Review

Discoveries and biological implications of mammalian 45S rDNA variants and non-structural rRNAs

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Abstract: Repetitive nature of the ribosomal DNA (rDNA) gene makes the sequencing of hundreds copies of mammalian 45S rDNA (about 45-kb per copy) extremely difficult and its assembly is often excluded. Increasing evidence shows that 45S rDNA variations (copy number or single nucleotide), structural ribosomal RNA (rRNA) transcript variants, and non-structural rRNA transcripts (sense and anti-sense long noncoding rRNAs that include promoter rRNAs, and rRNA-derived fragments) play essential roles in mammalian development and diseases. Complete pictures of the hundreds copies of 45S rDNA and their rRNA transcripts require further innovation in sequencing techniques that include bioinformatics. The advancements in mammalian rDNA and rRNA sequencings and the discoveries of novel functions of the rDNA variants and rRNA transcripts are discussed here.

Keywords: Ribosomal DNA; ribosomal RNA; mammalian genome; intra- and inter-individual variations; development; diseases.

1. Introduction

Ribosomal DNA (rDNA) is conserved in prokaryotic and eukaryotic organisms, and is mapped to one or more chromosomal loci [1,2]. In mammals, nuclear 45S rDNA (Figure 1) is transcribed and processed into functional ribosomal RNAs (rRNAs) in the forms of structural canonical (18S, 5.8S, and 28S rRNAs) and non-structural non-canonical regulatory (such as sense or antisense long noncoding rRNAs that include promoter rRNAs, or small rRNA fragments) molecules [3-6]. Variations (copy number or single nucleotide) among hundreds or thousands of rDNA unit in mammalian genomes are commonly observed within or among individuals, and are also dynamic within individuals or pedigrees in light of instructive or selective preferences during ontogenic or disease processes, or via epigenetic or transgenerational effects [7-9]. A tandem repeat of the about 45-kb mammalian rDNA unit within multiple chromosomes makes whole genome sequencing and assembly very difficult and is often excluded from the genomic analyses. Similarly, rRNA transcripts, due to their overwhelming abundancy, are frequently depleted before RNA-sequencing [10,11]. Advancement of sequencing technologies provides new look of the landscape of the 45S rDNA loci and multifaceted functions of the structural and non-structural rRNAs. The intra-individual and inter-individual diversities of the rDNAs and rRNAs have important implications to mammalian development and disease susceptibility or causation. In this review, these diversities are categorized into following sections: rDNA intergenic sequence and pre-rRNA leader sequence variants, rDNA copy number variation, structural rRNA variants, sense or antisense regulatory long noncoding rRNAs (lnc-rRNAs), and small rRNA fragments.

2. The rDNA intergenic sequence and pre-rRNA leader sequence variants

The intergenic spacer (IGS, Figure 1), formerly called non-transcribed spacer (NTS) and about 30-kb in length in humans and mice, separates structural rRNA-transcribed regions and contains spacer and main promoter elements to regulate pre-rRNA tran-

scription [12]. Genome-wide or chromosome-specific sequence determinations have identified rDNA variants within or among individuals, including hundreds in the IGS region [13-15] and opening up the plethora of resources to study organismal development and gene-environment interaction. A mouse model has demonstrated that a protein-restricted or high-fat diet treatment during early life (from conception to weaning), but not post-weaning alone or combination of pre-weaning and post-weaning protein restriction, increased methylation at CpG -133 site of the rDNA in the A allele marked by a single nucleotide polymorphism at -104 position, and the allele-specific methylation correlated negatively with weaning body weight [16,17]. Allele-specific rDNA methylation has been also observed in the spacer promoter in another mouse model, in which paternal exposure to chemical stressors (chromium or acid saline) two-weeks before conception altered rDNA allele frequencies (defined by nucleotide polymorphisms within main promoter) and corresponding methylation during embryonic development and among tissues, body weight, and/or leptin profile in the offspring [18,19].

