Exploiting pre-rRNA processing in Diamond Blackfan anemia gene discovery and diagnosis

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Diamond Blackfan anemia (DBA), a syndrome primarily characterized by anemia and physical abnormalities, is one among a group of related inherited bone marrow failure syndromes (IBMFS) which share overlapping clinical features. Heterozygous mutations or single-copy deletions have been identified in 12 ribosomal protein genes in approximately 60% of DBA cases, with the genetic etiology unexplained in most remaining patients. Unlike many IBMFS, for which functional screening assays complement clinical and genetic findings, suspected DBA in the absence of typical alterations of the known genes must frequently be diagnosed after exclusion of other IBMFS. We report here a novel deletion in a child that presented such a diagnostic challenge and prompted development of a novel functional assay that can assist in the diagnosis of a significant fraction of patients with DBA. The ribosomal proteins affected in DBA are required for pre-rRNA processing, a process which can be interrogated to monitor steps in the maturation of 40S and 60S ribosomal subunits. In contrast to prior methods used to assess pre-rRNA processing, the assay reported here, based on capillary electrophoresis measurement of the maturation of rRNA in pre-60S ribosomal subunits, would be readily amenable to use in diagnostic laboratories. In addition to utility as a diagnostic tool, we applied this technique to gene discovery in DBA, resulting in the identification of RPL31 as a novel DBA gene.


Introduction

Diamond Blackfan anemia (DBA) is a bone marrow failure syndrome that usually manifests as a macrocytic anemia in the first year of life [1]. In addition to anemia, approximately 50% of patients with DBA may have a heterogeneous array of congenital anomalies including thumb, craniofacial, cardiac, and urogenital abnormalities [2]. Mutations or single copy deletions in 12 genes that encode proteins of the 40S (RPS7, RPS10, RPS17, RPS19, RPS24, RPS26, and RPS29) or 60S (RPL5, RPL11, RPL15, RPL26, and RPL35A) ribosomal subunits have been identified in patients with DBA [OMIM #612563, 613308, 613309, 612561, 612562, 612563, 105650, 610629, 613309, 603633, 612527, 612528, respectively] [3–14]. Many cases of DBA arise spontaneously as a result of a disruptive alteration in one of two copies of a ribosomal protein gene; in familial cases, transmission is typically autosomal dominant, indicating DBA is caused by haploinsufficiency for ribosomal proteins. Mutations of a nonribosomal protein, the erythroid transcription factor GATA1 [OMIM# 300835], have also been identified in patients with DBA [15,16]. Despite significant progress in DBA gene discovery, the underlying genetic defect remains unknown in 35–40% of patients.
Of the DBA patients whose genetic defect is unknown, it seems likely that some fraction of the affected genes may also target ribosomal synthesis as the nature of a putative gene variant is ambiguous. Pre-rRNA processing has been used to define the effects of ribosomal protein deficiencies on ribosome synthesis [17]. Suboptimal levels of a ribosomal protein interfere with ribosome assembly, which can be assayed by tracking the generation of mature ribosomal RNAs from a nascent polycistrionic transcript. Different steps in pre-rRNA processing may be affected depending on whether the ribosomal protein is a component of the large or small subunit, the nature in which it interacts with rRNA and other factors involved in its incorporation into the assembling subunit [6,10,18,19]. Thus, ribosomal protein deficiencies are associated with characteristic pre-rRNA processing signatures, which in turn can be used to infer their functions in ribosome biogenesis. This tool has most often been used retrospectively to support a gene assignment in DBA but, as we demonstrate, can also be used prospectively to guide gene discovery.

Here, we describe an infant presenting with a severe macrocytic anemia, neutropenia, and congenital anomalies both typical and atypical of DBA. Testing for common inherited bone marrow failure syndromes failed to identify a cause. Additional study revealed a deletion of DBA. Testing for common inherited bone marrow failure syndromes, neutropenia, and congenital anomalies both typical and atypical of DBA validated the nature in which it interacts with rRNA and other factors involved in its incorporation into the assembling subunit [6,10,18,19]. Thus, ribosomal protein deficiencies are associated with characteristic pre-rRNA processing signatures, which in turn can be used to infer their functions in ribosome biogenesis. This tool has most often been used retrospectively to support a gene assignment in DBA but, as we demonstrate, can also be used prospectively to guide gene discovery.

