Published in final edited form as:

Hum Mutat. 2012 July ; 33(7): 1037–1044. doi:10.1002/humu.22081.

Frameshift mutation in p53 regulator *RPL26* is associated with multiple physical abnormalities and a specific pre-rRNA processing defect in Diamond-Blackfan anemia

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Abstract

Diamond-Blackfan anemia (DBA) is an inherited form of pure red cell aplasia that usually presents in infancy or early childhood and is associated with congenital malformations in ~30-50% of patients. DBA has been associated with mutations in nine ribosomal protein (RP) genes in about 53% of patients. We completed a large scale screen of 79 RP genes by sequencing 16 RP genes (*RPL3*, *RPL7*, *RPL8*, *RPL10*, *RPL14*, *RPL17*, *RPL19*, *RPL23A*, *RPL26*, *RPL27*, *RPL35*, *RPL36A*, *RPL39*, *RPS4X*, *RPS4Y1*, and *RPS21*) in 96 DBA probands. We identified a *de novo* two-nucleotide deletion in *RPL26* in one proband associated with multiple severe physical abnormalities. This mutation gives rise to a remarkable ribosome biogenesis defect that affects maturation of both the small and the large subunits. We also found a deletion in *RPL19* and missense mutations in *RPL3* and *RPL23A*, which may be variants of unknown significance. Together with *RPL5*, *RPL11*, and *RPS7*, *RPL26* is the fourth ribosomal protein regulating p53 activity that is linked to DBA.

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Supporting Information for this preprint is available from the Human Mutation editorial office upon request (humu@wiley.com)

Keywords

Diamond-Blackfan anemia; ribosomal protein genes; *RPL26*; ribosome biogenesis

INTRODUCTION

Diamond-Blackfan anemia (DBA; MIM# 105650) is an inherited form of pure red cell aplasia [Diamond LK, 1938] that usually presents in infancy or early childhood and affects approximately 5 to 7 children per million live births, equally in both genders [Ball et al., 1996; Campagnoli et al., 2004; Willig et al., 1999b]. The disease is most often characterized by a mild-to-severe macrocytic anemia, a normocellular bone marrow with selective erythroid hypoplasia, and occasional neutropenia and/or thrombocytosis [Alter and Young, 1998; Vlachos et al., 2008]. Laboratory findings such as increased mean corpuscular volume (MCV), elevated erythrocyte adenosine deaminase activity (eADA), and hemoglobin F (HbF) are observed in a majority of DBA patients [Glader et al., 1983; Vlachos et al., 2008; Willig et al., 1999a]. Growth retardation and congenital anomalies, especially of the head, neck, upper limbs and urinary system, are present in approximately 30-50% of patients, indicating that DBA is a broad disorder of development [Alter and Young, 1998; Ball et al., 1996; Vlachos et al., 2008; Vlachos et al., 2001; Willig et al., 1999b]. Although most cases are sporadic, about 40-45% are familial and show autosomal, and commonly dominant inheritance [Orfali et al., 2004]. The anemia is initially responsive to prednisone in approximately 60-70% of patients; however, to maintain normal erythropoiesis, a majority of these patients require continued low dose steroid treatment [Alter and Young, 1998; Vlachos and Muir, 2010]. Patients who do not respond to steroid therapy are usually transfusion-dependent, although responses to androgen and Interleukin-3 (IL-3) have been reported [Ball et al., 1995; Dunbar et al., 1991; Gillio et al., 1993]. Bone marrow transplantation can be curative for transfusion-dependent patients if a histocompatible bone marrow donor is available [Alter, 1998; Vlachos et al., 2008; Vlachos and Muir, 2010]. Reports in the literature reveal an increase in the incidence of malignancies, particularly acute myelogenous leukemia and solid tumors, in patients with DBA [Aquino and Buchanan, 1996; Janov et al., 1996; Lipton et al., 2001; van Dijken and Verwijs, 1995].

DBA has been associated with heterozygous mutations in nine ribosomal protein genes; in six small subunit ribosomal protein genes *RPS7* (MIM# 612563), *RPS10* (MIM# 603632), *RPS17* (MIM# 180472), *RPS19* (MIM# 603474), *RPS24* (MIM# 602412), and *RPS26* (MIM# 603701), and in three large subunit ribosomal protein genes *RPL5* (MIM# 612561), *RPL11* (MIM# 612562), and *RPL35A* (MIM# 180468) [Cmejla et al., 2007; Doherty et al., 2010; Draptchinskaia et al., 1999; Farrar et al., 2008; Gazda et al., 2006; Gazda et al., 2008]. Mutations in these genes have been reported in about 53% of DBA patients indicating that DBA is a disorder of ribosomal biogenesis and/or function. All these ribosomal protein genes are necessary for production of the small or the large subunits. Mutations in DBA usually result in haploinsufficiency of the protein, which causes a defect in ribosome biogenesis that can be detected in patients' total RNA [Choesmel et al., 2007; Doherty et al., 2010; Farrar et al., 2008; Flygare et al., 2007; Gazda et al., 2008; Idol et al., 2007]; there is evidence for a dominant negative mechanism that may explain the pathogenesis in some patients with point mutations [Devlin et al.]. DBA is to date the only congenital disease associated with ribosomal protein gene mutations, while haploinsufficiency for ribosomal protein gene *RPS14* has been implicated in the pathophysiology of the 5q syndrome, a subtype of acquired myelodysplastic syndrome [Ebert et al., 2008].

