Ribosomal RNA analysis in the diagnosis of Diamond-Blackfan Anaemia

TITLE

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RUNNING TITLE

rRNA analysis in DBA

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SUMMARY

Diamond-Blackfan anemia (DBA) is an inherited disease characterized by pure erythroid aplasia. DBA has been tagged as a “ribosomopathy”, given that almost all genes involved in the pathogenesis encode for ribosomal proteins (RPs) required for pre-ribosomal RNA (rRNA) processing.

We report here a multi-center study focused on the analysis of rRNA processing of 53 Italian DBA patients using a capillary electrophoresis analysis of rRNA maturation of the 40S and 60S ribosomal subunits.

We found that the ratio of 28S/18S rRNA was higher in patients mutated in RPs of the small ribosomal subunit where 40S subunit biogenesis is affected and 18S rRNA is reduced. In contrast, patients mutated in RPs of the large ribosomal subunit (RPLs) have a lower 28S/18S ratio because of 60S subunit biogenesis defects lowering levels of 28S rRNA. In addition, a peak corresponding to 32S pre-rRNA is observed in almost all patients mutated in RPL genes.

The assay reported here would be amenable for development as a diagnostic tool. In fact, an initial functional screening using such a strategy could reduce time to diagnosis and cut diagnostic costs by limiting the number of genes to be analyzed to attain a genetic diagnosis.

KEYWORDS

Diamond Blackfan anemia, RP gene, mutation, rRNA, diagnosis

INTRODUCTION

Diamond Blackfan Anemia (DBA) is an inherited bone marrow failure syndrome (BMFS) that typically shows an onset in the first year of life in most cases and is characterized by a pure erythroid aplasia. Clinically the patients are very heterogeneous. One third of patients show congenital malformations. Some patients respond to steroids, while others require regular transfusions. Approximately 10% of patients undergoing treatment achieve clinical remission by unknown mechanisms (Vlachos*, et al* 2008).

Making a DBA diagnosis is challenging as it is based on a number of clinical features shared by other BMFS and often hinges upon exclusion of other BMFS. In sixty percent of DBA patients, heterozygous mutations in genes that encode for ribosomal proteins (RPs) of both small and large ribosomal subunit have been identified. So far 15 RP genes have been found mutated in DBA (*RPS7, RPS10, RPS17, RPS19, RPS24, RPS26, RPS27, RPS29, RPL5, RPL11, RPL15, RPL26, RPL27, RPL31, RPL35A*) (Boria*, et al* 2010, Farrar*, et al* 2014, Gazda*, et al* 2012, Landowski*, et al* 2013, Mirabello*, et al* 2014, Wang*, et al* 2015).

DBA is often considered a prototype of a new class of human diseases referred to as ribosomopathies. While the mechanistic link between RP haploinsufficiency and the erythroid phenotype of DBA patients remains unclear, virtually all mutations in RP genes known to cause DBA have been shown to disrupt ribosome biogenesis. Ribosome biogenesis is a complex process involving RPs and RNAs, which comprise the structural components of the 40S and 60S ribosomal subunits, and numerous extra-ribosomal factors. Reduced amounts of RPs of the 40S and 60S subunits disrupt the biogenesis of their respective subunits by interfering with the maturation of RNA components of either subunit. Disruption of pre-ribosomal RNA (rRNA) processing can lead to specific reductions in the mature RNAs found in either 40S or 60S ribosomal subunits.

On this basis, we were prompted to ascertain whether analysis of mature rRNA species could be used to monitor RP haploinsufficiency and whether this approach might be amenable for development as a diagnostic tool.

We report here a multi-center study focused on the analysis of rRNA of 53 Italian DBA patients.

MATERIALS AND METHODS

Patients

The patients involved in this study were all included in the Pediatric Haemato-Oncology Italian Association (AIEOP) DBA Registry. Diagnoses were made following criteria suggested by the DBA International Clinical Consensus Consortium (Vlachos, *et al* 2008). Control RNAs were obtained from healthy individuals.

Written informed consent was obtained from patients and controls and/or their legal guardians.

This study was planned according to the guidelines of the local ethical committee.

Clinical and molecular data are reported in **Supplementary Table I**.

