data among studies regarding rDNA CNVs in health, disease, and aging seem to result from the complicity of rDNA quantification [33,43–45,63–67]. Quantitative rDNA analysis using genome sequencing results is critically dependent on the technical details of library preparation. For the same cell lines, data on rDNA content obtained from the analysis of several libraries in different centers differ [33]. rDNA is a very poor template for Taq polymerase. This is due to an anomalously high GC pair content, unequal methylation of different copies, tandem nature of the repeats, increased rDNA damage, and the presence of proteins tightly bound to rDNA, in DNA samples [43,63]. To adjust for the contribution of the aforementioned factors to rDNA quantification, we developed a variation of the hybridization technique using long non-radioactively labeled DNA probes (non-radioactive quantitative hybridization [NQH] technique). This method does

not depend on the level of oxidation and fragmentation of the DNA template [43]. With this approach, we revealed pronounced variation in the distribution of healthy subject DNA samples in different age groups (16–91 years old) by rDNA CN values [64]. We also found increased rDNA CN in patients with SZ [43–45,65] and cystic fibrosis (CF) [66,67].

SZ is a multifactorial disease with a heritable component of 70–80% [68]. CF is a monogenic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7 [69,70]. An association between the pathology and high levels of oxidative stress and DNA damage in primary and cultured cells of the patients has been reported for both SZ [45,71–74] and CF [75–77]. The elevated rDNA CN in the genomes of these completely different patient groups is intriguing and requires additional experimental corroboration and a greater understanding. There are no data in the literature on changes in rDNA CN in the same individuals over the course of their life. In addition, data on the possible heterogeneity of cells of different organs of one organism or cells of one cell population according to the rDNA CN parameter for humans are extremely scarce. In the present study, the following four groups were assessed. (1) We additionally quantified rDNA in 1124 DNA samples. Batches of DNA samples from different groups (healthy control (HC), SZ, and CF) were isolated from blood and analyzed within the same experiment to directly confirm the difference in rDNA CN in the genomes of controls and patients. (2) For the first time, rDNA CN was identified in the DNA isolated from the leukocytes of 105 long livers (90–101 years old). (3) For the first time, we monitored the changes in rDNA CN in leukocytes over several years in diseases that are accompanied by chronic oxidative stress (SZ and CF). (4) The variation in rDNA CN in eight different organs of the same patient and in the cells of the same fibroblast population was studied for the first time.

Finally, we conducted total comparative analysis of rDNA content in the genomes of the four groups for the entire sample of cases and HCs, whose DNA samples were assayed both previously and in the present study (N = 3264).

2. Materials and Methods

2.1 Patient Groups

2.1.1 Patients with CF (CF Group)

The CF group consisted of 322 patients with CF aged 0.21 to 66.7 years, who had been registered in the ‘Register of Cystic Fibrosis Patients in the Russian Federation’ database (https://mukoviscidoz.org/doc/registr/_Registre_2022.pdf). CF was diagnosed according to European Cystic Fibrosis Society best practice guidelines [78]. The age subgroups did not significantly differ by sweat test results or sex. We found that the proportion of patients with a ‘mild’ genotype increased with age, from 2.6% in the subgroup under 10 years old to 75% in the group of patients

older than 31. With increasing patient age, there was also an increase in the number of patients infected with *Pseudomonas aeruginosa* ($p < 0.001$) and an increase in the number of complications of the disease, while pulmonary function declined ($p = 0.009$).

2.1.2 Patients with SZ (SZ Group)

The tested cohort included 1489 inhabitants of Moscow city and the Moscow region (Table 1). The data for 956 patients were previously published [44]. Within the framework of the present study, the DNA samples of 533 patients were analyzed. The patients were hospitalized in N.A. Alexeev Clinical Psychiatric Hospital #1 because of SZ aggravation. Patients with SZ included those with long-term antipsychotic therapy for a chronic disease and first-episode drug-naïve cases. The patients were assessed using the structured Mini International Neuropsychiatric Interview. According to International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) criteria, paranoid SZ (F20.00 or F20.01) was diagnosed. The diagnoses were validated according to Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria. Quetiapine, risperidone, paliperidone, haloperidol, chlorpromazine, clozapine, olanzapine, and ziprasidone were the standard antipsychotics administered for the treatment of acute episodes.

The group of children with very early SZ onset was recruited using the clinical psychopathologic approach from the Child Psychiatry Department of the Federal State Budgetary Scientific Institution “Mental Health Research Center” (Moscow Russia). The group was described in detail in our previous publication [79]. DNA samples isolated from various tissues of a patient with SZ (male, 54 years old, chronic form of paranoid SZ) were obtained from a SZ DNA sample collection from the Molecular Biology Laboratory of the Research Centre for Medical Genetics (RCMG; Moscow, Russia).

