5’UTR Variants of Ribosomal Protein S19 Transcript Determine Translational Efficiency: Implications for Diamond-Blackfan Anemia and Tissue Variability

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Abstract

Background: Diamond-Blackfan anemia (DBA) is a lineage specific and congenital erythroblastopenia. The disease is associated with mutations in genes encoding ribosomal proteins resulting in perturbed ribosomal subunit biosynthesis. The RPS19 gene is mutated in approximately 25% of DBA patients and a variety of coding mutations have been described, all presumably leading to haploinsufficiency. A subset of patients carries rare polymorphic sequence variants within the 5’ untranslated region (5’UTR) of RPS19. The functional significance of these variants remains unclear.

Methodology/Principal Findings: We analyzed the distribution of transcriptional start sites (TSS) for RPS19 mRNAs in testis and K562 cells. Twenty-nine novel RPS19 transcripts were identified with different 5’UTR length. Quantification of expressed w.t. 5’UTR variants revealed that a short 5’UTR correlates with high levels of RPS19. The total levels of RPS19 transcripts showed a broad variation between tissues. We also expressed three polymorphic RPS19 5’UTR variants identified in DBA patients. The sequence variants include two insertions (c.-147_-146insGCCA and c.-147_-146insAGCC) and one deletion (c.-144_-141delTTTC). The three 5’UTR polymorphisms are associated with a 20–30% reduction in RPS19 protein levels when compared to the wild-type (w.t.) 5’UTR of corresponding length.

Conclusions: The RPS19 gene uses a broad range of TSS and a short 5’UTR is associated with increased levels of RPS19. Comparisons between tissues showed a broad variation in the total amount of RPS19 mRNA and in the distribution of TSS used. Furthermore, our results indicate that rare polymorphic 5’UTR variants reduce RPS19 protein levels with implications for Diamond-Blackfan anemia.

Introduction

Diamond-Blackfan anemia (DBA; OMIM #205900) is a rare congenital bone marrow failure characterized by decreased numbers or absence of erythroid precursor cells [1]. Approximately 50–60% of DBA patients carry a mutation in one of nine ribosomal protein (RP) genes of which RPS19 mutations account for 25% [2]. A series of >100 coding mutations in the RPS19 gene have been identified ranging from deletions and insertions of various sizes, single base substitutions resulting in both non-sense and missense mutations and splice site mutations [3]. A large proportion of mutations presumably result in functional haploinsufficiency for RPS19 by removing transcription from one allele (deletions or insertions) or by nonsense mediated mRNA decay (splice site, non-sense and missense mutations) [4]. A translated RPS19 protein variant can also mediate haploinsufficiency due to reduced stability, inappropriate localization to the nucleoli, reduced affinity to interacting partners and failure to assemble into the pre-ribosome (missense mutations) [5–7]. Haploinsufficiency for a ribosomal protein leads to perturbed ribosome subunit synthesis [8–10] followed by increased cellular stress, cell cycle arrest and apoptosis [11]. The precise mechanism by which RP mutations mediate the erythroid specific phenotype in DBA is still unclear. It has been hypothesized that erythropoiesis is particularly sensitive to ribosomal protein insufficiency and cellular stress because of a high proliferative and protein synthesis rate [12].

A few gene variants have been described in the non-coding 5’UTR of the RPS19 gene, i.e. in the first exon upstream of the ATG start codon [13–15]. These variants were initially identified in a subset of DBA patients and later in healthy individuals but at a low frequency [14,15]. In addition, targeted resequencing of the entire RPS19 gene in DBA patients has revealed a number of non-coding sequence variants and rare polymorphisms localized to introns and flanking sequences [13,14,16,17]. One of the 5’UTR
variants (c.-147_-146insGCCA) has been associated with rRNA processing defects but the RPS19 protein levels appeared unchanged in erythroid cells from a patient with this variant [14]. However, it is still unclear if this non-coding sequence variant is transcribed and if it interferes with the translation of RPS19. In addition, the distribution of TSS of w.t. RPS19 have not been carefully analyzed.

The regulation of ribosomal protein expression is critical for cellular adaptation to different requirements. It is well established that the 5’UTR of mRNAs is of importance for gene expression by influencing mRNA stability, subcellular localization, accessibility to the ribosomes and interaction with the translational machinery [18,19]. Thus, the 5’UTR mediates the adjustment of protein levels to developmental stages, tissue types and growth rate [20]. Conversely, inappropriate expression of 5’UTRs can contribute to abnormal developmental phenotypes and disease [21-23]. Furthermore, the 5’UTR of mRNAs encoding ribosomal proteins contains a 5’TOP sequence which enables fast up- or down-regulation of RP levels [24,25].