RNA extraction

Total RNA was obtained from mononuclear cells isolated from peripheral blood by Ficoll density gradient centrifugation and grown for three days in IMDM supplemented with 10% FCS, PHA (10 µg/ml). RNA was extracted using the RNeasy kit (Qiagen) following manufacturer's instructions.

Rapid rRNA analysis by electrophoresis

Total RNA was run on the Bioanalyzer 2100 (Agilent Technologies) capillary electrophoresis system using the RNA Nano chip (Agilent Technologies) according to the manufacturer's protocol. RNA integrity number (RIN) and RNA concentration (ng/uL) were obtained for each sample. Peak integration was performed using 2100 Expert (Agilent Technologies) software in fragment analysis mode using software-defined 18S and 28S peaks; the 30S or 32S peak was manually selected as the minor peak just larger than 28S rRNA.

Mutation detection

Mutation screening of the RP genes (*RPS17, RPS19, RPS24, RPS26, RPL5, RPL11, RPL35A*) with a combination of sequencing and Multiplex Ligation Probe Amplification (MLPA) analysis was performed as previously described (Quarello*, et al* 2012, Quarello*, et al* 2010).

To determine whether the sequence changes were polymorphic variations, we verified that none was reported in the Single Nucleotide Polymorphism database (dbSNP at *www.ncbi.nlm.nih.gov/SNP*) and we predicted their effect using PolyPhen-2 (*http://genetics.bwh.harvard.edu/pph2/*) and SIFT (*http://sift.jcvi.org*).

Statistical analysis

The data are summarized in terms of frequencies and percentages for categorical variables. Group comparison was made with the Mann-Whitney U-test. Statistical significance was considered achieved at a value of $p \leq 0.05$. Statistical analysis were done using NCSS 10 (Number Cruncher Statistical System, Kaysville, UT).

RESULTS/DISCUSSION

A total of 53 patients with DBA were included in this study (**Supplementary Table I**). Twenty (38%) had mutations in genes encoding RPs of the 40S ribosomal subunit: group 1; *RPS17* (3 patients), *RPS19* (11 patients), *RPS24* (2 patients), and *RPS26* (4 patients). Eighteen patients (34%) had mutations in genes encoding RPs of the 60S ribosomal subunit: group 2; *RPL5* (11 patients), *RPL11* (5 patients) and *RPL35A* (2 patients). We also studied 15 DBA patients with a classical diagnosis of DBA without an identified causal mutation (group 3).

The 40S ribosomal subunit is comprised of a single 18S rRNA whereas the large subunit is comprised of three rRNAs, 28S, 5.8S, and 5S rRNA. The 28S and 18S rRNAs are the major peaks resolved by capillary electrophoresis and a ratio of their relative amounts is included in calculating a RIN used to assess the quality of RNA preparations. We reasoned that changes in the amount of one ribosomal subunit relative to another caused by haploinsufficiency for a RP would alter the ratio of 28S to 18S rRNAs relative to controls. As shown in **Supplementary Table I** and in **Figure 1A** the ratio of 28S/18S rRNA was significantly higher in patients with mutations in RPS genes

where 40S subunit biogenesis is affected and 18S rRNA reduced (group 1; median 2, range 1.9-2.5) than in patients with mutations in RPL genes (group 2, median 1.4, range 1.1-1.6) (*p<0.0001*) or the control group (median 1.7, range 1.6-1.8) (*p<0.0001*). Conversely, the ratio of 28S/18S rRNA was significantly lower in patients with RPL mutations as compared to the control group (*p<0.0001*) (**Fig 1A**). This is most likely explained by the fact that 60S subunit biogenesis is affected in RPL mutant cases, lowering levels of 28S rRNA.

In addition to the mature rRNAs of the 40S and 60S ribosomal subunits, a prominent peak corresponding to 32S pre-rRNA is frequently observed in the analysis of RNAs from most patients with RPL mutations (**Fig 1B**). Previous studies revealed a significant increase in the ratio of 32S/28S rRNA in RNA derived from DBA patients with mutation in RPL genes (Farrar*, et al* 2014). This ratio was also assessed in the patient cohort analyzed here. An increase in 32S/28S RNA was evident in most patients with mutations in genes encoding large subunit RPs (group 2, median 0.08, range 0.03-0.13) relative to the control group (median 0.03, range 0.02-0.04) (*p<0.0001*). An outlier in this analysis was two patients with mutations in *RPL35A* that exhibited low 28S/18S ratios without a corresponding increase in 32S/28S ratio.