2.2 Patients with MCI

The group of patients with MCI comprised 93 participants aged 61–91 years. All patients participated in the Rehabilitation Program “Memory Clinic” developed at N.A. Alekseev Psychiatric Hospital #1. The results of the program [80] and data obtained for this patient group [42] have been published.

2.3 HCs

DNA samples from HCs were obtained from the Molecular Biology Laboratory of the RCMG. This cohort included mentally healthy subjects aged 3–91 years ($N = 1260$), who were not affected by any genetic pathology, cognitive impairment, or mental illness. Blood samples of long-livers (91–101 years old, $N = 105$) were obtained from the inhabitants of the Moscow Nursing Home for War Veterans.

2.4 Quantification of rDNA CN

2.4.1 DNA Isolation

DNA isolation from blood cells and the NQH technique were performed as previously described [43]. Isolated leukocytes were used for most of the tests. A small amount of DNA samples from patients with SZ (~15%) were isolated from whole blood. Briefly, DNA was isolated after cell lysis via extraction with organic solvents (2% sodium lauryl sarcosylate (L9150, Sigma-Aldrich, St. Louis, MO, USA), 0.04 M EDTA (E8008, Sigma-Aldrich, St. Louis, MO, USA)) and subsequently treated with 150 $\mu\text{g/mL}$ RNAase A (45 min, 37 °C; Sigma-Aldrich, St. Louis, MO, USA) and 200 $\mu\text{g/mL}$ proteinase K (24 h, 37 °C; Promega Co., Madison, WI, USA). Proteinase K treatment is critically important for maximum effectiveness of rDNA isolation [65]. The lysate samples were extracted with an equal volume of phenol (77608 Merck, Kenilworth, NJ, USA)/chloroform (Chimmed, Moscow, Russia)/isoamyl alcohol (Chimmed, Moscow, Russia) (25:24:1), phenol and chloroform/isoamyl alcohol (24:1). DNA was precipitated after adding 1/10 volume of 3 M sodium acetate (GRM2966, NeoFroxx GmbH, Einhausen, Germany) (pH 5.2) and 2.5 volume of ice-cold ethanol. Only freshly distilled solvents were applied for extraction, and 8-hydroxyquinoline (252565, Sigma-Aldrich, St. Louis, MO, USA) was used to stabilize the phenol. Finally, the DNA was centrifuged at 10,000 g at 4 °C for 15 min, washed with 70% ethanol (v/v), dried and dissolved in water. The efficiency of NQH is mostly determined by the accuracy of DNA quantification. We conducted quantification by obtaining a crude estimation of the initial DNA amount by ultraviolet spectroscopy, and performing final DNA quantification fluorimetrically using the PicoGreen dsDNA Quantification Reagent (Invitrogen Molecular Probes, Carlsbad, CA, USA). DNA solutions with the same concentration (20 ng/ μL) were prepared for the NQH method.

2.4.2 NQH method

For human rRNA detection, the p(ETS-18S) probe was used, which was a 5.8 kb long rDNA fragment that encompassed a region from position –515 to position 5321 with reference to the transcription initiation point (HSU 13369; GenBank accession No. U13369). It was cloned into EcoRI site of pBR322 vector (<https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/pbr322>) and biotinylated using a nick translation kit (Biotin NT Labeling Kit; Jena Bioscience GmbH, Jena, Germany). The membrane (ExtraC) was wetted using a solution of 10× saline sodium citrate (SSC) and dried. Then 1 μL of 1 M sodium hydroxide was added to 10 μL of the DNA solution at 0 °C in TE buffer (2339, NeoFroxx GmbH, Einhausen, Germany). The solution was stirred and incubated at 0 °C for 10 min, and neutralized with 11 μL of 20× SSC (pH 5.0). Then the de-

Table 1. Demographic measures and descriptive statistics for rDNA CN in HCs and patients.

Index		HC	SZ	CF	MCI	
N		1360	1489	322	93	
Sex (men)		62%	59%	49%	29%	
Age	Mean \pm SD	40 \pm 26	32 \pm 13	18 \pm 12	76.6 \pm 6.5	
	Range	3–101	5–83	0.2–67	61–90	
	Median	32	29	19	76	
rDNA copy number	All	Mean \pm SD	422 \pm 104	534 \pm 108	567 \pm 100	330 \pm 60
		Range	171–796	202–986	289–932	220–541
		Median	418	528	556	314
		CV	0.25	0.20	0.18	0.18
	Group 0–16 y.o.	N	277	44	142	
		Mean \pm SD	438 \pm 92	565 \pm 166	587 \pm 118	
		Range	171–751	303–986	289–932	
		Median	434	519	585	
		CV	0.21	0.29	0.20	
	Group 17–40 y.o.	N	530	1130	169	
		Mean \pm SD	422 \pm 117	534 \pm 108	547 \pm 78	
		Range	171–796	202–977	350–892	
		Median	414	531	547	
		CV	0.28	0.20	0.14	
	Group 41–72 y.o.	N	340	304	12	28
		Mean \pm SD	423 \pm 112	527 \pm 98	604 \pm 93	319 \pm 54
		Range	205–699	229–896	461–790	221–460
		Median	421	522	609	310
		CV	0.27	0.19	0.15	0.17
	Group 73–89 y.o.	N	118	11		65
		Mean \pm SD	396 \pm 63	531 \pm 79		336 \pm 63
		Range	198–541	371–696		220–321
		Median	392	528		319
		CV	0.15	0.15		0.19
	Group 90–101 y.o.	N	105			
		Mean \pm SD	402 \pm 55			
		Range	289–519			
		Median	401			
		CV	0.14			