The RPS19 coding sequence and its 5’UTR are highly conserved [7,13]. However, the significance of the rare polymorphic 5’UTR sequence identified in DBA patients is yet unknown. We examined the transcription of 5’UTR variants and we hypothesized that they affect RPS19 protein synthesis rate as a possible contributing mechanism in DBA. We show herein that the expression of three structural RPS19 5’UTR variants leads to a reduced translation into RPS19. Furthermore, RPS19 uses a broad range of TSS with effects on RPS19 translation and with tissue variations.

Materials and Methods

RPS19 expression constructs with different 5’UTR variants RPS19 cDNA clones with three w.t. variants of the 5’UTR (figure 1) were amplified and cloned into the reporter vector pAcGFP-N1 (Clontech) downstream of the CMV promoter. The resulting constructs encode fusion proteins consisting of a full length RPS19 linked to a fluorescent reporter at the C-terminus (Figure 2A). Three DBA associated 5’UTR variants (c.-147_-146insGCCA, c.-147_-146insAGGC and c.-144_-141delTTTTC) were generated by site directed mutagenesis from the pAcGFP-N1-382-S19-5’UTR clone (INTERMEDIATE clone) with Quick change II site directed mutagenesis kit (Stratagene) according to manufacturers recommendations.

Cell culture and transfection K562 [26], HeLa [27] and HEK293 [28] cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 20 IU penicillin/streptomycin (all Invitrogen) at 37°C with 5% CO2 in a humidified environment. Cells were transfected in 10 cm dishes with 5 μg of total RNA following manufacturer’s recommendations. Quantitative real-time PCR was performed in triplicates using platinum SYBR green qPCR supermix UDG (Invitrogen) according to the protocol supplied by the manufacturer. Primer sequences and PCR conditions used to quantify w.t. RPS19 mRNA with different 5’UTRs are available upon request.

5’ Rapid Amplification of cDNA ends (5’ RACE) 5’RACE was performed with 1 μg of total RNA using the GeneRacer® kit (Invitrogen) according to manufacturer’s recommendation. Initially, the RNA was treated with DNase I to clean the samples from any genomic DNA. First strand cDNA synthesis was carried out with GeneRacer Oligo(dT) primer and Superscript RT III RACE ready cDNA kit. For amplification of the cDNA end we used the 5’GeneRacer forward primer included in the kit and RPS19 specific reverse primer. The PCR product was cloned into a TOPO-TA vector (Invitrogen) and randomly picked individual clones were sequenced.

Western blotting HEK293T and K562 cells were lysed in RIPA buffer supplemented with MG132 proteasome inhibitor (SIGMA), phosphatase inhibitor cocktail 1 (SIGMA), 0.1 mM Sodium vanadate (SIGMA) and protease inhibitor cocktail (SIGMA). Cell lysates were separated on a 10% Bio-Trio SDS-PAGE (NuPage gel; Invitrogen), and transferred to PVDF Immobilon-FL membranes (Millipore). Membranes were hybridized with primary antibodies against GFP (Clontech) and β-actin (Abscam). Proteins detected by the antibodies were visualized using Alexa Fluor 680 (γ-rabbit) and IRD 800 labeled (γ-mouse) secondary antibodies (Molecular probes and LiCor Bioscience, respectively). Western blots were analyzed using the Odyssey® infrared imaging system determining integrated intensities, using β-actin as a normalization control as described previously [29].

Results

Multiple transcript variants of the RPS19 gene The RPS19 gene spans a genomic region of 11.5 Mb on chromosome 19q and consists of 6 exons [13]. The first exon is untranslated and the start codon is located in the immediate beginning of exon 2. Ribosomal protein S19 exists in one single form consisting of 145 amino acids. Six variants of the RPS19 transcript have been described with differences only in length of the 5’UTR from the use of alternate transcription initiation sites [7,13,30,31]. This prompted us to search for additional RPS19 transcript variants and to analyse their effect on RPS19 expression. We performed cDNA 5’RACE with RNA from K562 cells and testis and we determined the transcript sizes and their transcriptional start sites. We identified altogether 31 alternative RPS19 transcripts with 5’UTRs ranging from 32 to 467 nucleotides. Twenty-nine transcript variants are yet undescribed (figure 1A). The distribution of the RPS19 5’UTR length appeared to be different when comparing randomly picked clones from K562 cells and testis, respectively (figure 1). Clones from testis had a distribution towards longer 5’UTRs with the longest clone spanning 467 nucleotides, extending about 100 nucleotides beyond the previously reported longest RPS19 transcript (Gene bank #BC018616). Longer RPS19 5’UTRs appear highly structured as predicted by the RNA secondary structure prediction program Mfold (data not shown).