**Methods**

Array comparative genomic hybridization (aCGH). aCGH of peripheral blood DNA was performed in a clinical laboratory using a custom 180,000 probe microarray (Agilent Technologies). The positive deletion was confirmed in the proband, and excluded in the parents, by metaphase FISH analysis of peripheral blood using probe RPL31-532P9. Deletion and phenotype data regarding the index RPL31 DBA case were submitted to the DBA Mutation Database at http://www.dbagenes.unito.it [21,22].

Pre-rRNA processing studies. This DBA study is registered at ClinicalTrials.gov (NCT00106015). Non-DBA patient RNA was obtained from pediatric hematology/oncology (age 2–17 years) patients without a suspected diagnosis of bone marrow failure syndrome (hemoglobin SS, SC, or S+ thalassemia, acquired immune thrombocytopenia, iron deficiency anemia or history of oncologic disorder more than 1 year off therapy). Informed consent was obtained from patients or guardian and family members under participating institutional protocols according to the Declaration of Helsinki. Concordavalin A-stimulated (ConA) peripheral blood mononuclear cells (PBMC) were prepared as described previously [7]. Total RNA was isolated using RNeasy kits (Qiagen). RNA was fractionated on a 1.5% formaldehyde–agarose gel for Northern analysis, gel–ethidium bromide stained gels were visualized. For Northern analysis, RNA was fractionated on a 1.5% formaldehyde–agarose gel (PBMC) were prepared as described previously [7]. Total RNA was isolated using RNeasy kits (Qiagen). RNA was fractionated on a 1.5% formaldehyde–agarose gel for Northern analysis, gel–ethidium bromide stained gels were visualized.

Flow cytometric analysis. Cells were analyzed by FACS Calibur 1 flow cytometer (BD Biosciences) after staining with FITC-conjugated anti-CD235 and PE-conjugated anti-CD41 antibodies (BD Biosciences). Data were analyzed in FlowJo Version 8.8.6 (TreeStar).

Quantitative RT-PCR (qRT-PCR). Total RNA was reverse transcribed using Quantitect RT kit (Qiagen). The level of RPL31 mRNA was measured by qRT-PCR on a 7500 real-time system (Applied Biosystems) using Power Syber green mix (Applied Biosystems) for detection of PCR products. cDNA (1 μl) was used in a 25 μl final volume reaction with 400 nM primers. RPL31 was internally normalized against GAPDH and compared in reference to cells transduced with luciferase shRNA.

**Results**

Identification of copy loss involving RPL31

A female infant of Hispanic and Caucasian ancestry presented at 4 months of age with severe, macrocytic anemia (hemoglobin 2 g/dL, MCV 130 fL) and neutropenia (absolute neutrophil counts [ANC] averaging 300/μl) with variably elevated platelet counts (range 3 × 10⁸–8 × 10⁸/μl). Bone marrow evaluation revealed decreased cellularity for age (50–60%) with an absence of erythroid precursors, myeloid hypoplasia with maturation arrest, increased numbers of megakaryocytes, lymphocytosis, and eosinophilia (Fig. 1A,B). The bone marrow karyotype was normal. She had multiple congenital anomalies including bilateral proximal radioulnar synostosis (RUS, Fig. 1C), a left triphalangeal thumb (Fig. 1D) and right thenar hypoplasia. She had normal cardiac anatomy by echocardiography, but abdominal ultrasound at 4 months demonstrated a small spleen (reference mean length and standard deviation for age is 52.0/4.7 cm), measuring 2.5 cm [25].