CV, coefficient of variation; CF, cystic fibrosis; HC, healthy control; MCI, mild cognitive impairment; rDNA, ribosomal DNA; SZ, schizophrenia; y.o., year old; CN, copy number.

natured DNA samples were dotted onto a membrane. To each dot, about 1.5–2 μ L (20 ng) sample was applied, and 3–6 parallel dots of each sample were made. As a reference, standard genomic DNA samples were applied to the same membrane. Then the membrane was heated for 2 h at 80 °C in a vacuum. A special instrument was used for hybridization, namely, a hybridization furnace (BINDER GmbH, Tuttlingen, Germany) with adjustable temperature and a swaying table (Biosan, Riga, Latvia) for stirring the solution above the membrane. The membrane was fixed in 10 \times Denhardt's solution (0.05 M phosphate buffer, pH 7.0; 0.5 M sodium chloride [NaCl]; 50% formamide (1103, NeoFroxx GmbH, Einhausen, Germany); 100 μ g/ μ L *Escherichia coli* tRNA (R1753, Sigma-Aldrich, USA)) at 42

°C for 5 h. Afterwards, the denatured DNA probe at a concentration of 50 ng/mL was added. The hybridization proceeded for 16 h at 45 °C. The membrane was washed with 2 \times SSC, 0.1% sodium dodecyl sulfate (SDS; 2 \times 15 min, 25 °C), 0.01 \times SSC, 0.1% SDS (20 min, 65 °C), and 2 \times SSC (10 min, 25 °C). Then the membrane was fixed for 30 min at 37 °C in a special solution (0.1% gelatin; 0.1% fat-free milk; 0.1 M NaCl; Tris-HCL buffer, pH 7.5), treated with alkaline phosphatase-conjugated streptavidin (1 μ g/ μ L; Sigma) in a solution (0.1 M Tris-HCL buffer, pH 7.5; 0.005 M magnesium chloride [MgCl]; 0.1 M NaCl) at 25 °C for 20 min, and washed (3 \times 10 min) with a solution (Tris-HCL buffer, pH 7.5; 0.1 M NaCl; 0.005 M MgCl). Then the membrane was incubated with a solution contain-

ing substrates for alkaline phosphatase (Tris-HCL buffer, pH 9.5; 0.005 M MgCl₂; 0.1 M NaCl; NBT (1267, NeoFroxx GmbH, Einhausen, Germany) [4.4 µg/µL]/BCIP (1265, NeoFroxx GmbH, Einhausen, Germany) [3.3 µg/µL] color development substrate). The reaction was carried out at 25 °C in the dark until violet spots appeared visually. After washing and drying the membrane, it was scanned. The results were quantified using the in-house software application “Imager 6.0” (developed by R. Veiko, an employee of RCMG (Moscow, Russia)); this computer program determines the location of each spot and the adjacent background signal, calculating the area and integral intensity of the spot. The signals for a single sample were averaged and the mean and standard error of the mean were calculated. The rDNA CN in each sample was determined for the entire 13,334 bp long transcribed region using a calibration curve equation. To plot a calibration curve for the signal of rDNA CN dependence, six standard samples of genomic DNA (20 ng per dot) with a known rDNA content were applied to the same membrane. The known concentration of rDNA in the standard calibration samples was determined as previously described [65]. The calibration samples with high rDNA content (exceeding that in the human genome) were obtained by adding the model rDNA (insert 5836 bp long isolated from plasmid p(ETS-18S)) to human DNA samples in the amount of several picograms per nanogram of DNA. Non-homologous DNA (20 ng/dot) was also added to the same filter to control the non-specific signal. Through a series of independent tests, the ratio of rDNA CN to the relative hybridization signal was well reproduced.

2.5 Cell Heterogeneity Analysis of Human Skin Fibroblasts

HSF-61 human skin fibroblasts (HSFs) were suspended and seeded into wells (N = 40) at a density of 10–20 cells per well, and cultured for 3 weeks with regular replenishment of the culture medium. Then DNA was isolated from the 32 wells and quantified. We further estimated the rDNA abundance in the samples from each well using NQH.