Single nucleotide variations in the pre-RNA leader sequences have been observed in mouse and human cells. The +139 (T/C) correlated negatively with the level of a sense long noncoding rRNA originated from about -1000 upstream and its transcript abundance was preferentially incresed in human lung cancer cells compared with normal line [20]. At least 7 pre-rRNA leader sequence variants have been identified from several mouse strains and differential expression of variant transcripts among tissues and preimplantation embryos, and in particular, learning-induced variant IV expression that enhances memory have been demonstrated [21-23].

3. The rDNA copy number variation (CNV)

The copy number of rDNA has been estimated to be from tens to thousands in humans and tens to hundreds in mice [24,13]. An estimation of about 50 rDNA copies in a single human chromosome 21 was reported in a mouse-human hybrid cell line [14] and of 1 to 20 rDNA copies in a single human chromosome 22 were observed in several clones depending on the origin of isolated chromosome [15]. From an analysis of 2546 human genomes, 19 of 26 ethnic populations are differentiable according to the number of rDNA copy [13]. CNV is also observed among tissues and increases with age [25-27]. Losses of rDNA copy number are frequently detected in some cancers and schizophrenia [28-30]. However, selective cancers and neurodegenerative diseases, such as dementia with Lewy bodies (DLB), mild cognitive impairment (MCI), and Alzheimer's disease (AD), have been associated with increase of rDNA copy number or content [31,32].

Estimation of the rDNA copy number often rely on techniques including computational analyses of genome sequencing data, quantitative or droplet digital polymerase chain reaction (qPCR or ddPCR), pulsed-field gel electrophoresis, and in-situ hybridization. Recently, questions for the accuracies of these techniques to quantify the rDNA copy number have been raised [33,34]. The library preparation and read depth in genome sequencing affect the accuracy of copy number estimation [34]. Classical qPCR or new ddPCR requires co-amplification of presumably single-copy reference gene. Pulsed-filed gel electrophoresis or in-situ hybridization is not high-throughput and is not intended to resolve small differences, such as tens of copies. Furthermore, rDNA pseudogenes, representing highly degenerated 45S or IGS adjacent to canonical rDNA loci and rDNA-like sequences of 20 to 117 bp across human and mouse genomes, have been detected [35]. Extent of the impact from these rDNA pseudogenes and rDNA-like sequences to rDNA copy number estimation is currently unclear.

4. The structural rRNA variants

Sequencing and computational analyses of human and mouse rDNA loci directly or rRNA pools have identified intra- and inter-individual variants within the canonical structural 18S, 5.8S, and 28S rRNAs [36,13,14]. The sequence variations within the 18S rRNA, component of the 40S ribosomal unit, and within the 5.8S and 28S rRNAs, components of the 60S ribosomal unit, represent multiplicity of ribosomes. It has been pro-

posed that each ribosome or group of ribosomes is specialized to carry out translation of specific mRNAs [37,4]. Massive parallel-screening of mRNAs with specific composite ribosomes identified unique group of mRNAs [38] and thus supports the notion that a specialized ribosome is assembled as target-specific translational machinery. Also, in zebrafish model, observations of replacement of maternal rRNAs with somatic rRNAs during embryogenesis and preferential affinities of ES6 and ES3 of the 18S rRNA to corresponding somatically expressed and maternally expressed mRNA, respectively [39], further support the specialized ribosome notion.