Chromosome breakage assay for Fanconi anemia (FA) was within normal limits and sequence analysis revealed no mutations in the Shwachman Diamond syndrome (SDS) gene (SDSB). Nine ribosomal protein genes known to be involved in DBA and available for clinical testing were sequenced, revealing only two synonymous single nucleotide polymorphisms, one each in RPS19 and RPL11. Red blood cell

**Lentiviral vector production.** 293T cells (2 × 10⁶) were plated in a 6-cm dish in DMEM supplemented with 10% FCS (HyClone), with 4 mM glutamine, 50 mg/ml penicillin, and 50 U/ml streptomycin. The cells were transfected with 3 plasmids (3 μg packaging construct, 1 μg envelope construct, and 4 μg pLKO with shRNA encoding lipofectamine and OPTI-MEM (Invitrogen). After 6 hr, the transfection medium was changed. Virus containing supernatant was collected after 48 hr, pooled, filtered through a 0.45 μm filter and stored at −80°C. The titer of lentivirus was evaluated by slot blot analysis of RNA extracted from virus containing medium compared to known numbers of infectious particles.

**Transduction of cells.** K562 cells were cultured in 12 well plates (2 × 10⁶ cells/well) DMEM (Invitrogen) + 10% FBS (HyClone). For transduction, one half volume of medium was replaced with virus-containing medium with polybrene added to a final concentration of 2 μg/ml. After 24 hr, fresh media was added containing 1.5 μg/ml of puromycin. The media was replaced after 48 hr in selection and cells were harvested for RNA analysis at 96 hr after transduction. CD34⁺ human peripheral blood stem and progenitor cells were a kind gift from Harry Malech (NIAID). CD34⁺ cells were cultured in 12 well plates (1.5 × 10⁶ cells/well) for 12 hr (hr 0–12 after plating) in proliferation media consisting of StemSpan Serum-Free Expansion Medium (Stemcell Technologies) supplemented with 100 U/ml penicillin/streptomycin (Invitrogen), 2 mM glutamine (Invitrogen), 40 μg/ml human LDL (Stemcell Technologies), 100 ng/ml stem cell factor, 50 ng/ml thrombopoietin, 10 ng/ml IL-3, 10 ng/ml IL-6 (all Peprotech), and 0.5 U/ml erythropoietin (Stemcell Technologies). For transduction, one half volume of medium was replaced by virus-containing medium and polybrene was added to a final concentration of 2 μg/ml. After 24 hr (hr 0 following plating), fresh proliferation media was added containing 1.5 μg/ml of puromycin. After 48 hr in selection (84+ hr after plating), cells were cultured for 7 days in differentiation media consisting of proliferation media supplemented to a final concentration of 3 U/ml erythropoietin.

**Lentiviral vector construction.** Oligonucleotides encoding RPL31 shRNA (TRCClone ID TRCN0000291963 [23]) and luciferase shRNA were phosphorylated by T4 polynucleotide kinase, annealed, and ligated into pLKO.1 vector digested with EcoRI and AgeI [24].

**Lentiviral vector production.** 293T cells (2 × 10⁶) were plated in a 6-cm dish in DMEM supplemented with 10% FCS (HyClone), with 4 mM glutamine, 50 mg/ml penicillin, and 50 U/ml streptomycin. The cells were transfected with 3 plasmids (3 μg packaging construct, 1 μg envelope construct, and 4 μg pLKO with shRNA encoding lipofectamine and OPTI-MEM (Invitro
transfusions precluded an accurate determination of erythrocyte adenosine deaminase activity, which is elevated in 85% of patients with DBA [26].

The patient remained red cell transfusion-dependent throughout her first year of life, with hemoglobin concentrations typically falling to < 6 g/dL within 3 weeks following transfusion. Although neutropenic at baseline and requiring hospital admission and empiric antibiotic therapy on several occasions for fever with neutropenia, she did not develop clinically significant bacterial infections during this time. She subsequently achieved acceptable peripheral neutrophil counts (average ANC 3,000/l) with granulocyte colony-stimulating factor administered at 5 mg/kg thrice weekly beginning at 7 months of age. She was vaccinated in the first year of life according to Advisory Committee on Immunization Practices guidelines, including three doses of conjugated pneumococcal (PCV13) vaccine. At 12 months of age she presented with, and subsequently died as a result of, overwhelming S. pneumoniae sepsis. The family declined post-mortem examination.