2.6 Cell Line Authenticity Statement

All cell lines were validated by short tandem repeat profiling and tested negative for mycoplasma.

2.7 Statistical Analyses

The relative standard error for NQH was a mere 5 ± 2% (detection error when analyzing 3–6 spots on one filter). The total standard error was 11 ± 8% in independent experiments (spread of the results was assessed from the stages of DNA extraction and quantification to hybridization on different filters). For each DNA sample, 3–6 parallel dots were applied in one experiment. Each test was repeated at least twice as independent biological replicates. Table 1 shows the descriptive statistics for rDNA CN. The significance of difference was analyzed by analy-

sis of variance. Differences in rDNA distributions between samples were tested using Kolmogorov–Smirnov statistics. MedCalc (<https://www.medcalc.org/manual/roc-curves.php>) produced a complete sensitivity/specificity report (receiver operating characteristic [ROC] curve analysis). Each point of such a curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) shows the parameter difference between two compared groups (e.g., healthy vs. patients). Data were processed using StatPlus 2007 software (<http://www.analystsoft.com/>). All *p* values were two-sided and considered statistically significant at *p* < 0.001.

3. Results

3.1 General Description

In this study, rDNA was quantified in 1124 DNA samples (Fig. 1B) isolated from the blood leukocytes of HCs (N = 546, including 105 long livers ≥90 years), SZ (N = 533), and CF (N = 45) cases. An assay of rDNA CN was performed using the NQH technique [43]. For hybridization, a biotinylated DNA probe that carried a fragment of the ribosomal repeat (see scheme in Fig. 1A) was used. DNA samples from patients with SZ and CF contained considerably more rDNA copies than DNA from HCs (*p* < 10^{−18}). The total analysis included additional data, which were obtained and published for all of the aforementioned groups [43–45,64–67] as well as a group of patients with MCI [42]. Fig. 1C presents the integrated dataset of rDNA abundance in DNA samples isolated from the blood of 3264 subjects of various ages (0.2–101 years old). All study participants were divided into four groups: HC group (N = 1360), SZ group (N = 1489), CF group (N = 322), and a group of elderly individuals with MCI (MCI group, N = 93). Table 1 describes the participants and descriptive statistics for their rDNA CN. Fig. 1D shows the rDNA CN distributions for the four groups.

3.2 rDNA CNVs in Health and Disease

Table 1 and Fig. 1 show that the rDNA CN in the four groups was in the order of: CF group > SZ group > HC group > MCI group. The groups significantly differed in rDNA CN (Df = 3, F = 405.8, *p* < 10^{−20}). DNA samples from the CF group contained maximum rDNA CNs, whereas samples from the MCI group contained minimum rDNA CNs. ROC analysis confirmed the largest difference between the CF and HC groups (AUC = 0.85; Fig. 1E). In the following sections, the rDNA CNVs are considered for each group in more detail.

3.3 rDNA CNVs in the HC group

We divided the HC group into five age subgroups: <17, 17–40, 41–72, 73–89, and 90–101 years (Fig. 2A). In the age subgroup of long livers (90–101), rDNA CN varied in a narrow range of 300 to 500 rDNA copies per diploid genome (Fig. 2A). The age subgroups <72 years