We then analyzed the relative expression of three groups of RPS19 transcripts with different 5’UTRs on mRNA from a panel using random hexamer primers and 2 μg of total RNA following manufacturer’s recommendations.
of human tissues (Stratagene) as well as the cell lines K562 and HeLa by quantitative real-time PCR. Three specific primer pairs were designed that generate amplicons from the RPS19 5′UTR corresponding to nucleotide positions −63 to +49 (amplicon A), −350 to −239 (amplicon B) and −449 to −354 (amplicon C), respectively (figure 1B). Quantification of the RT-PCR products revealed different patterns of 5′UTRs when comparing different tissues and cell lines (1C and D). Transcripts detected by the shortest amplicon (amplicon A) are predominant in all tissues investigated. The expression of longer 5′UTRs defined by the amplicons B and C constitute from 1% to 17% of the total amount of RPS19 mRNA (figure 1D). No strong correlation was observed between the total amount of RPS19 mRNA and the relative proportions of longer and short 5′UTR.

Short RPS19 5′UTRs show high translational activity

We made three constructs with RPS19 5′UTRs of 35 nucleotides (SHORT; containing the 5′TOP sequence), 382 nucleotides (INTERMEDIATE) and 467 nucleotides (LONG), respectively. Each construct expresses a full-length RPS19. The constructs were fused with Green fluorescent protein at the carboxy-terminus (figure 2A) and analyzed when transiently transfected into HEK293T and K562 cells. The SHORT 5′UTR variant is translated 4–6 fold more efficient than the variant with 5′UTRs of 382 nt and >10 fold more efficient than RPS19 with the 467 nt 5′UTR (figure 2B–C). The results were similar for both cell lines.

DBA associated 5′UTR variants affect translational activity

We next investigated the effect on translation of three distinct polymorphic sequence variants in the 5′UTR of RPS19 found in a subset of patients with DBA. We introduced two insertions (c.-147_-146insGCCA, c.-147-146insAGCC) and one deletion (c.-144_-141delTTTC) into the INTERMEDIATE construct (Figure 3A) followed by a transient transfection into K562 and HEK293T cells. The three DBA associated 5′UTR variants reduced the RPS19 levels by approximately 20–30% when compared to the wild type INTERMEDIATE construct (figure 3B and C). A marked reduction in expression (32%) was observed for the TTTC deletion in both cell lines. The AGCC insertion showed a 30% reduced expression in HEK293 cells and a 26% reduction in K562 cells. The GCCA insertion was associated with a 20% reduction in expression in both cell lines (figure 3B and C). The RPS19 mRNA levels were similar when comparing cells transfected with the rare variant constructs to cells transfected with the wild-type construct.

Discussion

The 5′-untranslated region (5′UTR) of an mRNA is an important regulator of translation by influencing e.g. mRNA stability, sub-cellular localization and translational efficiency [32–34]. Furthermore, multiple transcriptional start sites and 5′UTRs expressed from a single gene encoding one and the same protein may regulate gene expression through differential expression with respect to developmental stages, tissue type and in response to stimuli [20,35,36]. One element that enables fast up- or down-regulation of ribosomal proteins in response to nutrient supply is the 5′TOP sequence, a stretch of 4 to 14 pyrimidines following a Cytosine as the first nucleotide located at the 5′end of an mRNA [37]. This 5′TOP sequence is contained in the SHORT 5′UTR variant used for expression analysis in our study and possibly responsible for fast adaptation of RPS19 levels. The heterogeneous 5′UTRs of mRNAs transcribed from a single gene arise from the use of alternate transcriptional initiation sites and differential RNA processing [38]. It has been estimated that 10–18% of genes express alternate 5′UTRs by multiple promoter usage. Alternate untranslated regions determine tissue specific function and their inappropriate expression can contribute to the development of abnormal phenotypes and disease [39].