Comparative genomic hybridization (CGH) and subsequent confirmatory fluorescence in situ hybridization (FISH) revealed a 3.2 Mb deletion on chromosome 2 (2q11.2, chr2:99,028,236—102,253,369, GRCh37/hg19) that was not found in either parent. The deletion at 2q11.2 involves 30 genes annotated in the NCBI RefSeq database (Fig. 1E) and includes RPL31, a 60S ribosomal subunit gene, suggesting haploinsufficiency for RPL31 as a potential explanation for her syndrome. However, as RPL31 has not been previously identified as a DBA gene, we addressed whether the 2q11.2 deletion was associated with a defect in ribosome biogenesis in the proband.

Pre-rRNA processing defects in RPL31-deficient primary cells

To determine whether the 2q11.2 deletion is associated with defective ribosome biogenesis, we analyzed pre-rRNA processing in ConA-stimulated PBMC by Northern blot analysis of total RNA derived from the proband, her parents, and a healthy control. Because RPL31 encodes a protein of the 60S ribosomal subunit, we initially assessed pre-rRNA processing using an oligonucleotide probe complementary to sequences within an internal spacer, ITS2 (Supporting Information Fig. S1). We identified a prominent increase in 32S pre-rRNA in the proband as compared to parents and control (Fig. 2A). The 32S pre-rRNA contains the mature 5.8S and 28S RNAs of the 60S ribosomal subunit separated by ITS2. Cleavage within ITS2 ultimately gives rise to the 3’-end of 5.8S RNA and the 5’ end of 28S RNA (Supporting Information Fig. S1). Thus, a pre-rRNA processing defect affecting the 32S pre-rRNA and the subsequent production of mature 28S RNAs is consistent with haploinsufficiency of RPL31, a large subunit ribosomal protein gene.

We also investigated the pre-rRNA processing steps associated with maturation of the 40S subunit using an oligonucleotide
complementary to sequences within ITS1, which hybridizes to rRNA precursors of the small ribosomal subunit (Supporting Information Fig. S1). These studies demonstrated modestly decreased 30S pre-rRNA in the proband relative to controls. Unexpectedly, the proband also had increased levels of 18SE pre-rRNA, the immediate precursor to mature 18S rRNA (Fig. 2B). This late step in pre-rRNA maturation occurs after 40S subunit precursors are transported from the nucleus to the cytoplasm [27]. Since it seemed unusual for the reduced expression of a large subunit ribosomal protein to affect such a specific step in the maturation of small ribosomal subunits, we considered the possibility that the proband may have more than a single defect in ribosome synthesis, potentially resulting from an unidentified mutation in another ribosomal protein gene or in other genes critical for ribosome synthesis.

Depletion of RPL31 mimics the pre-rRNA processing abnormality of patient cells

To determine whether the effects observed on pre-rRNA processing in the proband could be exclusively attributed to haploinsufficiency for RPL31, we examined pre-rRNA processing in human K562 cells depleted of RPL31 mRNA by RNA interference. These studies revealed a virtually identical pre-rRNA processing pattern as compared to that of the proband (Fig. 2C,D). Thus, the complex pre-rRNA processing phenotype observed in the proband can be attributed to haploinsufficiency for RPL31 alone (Fig. 2E).