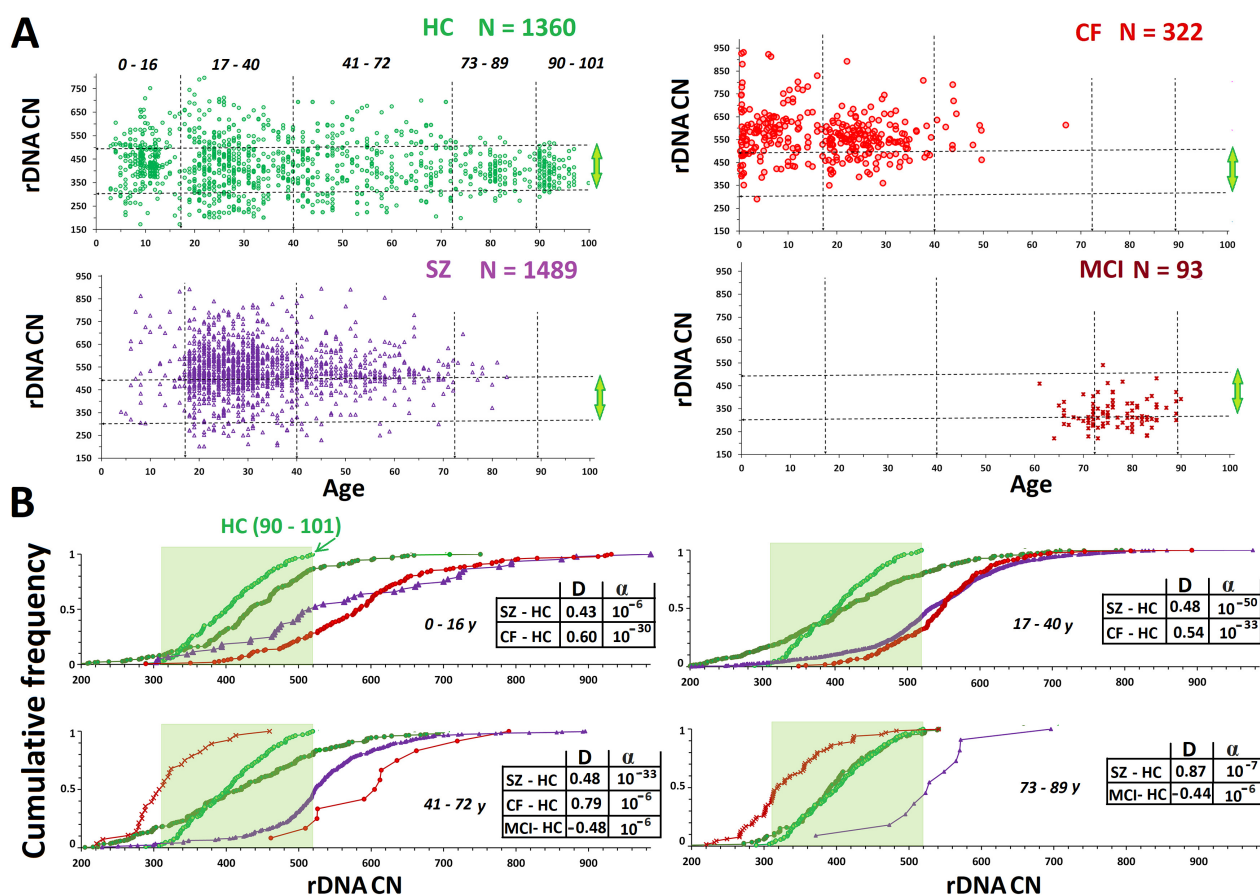


Fig. 2. Analysis of rDNA copy number variants (CNVs) in the HC, CF, SZ, and MCI groups. (A) Analysis by age of rDNA CN in the HC, CF, SZ, and MCI groups. The vertical dotted lines divide the five age subgroups. The horizontal lines divide the subgroups with high (>500 copies), medium (300–500 copies), range of long livers (area indicated by green arrow), and low (<300 copies) rDNA CN. (B) Comparison of rDNA CN distributions in various age subgroups. The area for the long livers is marked in green. The Kolmogorov–Smirnov (D and α) test statistics are presented in the tables.

contained 20–25% of DNA samples with rDNA CN more than 500 and 10–18% samples with rDNA CN less than 300 (Fig. 2B). The mean rDNA CN values did not differ among subgroups. However, the ranges and coefficients of variation (CVs) of rDNA CN were found to be decreased in the two oldest subgroups (Table 1). In our previous study, a trend of reduced rDNA CN in male versus female DNA was established in 17- to 78-year-old HCs (N = 122) [64]. However, no significant difference in rDNA CN between male and female subjects was revealed after 1360 DNA samples had been analyzed in the current study.

3.4 rDNA CNVs in the CF Group

The CF group (N = 45) had significantly higher rDNA CNs than the HC group (Fig. 1B). This finding corroborates our previous findings [66,67]. In the CF group (N = 322, aged 0.21–66.9 years), only one DNA sample with rDNA CN less than 300 was found (289 copies in a patient aged 3 years) (Fig. 2A). Genomes with rDNA CN less than 350 copies were seldom found among CF patients; 60–90% of DNA samples in the different age subgroups harbored 500+

rDNA copies (Fig. 2B). There was no association between rDNA CN and the patient's sex. The maximum range and maximum CVs were observed for the younger CF subgroup (0.21–16 years old) (Table 1). The maximum rDNA abundance (880–932 copies) was found in DNA samples from patients under 7 years old (Fig. 2). The 0.21- to 16-year-old CF subgroup had higher rDNA CNs than the 17- to 40-year-old CF subgroup ($p < 0.001$). The rDNA CN ranges and CVs were considerably decreased in patient subgroups older than 17. The 41- to 66.9-year-old CF subgroup contained no DNA sample, with rDNA CN either more than 790 or less than 460. Thus, we discovered the same regularities in CF patients as in the controls, namely, increased age was negatively correlated with rDNA CN ranges and CVs.