A study combining transformation-associated recombination (TAR) cloning, and short- and long-read sequencings of a single human chromosome 21 identified 101 variants, including 19 in 5'ETS, 4 in 18S, 14 in ITS1, 0 in 5.8S, 13 in ITS2, 43 in 28S, and 8 in 3'ETS [14]. Within the 28S rRNA, 42 of 43 variants are mapped to the expansion segments (ESs) region, predominantly at the ES27 site and few in ES7 and ES15 sites. Using the same techniques, 54 variations were mapped to the transcribed region of chromosome 22 rDNA units, including predominantly the ES27 site and two single nucleotide polymorphisms (SNPs), G59A in 28S rRNA and U682C in 18S rRNA, in which the latter is not observed in human chromosome 21 [15]. Analyses of whole genome sequencing data from the 1000 Genomes Project have identified rDNA variant alleles, 630 in 18S, 1069 in 28S, and 50 in 5.8S rRNA regions [13]. Among those variants, the G928A within the helix 22 and G1233A within the helix 30 of 18S rRNA have maximum intra-individual allele frequencies of 3.7% and 4.6%, respectively. The C462T and A1183G variants of 18S rRNA with the intra-individual allele frequencies of 61% and 27%, respectively, correspond to the sites for a post-transcriptional modification. Other rRNA variants with over 20% intra-individual allele frequencies are localized to the 40S-60S intersubunit bridge elements or to the binding sites of ribosomal proteins. Of 44 variants that highly correlate with signatures of 26 populations, 32 are present in the 18S rRNA and 12 in the 28S rRNA, including the A2538G 28S variant. The C543T within the helix 16 and the G480A within helix 5 of the 18S rRNA, and the G1764A within helix 38 of the 28S rRNA are also detected in the analyses of 32 mouse strains. RNA-sequencing of mouse tissues also confirmed variants across the 18S, 28S, and 5.8S rRNAs. Variants having highest estimated differences between tissues were localized in ESs, preferentially at ES27.

Interestingly, specific regions of 18S and 28S rRNAs have recently been identified as binding sites of NSP1 and NSP8 of SARS-COV-2 (COVID-19) viruses, providing mechanistic explanations how coronaviruses hijack translational machinery in the host cells. Structural analysis of in vitro reconstituted Nsp1-40S detected bindings of the NSP1 C-terminal domain to the mRNA entry channel in collaboration with ribosomal proteins uS5 of the body and uS3 of the head of 40S subunit, likely including helix 16 of the 18S rRNA, and also to helix h18 of the body, near helix 34 of the head of 40S subunit, to block retinoic acid-inducible gene I (RIG-I)-dependent innate immune responses [40]. In a cellular study, it was found that NSP1 binds to a 37-nt region of 18S rRNA, within the helix 18 and adjacent to the mRNA entry channel, resulting in the disruption of 40S mRNA scanning and translation initiation of immune-responsive transcripts, such as IFN-ß mRNA and endogenous IFN-ß-responsive mRNAs [41]. Also, NSP8 has high affinity to 28S rRNA at 3,017-nt to 3,529-nt region, corresponding to the ES27 (2,889-nt to 3,551-nt) expansion segments that interact with the ribosome exit tunnel of the 60S subunit. These findings open up the opportunities to explore questions whether the intra- and inter-individual variations of the structural rRNAs described above may explain the susceptibility and severity of coronavirus-related complications. Furthermore, the promising of mRNA vaccine relies on the design at 5'-untranslated regions (UTRs) for ribosomal entry [42], highlighting the urgency to decipher the roles of structural rRNA variants in antiviral therapies.

5. The sense or antisense regulatory long noncoding rRNAs (lnc-rRNAs)

The sense and antisense lnc-rRNAs are transcribed upstream and downstream, respectively, from canonical transcription start site where RNA polymerase I initiates the

synthesis of pre-rRNA which is then processed to produce mature 18S, 5.8S, and 28S rRNAs (Figure 1), and thus sense and antisense lnc-rRNAs overlap or are complementary to the pre-RNA. In contrast to the canonical pre-rRNA synthesis, lnc-rRNAs are generated by RNA polymerase I or II and are hundreds to thousands nucleotides in length acting as regulatory elements to control canonical pre-rRNA transcription and processing, and other cellular events. Applications of global RNA sequencing techniques have facilitated the discoveries of novel lnc-rRNAs.