Haploinsufficiency for RPL31 impairs erythroid differentiation

To assess the effects of reduced amounts of RPL31 on erythropoiesis, we transduced normal human CD34+ hematopoietic progenitors with lentiviral vectors expressing shRNA against RPL31 or luciferase mRNA and cultured in conditions promoting erythroid differentiation. Under these conditions, most differentiating progenitors transduced with control shRNA undergo erythroid differentiation as measured by CD235 (glycophorin A) expression (Supporting Information Fig. S2A). Erythroid differentiation is markedly inhibited in hematopoietic progenitors following RPL31 mRNA knockdown (Supporting Information Fig. S2B), with a 100-fold reduction in the proportion of CD235+ cells and a marked decrease in the erythroid (CD41+/CD235+) /megakaryocyte (CD41+/CD235+) ratio (Supporting Information Fig. S2C). Thus, reduced levels of RPL31 inhibit erythroid differentiation in a manner consistent with that of the previously established DBA-associated ribosomal protein genes.

RPL31 alterations are rare in DBA

Due to phenotypic similarity to the RPL31 index patient, we studied a previously reported DBA patient who lacked detectable alterations in the known DBA genes. Like the index case, this patient had proximal RUS (reported in [28]) and later developed intermittent neutropenia and thrombocytosis. We identified a de novo, intron 1 splice donor site mutation (c.1-386G>A) in this proband (Supporting Information Fig. 3). Although inclusion of intron 1 in the mature mRNA does not disrupt the coding sequence, it is predicted to generate 2 open-reading frames (ORF) upstream of the RPL31 ORF and thus may impair translation of RPL31 mRNA.

To further assess the frequency of RPL31 mutations, we sequenced the coding region, intron/exon boundaries and untranslated regions of RPL31 in 75 DBA probands as a part of the Diamond Blackfan Anemia Registry ribosomal protein gene-resequencing project. We were unable to identify additional putative disease-causing single nucleotide or small insertion/deletion variants of RPL31 in this cohort of patients (0/75; 95% CI 0–5.8%). Primer sequences and all detected sequence variants are shown in the Supporting Information Materials (Supporting Information Tables S1 and S2). We were similarly unable to identify deletions involving RPL31 by SNP microarray genotyping in 75 DBA probands who lack a known DBA gene mutation.

Elevated 32S pre-rRNA is a consistent feature in DBA patients with mutations in the commonly mutated large ribosomal subunit genes

As noted above, 32S pre-rRNA is increased in PBMC from a DBA patient where RPL31 was lost by deletion. Remarkably, increased 32S...
Elevated levels of 32S pre-rRNA, which are clearly evident by Northern analysis using a probe to ITS2 in RPL31-deleted proband RNA (A, upper panel), are also evident on visualization of the ethidium bromide-stained RNA gel (A, lower panel) from peripheral blood mononuclear cells after culture for 96 hr in ConA. Similar results were observed in DBA specimens harboring other large subunit alterations (B, demonstrating two independent RPL5 mutations) but not normal control RNA. Ethidium bromide-stained and Northern blot analysis of a larger sample of representative DBA patient RNA demonstrates a prominent 32S band in a patient with a known RPL11 mutation as well as a patient (DBA2) with an unknown gene mutation, 32S pre-rRNA is not elevated in control RNA or in a patient with mutation of the small ribosomal subunit gene RPS26. Another patient with unknown causative mutation (DBA1) lacks clear evidence for either large or small subunit processing defect. Control lanes consist of RNA obtained from unaffected parents of the DBA proband (Panels A & B) or non-DBA healthy controls (Panel C).

Figure 3. Elevated levels of 32S pre-rRNA, which are clearly evident by Northern analysis using a probe to ITS2 in RPL31-deleted proband RNA (A, upper panel), are also evident on visualization of the ethidium bromide-stained RNA gel (A, lower panel) from peripheral blood mononuclear cells after culture for 96 hr in ConA. Similar results were observed in DBA specimens harboring other large subunit alterations (B, demonstrating two independent RPL5 mutations) but not normal control RNA. Ethidium bromide-stained and Northern blot analysis of a larger sample of representative DBA patient RNA demonstrates a prominent 32S band in a patient with a known RPL11 mutation as well as a patient (DBA2) with an unknown gene mutation, 32S pre-rRNA is not elevated in control RNA or in a patient with mutation of the small ribosomal subunit gene RPS26. Another patient with unknown causative mutation (DBA1) lacks clear evidence for either large or small subunit processing defect. Control lanes consist of RNA obtained from unaffected parents of the DBA proband (Panels A & B) or non-DBA healthy controls (Panel C).
Discussion