3.5 rDNA CNVs in the SZ Group

Expansion of the SZ cohort by an additional 533 participants did not change the results previously reported [43–45]. The patients harbored more rDNA copies than the HCs (Figs. 1, 2 and Table 1). In the SZ group, similar to the HC

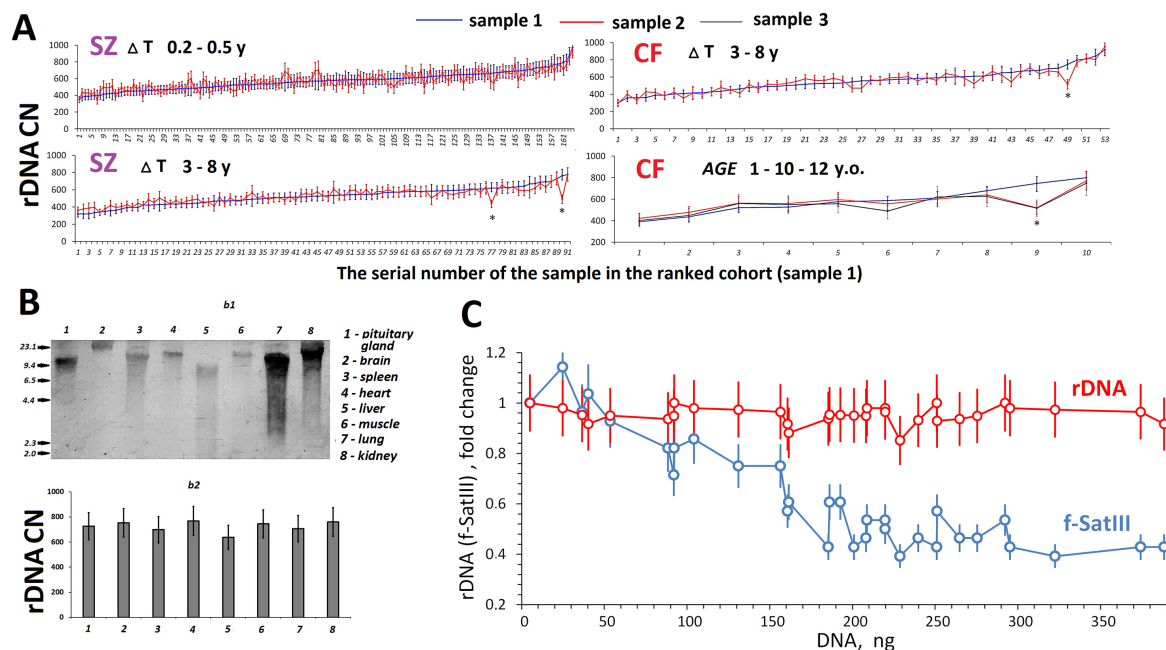


Fig. 3. Time dynamics of rDNA copy numbers (CN) in leukocytes and variation of rDNA CN in different cells from one subject or cell culture. (A) Changes in rDNA CN in DNA samples isolated from blood cells of the same subjects at various time intervals (ΔT). The data are ranked by DNA isolated from the earliest blood sample (sample 1). * $p < 0.01$ (four DNA samples with relatively high rDNA content lost some copies within a few years). (B) *b1*: Sizes of rDNA fragments (Southern blot with biotin-p(ETS-18S)) from DNA samples isolated from eight different tissues (4 h after death) of the body of a patient with SZ. *b2*: rDNA CN in DNA isolated from different tissues. (C) Dependence of rDNA CN (red line) in the genomes of HSF-61 on total cell DNA content in the clones. For comparison, the plot also shows the data for f-SatIII repeat (blue) for the HSF-61 strain. HSF-61, human skin fibroblast strain 61.

and CF groups, the range and CVs of rDNA CN decreased with age. The mean rDNA CNs did not differ between the older and younger SZ subgroups. Maximum rDNA CNs (890–986) were found in the younger SZ subgroup (5–16 years old). The rDNA CNs were the same between the two sex subgroups.

3.6 rDNA CNVs in the MCI Group

For comparison, Figs. 1,2 present published data for the MCI group (61–91 years old) [42]. The group of elderly patients with MCI had lower rDNA CN than the other three groups. The genomes of 35% of patients harbored less than 300 rDNA copies, whereas only one patient harbored more than 500 rDNA copies. In the control age-matched HC subgroup (61–91 years old), merely 7% of genomes harbored less than 300 rDNA copies. The rDNA CN of the MCI group did not depend on the patient's sex.

3.7 Changes in rDNA CN in Leukocyte DNA During a Patient's Lifetime

Fig. 3A shows data on the rDNA contents in DNA samples isolated from the blood leukocytes of the same SZ and CF patients, but at different times. The samples were stored at -70°C and the DNA samples from the same individual were assayed simultaneously. For 255 patients with SZ and 63 patients with CF, blood was sampled two or three

times, with an interval of several months (before and after treatment) to several years. Of the 311 DNA sample pairs obtained from patients with SZ or CF, as few as four pairs (1.3%) demonstrated a decrease in rDNA content over time compared to the baseline content in the first sample. No rDNA amplification was revealed in all of the cases.

