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Exploiting Pre-rRNA Processing in Diamond Blackfan Anemia Gene Discovery and Diagnosis

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Abstract

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

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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.

Keywords

Diamond Blackfan anemia; rRNA; ribosomal protein; diagnostic hematology; inherited bone marrow failure; *RPL31*

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 twelve 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, 612527, 105650, 610629, 613309, 603633, 612561, 612562, 615550, 614900, and 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 non-ribosomal 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 ribosome synthesis whereas others may not. In principle, it should be possible to functionally categorize those with ribosomal defects even when the gene is unknown or if 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

polycistronic 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 on chromosome 2 encompassing *RPL31,* a gene encoding a protein of the 60S ribosomal subunit which has not been previously linked to DBA [20]. The effects of decreased expression of this gene on ribosome synthesis and erythroid differentiation validate *RPL31* as a new DBA gene. Importantly, these studies also revealed a novel feature of the 60S subunit pre-rRNA processing pathway that can be readily evaluated in diagnostic laboratories and therefore be a useful adjunctive test in establishing a diagnosis in as many as 20-25% of patients with DBA.

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 putative deletion was confirmed in the proband, and excluded in the parents, by metaphase FISH analysis of peripheral blood using probe RP11-352P9. 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 (Hemoglobin SS, SC, or S-β 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. Concanavalin 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 and ethidium bromide stained gels were visualized. For Northern analysis, gel-fractionated RNA was transferred to zeta-probe membranes (Bio-Rad). Oligonucleotide probes complementary to ITS1 and ITS2 were labeled with ^{32}P using T4 polynucleotide kinase. Probes were hybridized overnight with membranes at 37° C in ULTRAHyb-Oligonucleotide hybridization buffer (Ambion). Membranes were washed at 37° C with 6XSSC and subjected to phosphorimaging analysis.

For the Bioanalyzer studies, 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. Peak integration was performed using 2100 Expert (Agilent Technologies) software in fragment analysis mode using software-defined 18S and 28S peaks; the 32S peak was manually selected as the minor peak just larger than 28S rRNA. Interpretation was performed blinded to the identities of the individuals studied. Between-group 32S pre-rRNA data were analyzed using a two-tailed Mann Whitney test.

Lentiviral Vector Construction

Oligonucleotides encoding *RPL31* shRNA [TRC Clone 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 (2×10^6) 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 insert) using lipofectamine and OPTI-MEM (Invitrogen). After 6 hrs the transfection medium was changed. Virus containing supernatant was collected after 48 hrs, pooled, filtered through a 0.45μm 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 (2×10^5 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 hours, fresh media was added containing 1.5 μg/mL of puromycin. The media was replaced after 48 hours in selection and cells were harvested for RNA analysis at 96 hours 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 (1.5×10^5 cells/well) for 12 hrs (hour 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 hours (hour 36 following plating), fresh proliferation media was added containing 1.5 μg/mL of puromycin. After 48 hours in selection (84+ hours 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.

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 analyzed 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.

RPL31 Mutation and Deletion Analysis

RPL31 target regions were amplified and directly sequenced by PCR from peripheral blood DNA with primers as shown in supplementary table 1 on a 3730xl DNA analyzer (Applied Biosystems). Detected variants were submitted to NCBI dbSNP under batch ID RPL31-R109_ISDP1-20100921. SNP array for copy number analysis of peripheral blood DNA was performed as previously reported [7] and appended to the NCBI/Gene Expression Omnibus series record, GSE31575. Confidence intervals were calculated by the modified Wald method.

Results

Identification of Copy Loss Involving RPL31

A female infant of Hispanic and Caucasian ancestry presented at four 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 $3 \times 10^5 – 8 \times 10^5$/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 (**Figs. 1A** and **1B**). 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 5.2/0.47 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 (*SBDS*). Nine ribosomal protein genes known to be involved in DBA and available for clinical testing were sequenced, revealing only 2 synonymous single nucleotide polymorphisms, one each in *RPS19* and *RPL11*. Red blood cell 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 three 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/μL) with granulocyte colony-stimulating factor administered at 5 mcg/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 3 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 (**Supplementary Fig. S1**). We identified a prominent increase in 32S pre-rRNA in the proband 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 (**Supplementary 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 (**Supplementary 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 is it 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 compared to that of the proband (**Figs. 2C** and **2D**). 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 (**Supplementary Fig. S2A**). Erythroid differentiation is markedly inhibited in hematopoietic progenitors following *RPL31* mRNA knockdown (**Supplementary 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 (**Supplementary 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 (**Supplementary 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 Supplementary Materials (**Supplementary 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 pre-rRNA is evident in these patient-derived cells by ethidium bromide staining of RNA fractionated by agarose electrophoresis (**Fig. 3A**). We reasoned that this result could be of diagnostic utility if it were generalizable to other patients harboring mutations in ribosomal proteins of the 60S subunit.