Upon intrinsic or extrinsic stimulations, many sense and antisense lnc-rRNAs are upregulated in mammalian cells. They include RNA polymerase I-transcribed promoter-associated RNAs (pRNAs, 150-300 nt) after initiation of heterochromatic or late-replicating cell cycle state [43,44], and intergenic IGS16 and IGS22 (about 300 nt) and IGS28 (about 325 nt) transcripts during heat-shock and acidosis treatments, respectively [45,46], and RNA polymerase II-transcribed promoter and pre-rRNA antisense (PAPAS, 12 to 16 kb in size with essential nucleotides mapped to upstream -36/-160 region) under senescent or quiescent condition [47,48]. The pRNAs and PAPAS participate in chromatin remodeling to silence gene expression, including pre-rRNA. PAPAS is down-regulated in cancer cells that also overexpress pre-rRNA [47]. The IGS16, IGS22, and IGS28, corresponding to the regions at 16 kb, 22 kb, and 28 kb downstream from the pre-rRNA transcription start site, respectively, bind and sequester selected proteins inside the nucleolus, in particular, sequestration of VHL protein (a E3 ubiquitin ligase) by IGS28 under acidosis condition would allow hypoxia-inducible factors to evade proteasomal degradation [46].

Sense and antisense lnc-rRNAs of hundreds to thousands nucleotides have been also detected in human and mouse lung cells [20,49,50]. Human sense lnc-rRNAs are transcribed starting from approximately -1000 nucleotides upstream of the rRNA transcription start site (+1) and extending at least to +203 [20]. Mouse sense and antisense lnc-rRNAs are made from about -2547 upstream (near spacer promoter) and from about +12026 (near 3′-end of 28S), respectively, and massively parallel sequencing further identifies the sense lnc-rRNAs of up to about 10 kb of cleavage-ligation products carrying internal deletion of hundreds to thousands nucleotides [50]. Stabilization of these sense or antisense lnc-rRNAs by antisense oligonucleotides or transfection with in-vitro-synthesized lnc-rRNAs induces necrotic and apoptotic cell death, and/or autophagy, preferentially in cancer cells, depending on selected targets and treatments [49,50].

6. The small rRNA fragments (rRFs)

Through sequencing alignments of previously excluded small RNA fragments, rRNA fragments of about 20 to 50 nucleotides corresponding to 5′- or 3′-end of mature rRNAs were identified in human and mouse cells, and these rRFs were likely produced from the ANG RNase hydrolysis [51]. Further large-scale sequencing and computational analyses mapped the rRFs specifically to 18S, 5.8S, and 28S rRNAs, preferentially at the 5′- and 3′-ends, in mammalian cells [52,53]. These rRFs overlap each other by varying progressively with 1-base differences and RNAi knockdown of a 20-nt 28S fragment (hsa-rRF3-000001) induces apoptosis in a lung cancer cell line [52]. Some of these rRFs are called straddle (x-rRFs) because their 5′-ends contain additional 1 to 6 nucleotides not found in the 5.8S or 28S rRNAs, and rRFs are differentially expressed according to human population and gender [53}.

In addition, rRNA fragments functioning as PIWI-interacting RNAs (piRNAs, about 23 to 32 nt), endo-siRNAs (about 21to 24 nt, derived from convergent sense/antisense transcripts), QDE-2-interacting RNAs (qiRNAs, about 20 nt), and microRNA (miRNAs, about 20 nt) have been also detected in mammalian cells via large sequence databases and re-sequencing [54-57]. In mouse sperm cells (spermatozoa), more than 50% of the total reads of piRNAs and 37% of endo-siRNAs are mapped to rRNA sequences; in particular, the annotated PIR64428 corresponding to the cleavage product of the 5.8S rRNA is most frequently observed. In contrast, relative expression of piRNAs + endo-siRNAs (=

rasRNAs) originated from rRNAs decreased in oocytes (29% of total rasRNA reads) and zygotes (14% of total rasRNA reads) [54,55], indicating roles of these rasRNAs in the transition from paternally to maternally dominant gene controls. In the same study of rRNA-related qiRNAs, rRNA fragments of 18 to 30 nucleotides have been also mapped to mouse and human 18S, 5.8S, and 28S rRNAs, predominately in sense orientation, and are differentially expressed (upregulated in 4448 rRFs; downregulated in 3216 rRFs) between diabetic versus normal mouse liver [56]. Murine miR-712 (human mir-205 counterpart) and human mir-663, processed from ITS2 and ITS1 of pre-RNA, respectively, by XRN1 exoribonuclease and upregulated by disturbed flow in endothelial cells, downregulate tissue inhibitor of metalloproteinase 3 (TIMP3) expression, which in turn activates the downstream matrix metalloproteinases (MMPs) and a disintegrin and metalloproteases (ADAMs) molecules, consequentially pro-atherogenic responses, endothelial inflammation, and permeability [57], a possible cascade leading to atherosclerosis.