3.8 rDNA Abundance in Different Organs of the Same Body and Different Cells of the Same Cell Pool

Fig. 3B shows rDNA abundance (4 h after death) in different organs of the same 54-year-old patients with SZ. DNA was isolated from pituitary, brain, spleen, heart, liver, muscle, lung, and kidney cells. To measure the degree of rDNA damage, the isolated samples were assayed using blot hybridization with a biotinylated DNA probe (Fig. 3B, *b1*). The most fragmented rDNA was found in DNA samples isolated from the hypophysis and liver; the longest fragments did not exceed 9 kilobase pairs. The rDNA content was measured using NQH in the eight DNA samples and appeared the same (720 ± 43 copies), taking into account the standard error of experiment (Fig. 3B, *b2*). Thus, we did not find any significant variation in rDNA CNs in different organs of the subject.

To estimate the rDNA CNVs within the human skin fibroblast (HSF-61) strain, we conducted a cloning experiment. Fig. 3C [81] shows the dependence of rDNA CN

on the amount of cellular DNA (the number of cells). The rDNA CN was the same for all DNA samples ($N = 32$), taking into account the standard error of the method. For comparison, data for f-SatIII repeat (blue) obtained for this HSF-61 strain are plotted in the figure. We previously found that the lower the proliferative activity of cells in the well, the higher the f-SatIII content [81]. The current study showed that the HSF-61 population is heterogeneous by f-SatIII content, but not by rDNA abundance.

4. Discussion

This study generalizes rDNA CN data for 3264 individuals. The data were obtained for a decade using a modified NQH technique developed in our laboratory for the quantification of highly repetitive tandem repeats in human DNA samples of different origin and quality. Hori and colleagues [82] were the first to perform rDNA quantification in human DNA samples using a direct method of sequencing long DNA fragments (Oxford Nanopore Technology, Oxford, UK). The rDNA CN in a cohort of healthy controls varied from 250 to 700 (mean 430 ± 98 , $N = 26$). This result aligns well with our findings obtained using NQH in the present study and in previous studies. Of the 1360 DNA samples from the HC group, 95% harbored 250 to 700 rDNA copies (mean 422 ± 104). Sequencing data obtained as part of the “The Telomere-to-Telomere” project and rDNA analysis by droplet digital polymerase chain reaction (PCR) showed that the diploid CHM13 genome contains approximately 400 rDNA copies and 409 ± 109 rDNA copies, respectively [83]. Analysis of the 2546 sequenced human genomes [84] has shown that rDNA CN in humans vary from 61 to 1590 copies per subject, with a mean of 315 ± 104 . Some recent alternative methods for rDNA CN detection using fluorescence *in situ* hybridization analysis showed that HeLa cells contain 821 rDNA copies, of which 42% are active [13,14]. Thus, the NQH method determines approximately the same range of rDNA CN variation in human cells as other methods. We discovered two principle effects discussed below.

4.1 Some Cases with a Genetic Pathology Carry More rDNA Copies Than Controls

The fact that cases carried more rDNA copies than any HC was most pronounced for CF monogenic disease (Fig. 2A). Increased rDNA CN in the DNA of patients with SZ was described for the first time by our group in 2003 on a small sample. Expansion of the SZ cohort from 42 patients [65] up to 179 [43], 956 [44], and 1489 (current study) did not change the result, as a significant fraction of patients with SZ harbor many (above the normal average) rDNA copies in the genome. This finding was recently corroborated by a Japanese team, who determined the relative rDNA content (with quantitative PCR) in the genomes of brain and blood cells of Asian patients with SZ [46].

In the case of a monogenic disease with a known pathogenesis that does not affect the process of protein translation, such as CF, any idea that predisposition is caused by an increased rDNA CN should be excluded. The rDNA CN cannot be causative for the pathogenesis. Thus, analyses of our data and the data of other groups suggest only one hypothesis: provided that a genetic pathology exists in the embryo genomes, embryos with a large rDNA CN have better chances of survival. We suggest that a very large rDNA CN (>700) in the fetal genome compensate for the genetic pathology by providing the maximum level of ribosome biogenesis. A higher rDNA CN in the genome causes a more effective stress response [85] due to the capability to provide for a high level of protein synthesis (e.g., heat shock proteins). In addition, a higher rDNA CN as part of the nucleolus has been shown to stabilize the nuclear heterochromatin and hence reduce the probability of chromosomal rearrangements during stress [28–30].