Diamond Blackfan anemia is one of a number of inherited bone marrow failure syndromes (IBMFS), many of which have overlapping clinical features that can pose diagnostic challenges. In the absence of a known causative gene mutation, DBA is often considered as a diagnosis of exclusion after ruling out other IBMFS. The studies described here were initiated during the evaluation of a patient posing a significant diagnostic challenge in terms of differentiating amongst possible IBMFS.

The patient was an infant with an early, severe and persistent macrocytic anemia with skeletal abnormalities consistent with DBA or other IBMFS. She also had several features less frequently observed in DBA, including significant nonerythroid bone marrow abnormalities. In the context of bone marrow failure syndromes, radial ray abnormalities are generally associated with FA or thrombocytopenia absent radii (TAR) syndrome; a potential diagnosis of Fanconi anemia was excluded based on normal chromosome breakage studies. The presence of persistent neutropenia was potentially compatible with Shwachman Diamond syndrome, a diagnosis that was largely excluded based on normal SBDS gene sequence. Similarly, sequencing of the clinically available known DBA genes failed to define a genetic lesion. Further genomic analysis revealed a large deletion involving RPL31. We demonstrate here that RPL31 is required for steps in the maturation of 60S ribosomal subunits in patient cells and, importantly, that decreased expression of RPL31 in human CD34+ hematopoietic progenitors interferes with erythroid differentiation. These results strongly suggest DBA as the underlying diagnosis for this patient and identify RPL31 as a new DBA gene.

Several additional clinical findings in this case were initially confounding to establishing the diagnosis of DBA. The unusual phenotypic features might be attributable in part to the chromosomal deletion, with haploinsufficiency of multiple genes contributing to a contiguous gene syndrome. There are examples in the literature of patients with DBA resulting from genomic deletion who have unusual additional features, such as neurodevelopmental delays, that may reflect a contiguous gene syndrome [30]. However, recent findings that have expanded our understanding of ribosomopathy-associated disease, a clearer recognition of the broad phenotypic variations present even in genetically well-classified IBMFS, and the similarities between this proband and an additional DBA patient with an RPL31 splice donor mutation make it tempting to speculate that the majority of the phenotype is attributable to abnormalities of RPL31.

Neutropenia, although not a classical feature, is clearly prevalent in a subset of patients with DBA [31]. Thrombocytosis is frequently observed in the 5q-syndrome, a myelodysplastic syndrome that results from clonally acquired loss of the 40S ribosomal subunit protein, RPS14, in bone marrow progenitors [32,33]. Although radial defects are most typical of FA and TAR, radial hypoplasia along with triphalangeal thumbs were key phenotypic features in both DBA siblings described by Aase and Smith [34]. Radioulnar synostosis has also been associated with amegakaryocytic thrombocytopenia (RUSAT, OMIM #605032), a disorder caused by heterozygous frameshifting deletions in the homeodomain of HOXA11 [35]. Intriguingly, defective translation of Hox gene mRNA in mice has also been linked to abnormal skeletal development associated with haploinsufficiency of the non-DBA associated ribosomal protein gene, Rpl38 [36]. These observations suggest the possibility of aberrant Hox gene mRNA translation as a potential mechanism for skeletal manifestations of DBA. Finally, although the available clinical data are incomplete on this point, the development of fulminant, fatal pneumococcal sepsis and anatomically apparent hyposplenia are suggestive of a humoral immunologic abnormality. In this regard, there are reports of hypogammaglobulinemia or common variable immune deficiency in patients with DBA [6,37]. Isolated congenital asplenia (ICA, OMIM #271400), another genetically based and distinctive abnormality of the humoral immune compartment, has been linked to the ribosome through the identification of heterozygous mutations of RPSA in a subset of affected kindreds [38].