We examined a total of 6 patients with mutations in *RPL5* and 2 patients with mutations in *RPL11* (**Supplementary Table S3**), the most frequently mutated DBA genes encoding 60S subunit ribosomal proteins, estimated to cause 7 and 10% of all DBA cases, respectively [29]. We also analyzed one patient with a mutation in *RPL35A*, a less commonly mutated DBA gene. Elevated levels of 32S pre-rRNA are evident by ethidium bromide staining in all of these patient samples. Figure 3B shows the results from two independent specimens from patients with mutations in *RPL5,* both of which show elevated levels of 32S pre-rRNA.

Representative data for a larger series of patients with DBA, some harboring mutations in known DBA genes and other cases where the genetic lesions remain to be identified, are illustrated (**Fig. 3C**). The patient with a mutation in *RPL11* clearly shows elevated levels of 32S pre-rRNA evident by ethidium bromide staining as well as Northern blotting. The same is true for sample DBA2, from a patient whose gene is presently unknown, but clearly shows elevated levels of 32S pre-rRNA indicating a defect in the maturation of 60S ribosomal subunits. The two remaining samples include a patient with a mutation in *RPS26* and a second sample, DBA1, obtained from a patient whose gene is also presently unknown. The *RPS26*-mutated sample does not show a detectable increase in 32S pre-rRNA, but rather shows an accumulation of 18SE-pre-rRNA, as was previously shown for patients with mutations in *RPS26* [4]. DBA1, in contrast, shows no evidence of a processing defect affecting either ribosomal subunit in these analyses.

To date we have only identified a single patient with a 60S subunit ribosomal protein gene alteration (*RPL15*) that did not exhibit an increase in 32S pre-rRNA (**Supplementary Figs. S4A** and **S4D-E**). This result for *RPL15* was confirmed using shRNA to reduce *RPL15* expression in K562 cells (**Supplementary Figs. S4B-C** and **S4F**)*.* Our results for this patient are consistent with results recently published data for the index *RPL15* DBA patient where an increase in 32S pre-rRNA was not evident [12].

A Clinically Feasible Screening Assay for 60S Subunit Ribosomal Protein Gene Abnormalities

While agarose gels and ethidium bromide staining are routinely used in research laboratories, they are challenging to quantitate and unwieldy for clinical application. However, rRNAs are routinely quantified as a proxy for total RNA integrity for numerous downstream applications using capillary electrophoresis technology, suggesting a method by which these observations could be translated into a clinically tractable diagnostic assay. We reasoned it should also be possible to quantify the increase in 32S pre-rRNA that was observed in patients with DBA secondary to large ribosomal gene alterations.

We evaluated ConA-stimulated PBMC RNA from seven DBA patients with 60S subunit gene defects, five with 40S subunit defects and 19 non-DBA individuals. A minor peak corresponding to 32S rRNA was discernible in Bioanalyzer trace data from all but 2 non-DBA specimens, with a prominent 32S peak evident in DBA samples with a 60S subunit gene alteration (**Fig. 4**). The ratio of 32/28S rRNA was significantly higher in the 60S gene DBA group (mean 0.058, 95% confidence interval (CI) 0.047 – 0.069) than the 40S gene group (mean 0.017, 95% CI 0.004 – 0.027) or the control group (mean 0.009, 95% CI 0.006 – 0.012).