We have characterized the RPS19 5′UTR variants with respect to expression levels, tissue specificity, and translation efficiency. RPS19 is ubiquitously expressed and mutations in this gene are associated with DBA. The precise molecular mechanisms behind the disease remain unknown but we hypothesized that the expression of different RPS19 5′UTR variants may contribute to the regulation of RPS19 protein levels and, ultimately, to DBA. We determined the extent of the RPS19 mRNA 5′UTR by 5′-RACE on poly(A)+ purified mRNA from testis and K562 cells. The results show an extensive variation in the transcriptional start sites with >30 different 5′UTRs of which 29 are novel. The total amount of RPS19 mRNA varied considerably between tissues and we observed up to 10-fold differences. Interestingly, bone marrow shows relatively low level of total RPS19 transcripts when compared to several other primary tissues analyzed. We then investigated the distribution of 5′UTR variants in different tissues. Transcripts were divided into three groups containing a 5′UTR of at least 3 nt, 330 nt and 449 nt, respectively. Our data indicate clear differences in the distribution of RPS19 5′UTRs when comparing different tissues. The amplicon corresponding to the shorter 5′UTR (amplicon A) constituted between 83% to >99% of RPS19 transcripts but without correlation to the variation in total amounts of RPS19 mRNA.

To get a better insight into the translational regulation of RPS19 5′UTR variants we investigated the RPS19 levels expressed from constructs with three distinct 5′UTRs length. The SHORT 5′UTR variant, spanning a 35 bp 5′UTR, is translated four to ten fold more efficiently than the two longer variants with 5′UTRs of 382 bp and 467 bp, respectively. This is also consistent with the analysis of variable 5′UTR length of other genes [34,36]. A possible explanation is that the SHORT variant exhibits a less complex secondary structure, facilitating scanning by the transla-
The reduced translation from transcripts with longer 5' UTRs may be related to the more complex secondary structures, making the transcripts less accessible for translation. The functional significance of the longer RPS19 5' UTRs is unclear, but may be of importance for TOP independent translation providing baseline amounts of RPS19. The shorter variants could in this case be used for a fast adaptation of RPS19 levels in response to cellular needs. In combination, our observations suggest a large variation in RPS19 mRNA levels between tissues as well as in TSS used. The predominant and shorter 5' UTRs are more efficiently translated and may directly reflect the levels of RPS19.

We then analyzed the effect on translation of rare sequence variants in the RPS19 5' UTR found in subsets of DBA patients. We confirmed that the polymorphic 5' UTR variants are indeed transcribed and we hypothesized that these transcripts affect translational efficiency. We therefore investigated the RPS19 levels expressed from constructs with each of the two insertions (c.-147_-146insGCCA, c.-147_-146insAGCC and c.-144_-141delTTTC), respectively. All three variants result in significantly

![Figure 3](image-url)
reduced RPS19 levels when compared to the corresponding wild type sequence. A possible explanation is that the mutation causes the mRNA to adopt a more complex secondary structure that represses translation. It is noteworthy that the observed reduction in RPS19 levels in our cell-systems is related to a relatively large proportion of the ‘INTERMEDIATE’ 5′UTR of RPS19 transcripts containing each of the specific 5′UTR variants. Still, the observed effects of the three 5′UTR variants associated with DBA do not result in haploinsufficiency and, accordingly, the impact on RPS19 levels in vivo would depend on the relative amounts of longer RPS19 mRNAs. Although these 5′UTR variants may lead to suboptimal conditions for growth and differentiation of tissues sensitive to reduced RPS19 levels it is likely that additional factors are required for overt clinical forms of DBA. Our results are consistent with the increased ratio of 21S/18S pre-rRNAs associated with the c.-147_-146insGCCA variant observed previously [14]. Failure to detect reduced RPS19 levels in that study may be due to low abundance of longer transcripts and/or minor changes in RPS19 levels in the cells analyzed.

Our combined findings suggest complex regulatory mechanisms of RPS19. RPS19 uses a broad range of TSS with tissue specific differences and shorter 5′UTRs are more efficiently translated. We also show that DBA associated 5′UTR variants of RPS19 are less efficiently translated. Further investigations are now required to understand how RPS19 is regulated in different tissues both at the transcriptional and the translational level. These studies may clarify the distribution and levels of RPS19 5′UTR variants as well as RPS19 protein levels at different stages of erythropoiesis. Thus, analysis of the RPS19 TSS used in erythroid precursor cells may provide valuable information in search for molecular mechanisms behind DBA.

Author Contributions

Conceived and designed the experiments: ND JS. Performed the experiments: JB JS OG. Analyzed the data: JS JB ND. Contributed reagents/materials/analysis tools: JS JB ND. Wrote the paper: JS ND. JS made the statistical analyses and conducted the project together with ND.

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