4.2 Distribution of DNA Samples by rDNA CN is Narrower in Older Subgroups

In three groups with a wide age range and large number of DNA samples (CF, SZ, and HCs), we observed the same dynamics of rDNA CN changes with aging. The maximum variation of the parameter occurred in young/middle aged subgroups. For HC, this age was 3–72 years, whereas it was under 17 years old for the CF and SZ groups (Fig. 1). For the CF, SZ, and HC groups, we established a decrease in the range (from minimum to maximum) and coefficient of variation in the older subgroups. This effect was most pronounced in the HC group, apparently, due to the widest age range available (3–101 years). It was previously shown that the relative amount of active rDNA copies in lymphocytes change according to the same pattern as total rDNA CN, namely, in the oldest age group, the range and CV are decreased [86]. There are two possible explanations for this phenomena. (1) With aging, the cells undergo genomic rearrangements, which result in either loss or amplification of rDNA copies. Such events take place during carcinogenesis. Cancer cells in the tumor often differ from the ambient normal cells by altered rDNA CN [38,48–50]. The rDNA CN alterations are observed in various brain areas of patients with dementia [41]. (2) Healthy subjects with low (<300) or high (>500) rDNA CN do not survive after ≥ 70 years. Genetically unaffected subjects with low rDNA counts in their genomes have not only a shorter lifespan, but also susceptibility to some age disorders. In the groups of patients with genetic burden, subjects with low rDNA CN are eliminated by prenatal and early postnatal zygotic selection. Therefore, the affected groups appear to demonstrate elevated mean rDNA CN values.

Studying replicative senescence of HSFs, we revealed that the genomes of cells during final passages lost only hypermethylated copies of rDNA, which comprise, according to our estimates, no more than 10% of human DNA samples

[64]. These copies are transcriptionally inactive and their replication is complicated. The ionizing radiation-induced stress did not lead to changes in rDNA CN in cultured stem cells [87]. HSF cloning data (Fig. 3C) corroborated the same rDNA content in every cell of the cell pool. Quantification of rDNA in various areas of brain of a patient with SZ also showed the same content, whereas considerable changes in the contents of other tandem repeats (telomere and satellite III) were observed between the areas [44,88]. The rDNA abundance did not differ in the cells of different organs of a SZ patient (Fig. 3B). Within the framework of the present study, we analyzed changes in rDNA CN in 311 DNA samples obtained from the same subjects, but at different times (Fig. 3A). A decrease in rDNA CN, which was beyond the experimental error, was observed in as few as four DNA samples (1%). Perhaps the time interval between blood sampling from the same subjects (12-year maximum) was not long enough. Further studies are warranted with longer time intervals.

We did not register rDNA amplification in any of the cases. rDNA CN decreases down to 500 in the long liver subgroup can be partially explained by hypermethylated copy loss during the subject's lifetime (in approximately 10% of the genomes). However, the elimination of DNA samples with a low rDNA CN (<300) from the subgroups of older patients and controls can only be explained by higher death rates of the carriers (i.e., their shorter lifespans). The lower abundance of rDNA copies was linked to less effective stress response and chromatin instability [28–30,85,89].

Thus, the study corroborated our previous data [90] that rDNA CN is a stable individual trait in ontogenesis.

5. Conclusions

We believe that within one human organism or one cell population, rDNA CN is a fairly stable genetic trait statistically associated with some diseases and overall life expectancy, which however, can change in rare cases under conditions of chronic oxidative stress. We believe that the observed age- and disease-related differences between the studied groups in mean rDNA CN and its variance are caused by biased elimination of carriers of marginal (predominantly, low) rDNA CN values. Further research is needed to better understand the relationships of rDNA with normal cellular physiology and phenotypic variation.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

NNV, EIK, LNP, GPK, RAZ and SVK designed the research study. NNV, ESE, YLM, SAK, TPV and NVZ

formed the patient dataset. ESE conducted laboratory tests. NNV, ESE and SVK analyzed the data. NNV and LNP wrote the draft manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The investigation was carried out in accordance with the latest version of the Declaration of Helsinki. Studies on the SZ and HC groups were approved by the Independent Interdisciplinary Ethics Committee on Ethical Review for Clinical Studies (51 Leningradsky Prospekt, Moscow, 125468, Russia, 8 (915) 346-30-30); Protocol #4 dated March 15, 2019 for the scientific minimally interventional study ‘Molecular and neurophysiological markers of endogenous human psychoses’. The study design for the CF group was approved by the ethics committee of the Federal State Budgetary Scientific Institution ‘Research Centre for Medical Genetics’ (Protocol No. 6/4 dated 15.11.2016). The study design for the MCI group was approved by the Independent Interdisciplinary Ethics Committee on Ethical Review for Clinical Studies (Protocol #4 dated 15 March 2019). All participants or parents/legal guardians of participants provided written informed consent to participate in the study after the procedures had been completely explained.

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Conflict of Interest

The authors declare no conflict of interest.

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