While initial studies suggested that ribosome synthesis could be a common pathological target in other IBMFS [39], with the exception of DBA and possibly SDS, effects on ribosome synthesis appear to be secondary or disease modifiers rather than the primary pathological target of genetic lesions. The ribosome biogenesis defect in SDS appears late in the pathway involved in the maturation of 60S ribosomal subunits; specific effects on pre-rRNA processing similar to those observed in patients with DBA have not been reported in cells from SDS patients [40]. Thus, the pre-rRNA processing defects observed in cells from patients with DBA could potentially be specifically exploited for diagnostic purposes. The results reported here suggest a strategy for employing pre-rRNA processing analysis easily adaptable to a diagnostic setting. We found in both primary cells and knockdown models that loss of RPL31 leads to accumulation of 32S, an intermediate in the pre-rRNA processing pathway of the 5.8S and 28S rRNAs of the 60S subunit, and 18S pre-rRNA, a late intermediate of the 18S rRNA of the 40S subunit. The surprising finding of altered small subunit rRNA processing in DBA patients with large subunit gene abnormalities has not been previously reported, but is potentially significant as a unifying feature of large and small subunit gene abnormalities. In yeast, the ultimate cytoplasmic steps in 40S maturation, corresponding to 18SE cleavage, require interaction with mature 60S subunits [41]. Thus, it is possible that abortive large subunit processing may indirectly lead to aberrant 18SE processing. Although this finding has not been specifically reported in DBA patients, we note that knockdown of several DBA-associated LSU protein genes (RPL11, RPL19, RPL26, and RPL15) appears to lead to elevated levels of 18SE pre-rRNA [9,12].

We found that significant increases of 32S pre-rRNA is a general feature of nearly all DBA patients with mutations in genes encoding 60S subunit ribosomal proteins. Notably, we show that this increase in 32S pre-rRNA can also be detected in total RNA easily fractionated and quantified by automated capillary electrophoresis (e.g., Bioanalyzer or similar instrument), providing a functional readout for defects in the maturation of 60S ribosomal subunits. In addition to the patient described here with a genomic deletion involving RPL31, we show elevated levels of 32S pre-rRNA in patient samples with mutations in RPL5 and RPL11, two of the most frequently mutated genes encoding 60S subunit ribosomal proteins in DBA [10]. To date, we have only identified a single patient haploinsufficient for a protein of the 60S ribosomal subunit that did not show an increase in 32S pre-rRNA. Alterations involving this gene, RPL15, have been identified in only two patients with DBA (Supporting Information Fig. S4 and [12]).

Although the sample size in this study is relatively small and will require additional prospective study to better assess predictive value, these results suggest that deployment of this functional screening strategy in diagnostic laboratories would allow rapid identification of pre-rRNA processing defects in approximately 20–25% DBA patients based on current estimates of the frequency of mutations in genes encoding proteins of the large ribosomal subunit. 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 to attain a genetic diagnosis. In addition, this assay would allow functional characterization for those patients with amino acid or weak splicing consensus variants where, in contrast to nonsense and frameshift mutations, a pathogenic effect is uncertain. Alternative strategies for employing the Bioanalyzer more broadly in DBA diagnosis, including its use in patients with mutations in 40S ribosomal
protein genes, such as the frequently mutated \( RPS19 \) gene, are also currently under investigation.

These studies also demonstrate that pre-rRNA processing studies can be used to guide gene discovery as genome-wide approaches are adopted more broadly to identify novel genes in DBA. We present data on one patient whose affected gene is currently unknown that clearly demonstrates a defect in the maturation of 60S rRNA. We would predict that candidate genes identified should be consistent with this functional study. In addition, we have identified a patient with no discernible pre-rRNA processing defect. This patient likely joins an emerging group of DBA patients with mutations in genes that code for proteins with functions that do not directly impact on the biogenesis of either the 40S or 60S ribosomal subunit.

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