Among DBA patients without an identified causal mutation, 10 patients showed an elevated 28S/18S ratio, one patient had a reduced 28S/18S ratio with a prominent 32S peak and 4 patients had 28S/18S ratios comparable to controls.

The results reported here reveal that ratios of 28S to 18S rRNA differentiate patients with mutations in RP genes from healthy controls. Moreover, our data reveal that these ratios differentiate mutations in genes encoding proteins of the 40S ribosomal subunit from patients with mutations encoding proteins of the 60S ribosomal subunit . The relative ease of identifying underlying ribosome biogenesis defects in patient samples suggests that this approach may be amenable to further development as a diagnostic tool for ribosomopathies like DBA. Further, this analysis can potentially streamline the number of candidate genes that need to be evaluated for pathogenic mutations in patients suspected of having DBA.

Potential applications of this strategy are evident in patients within this cohort who currently lack identifiable casual mutations. Of these 15 patients, 10 had increased ratios of 28S to 18S rRNA suggesting a defect in 40S subunit biogenesis. Possible candidate genes for these patients would be novel RPS genes in which no mutations have been described yet or in which mutations are found in low frequencies in DBA patients and were not screened in the genetic analyses performed in this cohort. Alternatively, the genes affected in these patients might be non-ribosomal protein genes like TSR2 that play a role in 40S subunit biogenesis and that have recently been identified to play a role in DBA pathogenesis (Gripp*, et al* 2014).

Only one of the patients without a causal mutation has a decreased ratio of 28S to 18S ratio whose ribosome biogenesis defect is further re-enforced by the presence of 32S pre-rRNA and an increased ratio of 32S to 28S rRNA. Finally, four patients have 28S to 18S ratios comparable to

healthy controls. These patients could join an emerging group of DBA patients with mutations in genes that encode for proteins with functions that do not directly impact on the ribosome biogenesis (like GATA1) or in RPs that are not directly involved in rRNA processing (e.g. *RPS15, RPS25*) (Parrella*, et al* 2014, Robledo*, et al* 2008, Sankaran*, et al* 2012).

In conclusion, we propose that rRNA analysis, in particular the 28S/18S and 32S/28S ratios evaluated by capillary electrophoresis, could be integrated as a first step in a flowchart to be used for DBA diagnosis. In fact, an initial functional screening using such a strategy could reduce time to diagnosis and cut diagnostic costs, by limiting the number of potential genes to be sequenced or analyzed by MLPA to attain a genetic diagnosis.

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AUTHORSHIP

PQ, SRE and UR designed the study. PQ, CB, and UR selected DBA patients and analyzed clinical data. PF followed patients and helped in the data evaluation. EG, AC and AA were responsible for RNA extraction. EG and AC were responsible for rRNA analysis. EG, AC, CM, KDK were responsible for mutation detection. EG supervised the interpretation of all results. PQ performed statistical analysis. LF was responsible for cell cultures. PQ and SRE wrote the paper. ID, UR and KDK critically revised the manuscript.

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TABLES AND FIGURES

FIGURE 1

A. Comparison of 28S/18S ratio among DBA patients and healthy controls.

The 28S/18S ratio in DBA patients with 40S abnormalities was significantly higher than in patients with 60S subunit alterations and in healthy controls. Conversely, DBA patients with 60S gene alterations showed a significant lower 28S/18S ratio if compared to other groups.

B. Agilent Bioanalyzer 2100 electropherogram obtained from total RNA of peripheral blood mononuclear cells of DBA patients carrying RPS and RPL genes mutations and a healthy control.

The electropherogram data demonstrates two prominent peaks corresponding to 18S and 28S rRNA. A lower 18S peak was observed in DBA patients with 40S gene alterations in comparison to controls and to DBA patients with 60S gene abnormalities. Conversely, the latter group showed lower 28S RNA level associated with a minor peak corresponding to 32S pre-RNA.

Supplementary Table 1. Clinical and molecular data of DBA patients

Characterization of mutations was in accordance with the nomenclature proposed by the Human Genome Variation Society (http://www.hgvs.org). p.0? = probably no protein is produced.

Legend: ST steroid treatment, ^at diagnosis, SD steroid dependent, TD transfusion dependent, np not performed.