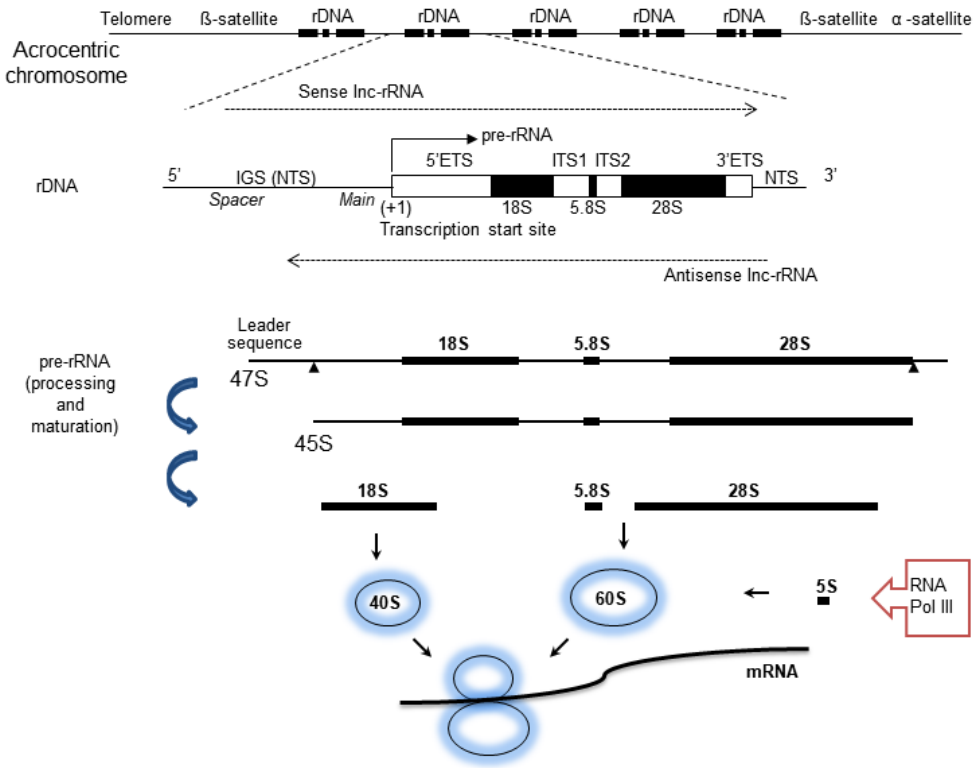

Figure 1. Mammalian rRNA biogenesis and ribosomal components. IGS: intergenic spacer (contains *Spacer* and *Main* promoter sequences); NTS: non-transcribed spacer; ETS: external transcribed spacer; ITS: internal transcribed spacer. The filled Δ indicates cleavage sites during processing of 47S pre-rRNA to 45S rRNA.

7. Conclusions

The state-of-the-art sequencing technologies that include bioinformatics have provided the opportunities to discover 45S rDNA and rRNA variations at genome-wide scale and their new functions from previously presumed noises or artifacts in mammalian genomes and transcriptomes. The plethora of 45S rDNA and rRNA variations coincides with cell-to-cell, tissue-to-tissue, individual-to-individual, and population-to-population diversities. Further innovation in this area is needed to test the hypotheses that organismal diversities or disease susceptibilities are originated from the rDNA and rRNA variations.

Supplementary Materials: Not applicable.

Author Contributions: Conceptualization, Y.H.S.; writing—original draft preparation, Y.H.S.; writing—review and editing, Y.H.S.; visualization, Y.H.S.

Funding: Not applicable.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The views expressed do not necessarily represent those of the US Federal agency.

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