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 non-erythroid 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 18SE 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 (**Supplementary 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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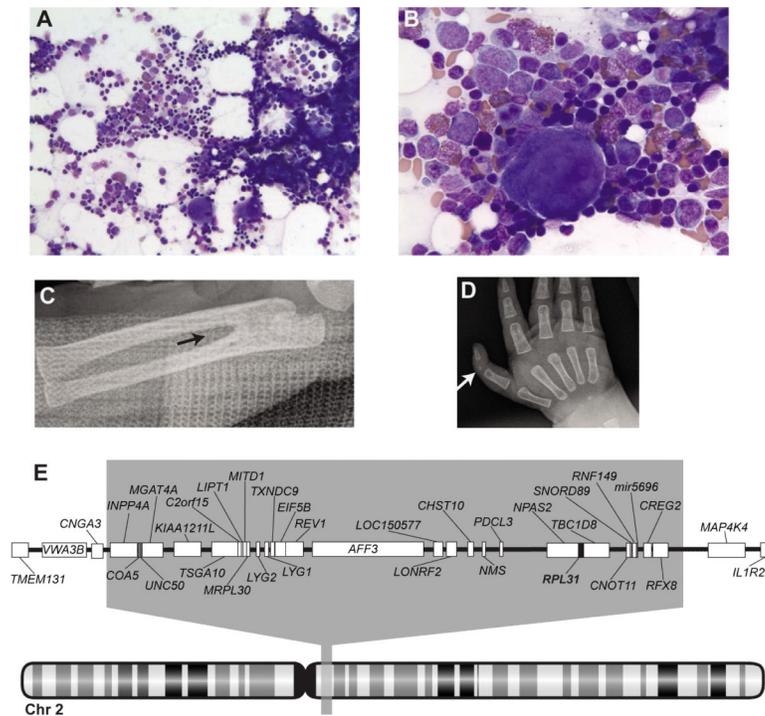
Figure 1.
Photomicrographs of bone marrow aspiration films from the proband obtained at 6 months of age demonstrate marrow that is hypocellular for age (50-60% cellularity) with increased numbers of morphologically normal megakaryocytes, erythroid aplasia, myeloid hypoplasia with maturation arrest, eosinophilia and lymphocytosis (**A**, original magnification 200X and **B**, original magnification 600X). Plain film radiographs demonstrate proximal radioulnar synostosis (**C**) and right triphalangeal thumb (**D**). The 2q11.2 deletion identified in the proband is illustrated (**E**). The involved region, extending from 99,028,236 – 102,253,369 (hg19), is displayed relative to chromosome 2 and enlarged above, with annotated RefSeq genes indicated by white boxes and labeled by gene symbol. A large ribosomal subunit gene, *RPL31* (black box, middle right), is included in the deleted region.
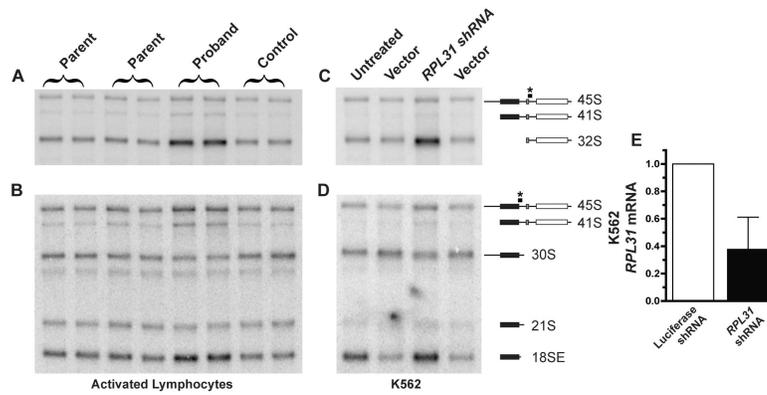
Figure 2.
Northern blot analysis of pre-rRNA processing was performed on primary peripheral blood mononuclear cells from the proband, her parents, and a normal unaffected individual after culture for 96 hours in ConA (**A** and **B**) and on K562 erythroleukemia cells (**C** and **D**) after transduction with lentivirus expressing shRNA to *RPL31* or luciferase (E). Membranes were interrogated with probes against ITS2 (**A** and **C**) and ITS1 (**B** and **D**), shown as * above schematic views of the pre-rRNA species to the right. In comparison to parents and a normal control, proband lymphocytes demonstrated a marked increase in 32S pre-rRNA abundance (**A**), indicative of a large subunit processing defect, as well as increased 45S and 18SE pre-rRNA, and diminished 30S pre-rRNA, suggesting involvement of small subunit processing (**B**). A similar pattern of abnormalities results from *RPL31* knockdown in K562 cells (**C** and **D**), indicating that *RPL31* haploinsufficiency alone is sufficient to produce these pre-rRNA processing abnormalities. Error bars indicate the SEM.
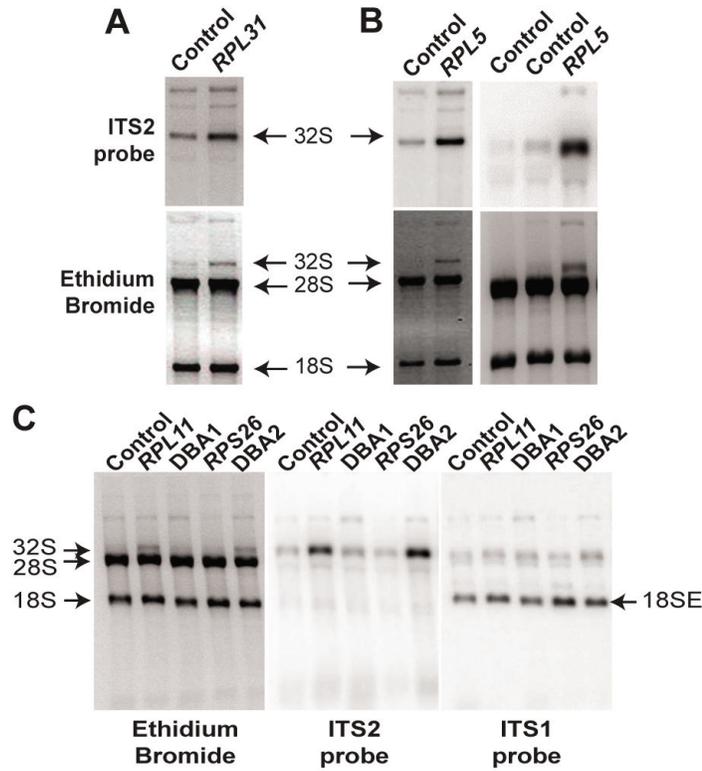
Figure 3.
Elevated levels of 32S pre-rRNA, which are clearly evident by Northern analysis using a probe to ITS2 in *RPL31*-deleted proband RNA (**A**, upper panel), are also evident on visualization of the ethidium bromide-stained RNA gel (**A**, lower panel) from peripheral blood mononuclear cells after culture for 96 hours 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. RNA was extracted from peripheral blood mononuclear cells after culture for 96 hours in ConA. Control lanes consist of RNA obtained from unaffected parents of the DBA proband (Panel A & B) or non-DBA healthy donors (Panel C).
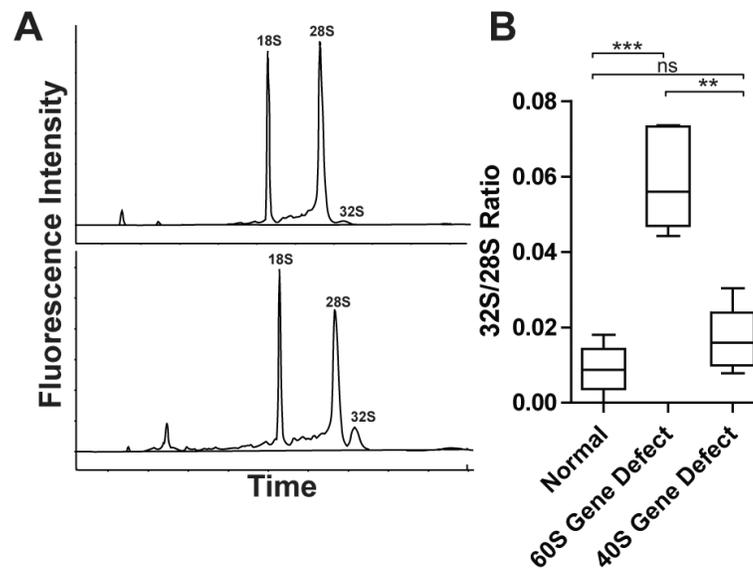
Figure 4.
Representative Agilent Bioanalyzer 2100 electrophoretogram data demonstrates prominent peaks corresponding to 18S and 28S rRNA, with a minor peak just larger than 28S rRNA corresponding to 32S pre-RNA, that was evident in analysis of total RNA from ConA-stimulated peripheral blood mononuclear cells (**A**). In comparison to non-DBA specimens (not shown) and DBA specimens with 40S gene alterations (**A**, upper panel), the 32S peak in DBA patients with 60S gene abnormalities is prominent (**A**, lower panel). The 32S/28S ratio in 7 tested DBA samples with 60S abnormalities is significantly higher than in 19 normal and 5 DBA specimens from patients with 40S subunit abnormalities (**B**). The two-tailed P-value of Mann Whitney, *** = 0.0001, ** = 0.0025, ns = 0.076.