The pathophysiological mechanisms of DBA remain elusive. Perdahl *et al.* demonstrated increased apoptosis in DBA erythroid progenitor cells [Perdahl et al., 1994]. Increased

apoptosis has also been shown in hematopoietic cell lines and in bone marrow cells with deficiency of RPS19 and RPL35A [Farrar et al., 2008; Miyake et al., 2008]. Haploinsufficiency of ribosomal proteins in mice does not recapitulate the hematological phenotype observed in humans, but rather induces various phenotypes which are suppressed by impairment of p53, suggesting that imbalance of the p53 family proteins is involved in DBA [McGowan et al., 2008]. Similar observations were made in zebrafish treated with morpholinos specific to *RPS19*, which displayed abnormal embryogenesis and anemia [Danilova et al., 2008]. Interestingly, it has been demonstrated that erythroid progenitor cells may be more affected by p53 activation upon decreased expression of the *RPS14* and *RPS19* genes, as compared with other hematopoietic lineages [Dutt et al., 2011], which provides a rationale for the specific sensitivity of this lineage in DBA. Several ribosomal proteins can up-regulate p53 activity by sequestering Hdm2, the E3 ubiquitin ligase that targets p53 to the proteasome, or by favoring p53 translation [Chen et al., 2007; Dai and Lu, 2004; Dai et al., 2004; Lohrum et al., 2003; Ofir-Rosenfeld et al., 2008; Zhang et al., 2010]. Haploinsufficiency of a ribosomal protein perturbs pre-rRNA maturation and incorporation of neo-synthesized ribosomal proteins into nascent pre-ribosomes, thereby increasing the pool of free ribosomal proteins available to stimulate p53 activation. In addition, defects in ribosome production may also stimulate the synthesis of ribosomal proteins [Fumagalli et al., 2009].

Here, we present the completion of the systematic sequencing of the ribosomal protein genes in a cohort of 96 patients. This work adds a tenth ribosomal protein gene mutated in a DBA, namely *RPL26* (MIM# 603704), and identifies rare polymorphisms of uncertain pathogenic significance in three other genes. *RPL26* mutation leads to a global pre-ribosomal RNA (pre-rRNA) maturation defect, which mainly affects formation of the large subunit, but also perturbs processing of the precursors to the 18S rRNA. Interestingly, RPL26 is a positive regulator of p53 activity, like RPL5, RPL11 and RPS7, which are also mutated in DBA.

MATERIALS AND METHODS

Patients

Ninety-six families participated in the study, the majority of whom were included in previous reports [Doherty et al., 2010; Farrar et al., 2008; Gazda et al., 2006; Gazda et al., 2008]. Twelve of them were multiplex DBA families, and 84 comprised of only one affected individual. Informed consent was obtained from all patients and their family members under a protocol at Children's Hospital Boston (Boston, MA, USA). The diagnosis of DBA in all probands was based on the findings of normochromic anemia, elevated erythrocyte adenosine deaminase activity (eADA), reticulocytopenia and a low number or lack of erythroid precursors in the bone marrow, often associated with congenital malformations. None of the 96 probands was previously found to have a pathogenic mutation and one proband had a variant of unknown significance in *RPS15* [Gazda et al., 2008].

DNA isolation and genomic DNA sequencing

Genomic DNA was isolated from blood samples using the Nucleic Acid Isolation System QuickGene – 610L (AutoGen, Inc., Holliston, MA, USA) according to the manufacturer's instructions. To complete our large scale screen of RP genes in a DBA population, genomic DNA samples from 96 unrelated DBA probands enrolled in the study were amplified by polymerase chain reaction (PCR) and sequenced for mutations in 16 RP genes: *RPL14*, *RPL7*, *RPL8*, *RPL35*, *RPL19*, *RPL23A*, *RPL26*, *RPL27*, *RPL17*, *RPS21*, *RPL3*, *RPL10*, *RPL36A*, *RPL39*, *RPS4X* and *RPS4Y1*, on chromosomes 3, 8, 9, 17, 18, 20, 22, X and Y. In total, together with the 63 RP genes sequenced previously [Doherty et al., 2010; Farrar et al., 2008; Gazda et al., 2006; Gazda et al., 2008; Gazda et al., 2004], we screened all 79 RP

genes in this cohort. Primers were designed using Primer3 software to amplify the coding exons and intron/exon boundaries of the above genes. PCR products, between 200 and 600 base pairs, were robotically prepared using a Hamilton MicroLab StarPLUS (Hamilton Company, Reno, NV), purified using Exo-SAP reagent (USB Corporation, Cleveland, OH) and sequenced using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The chromatograms were analyzed using Sequencher software version 4.7 (Gene Codes Corporation, Ann Arbor, MI). Duplicate, independent PCR products were sequenced to confirm the observed nucleotide changes in the probands. DNA samples from at least 200 control individuals, i.e. 400 chromosomes were sequenced to determine whether the observed sequence variations were nonpathogenic variants. Subsequently, DNA samples from family members were sequenced to determine whether the mutation co-segregated with the DBA phenotype within the pedigree.

Tissue culture and RNA isolation

Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCLs) were cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.292 mg/ml L-glutamine (all from Invitrogen, Carlsbad, California) at 37°C in 5% CO₂. Total RNAs were isolated from LCLs using RNA isolation kit (Qiagen, Valencia, CA, USA).

Western blotting

Western blotting of protein lysate from lymphoblastoid cell lines from the six family members with the *RPL19* mutation was performed as previously described [Gazda et al., 2004]. The RPL19 wt and mutated proteins were detected with the rabbit polyclonal anti-RPL19 antibody (ProteinTech Group, Inc, Chicago, IL) and anti-rabbit IgG (H+L) HRP conjugated antibodies (Bio-Rad, Hercules, CA). Proteins were visualized by enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL) on the Gel Doc XR + System (Bio-Rad) using Quantity One 1-D Analysis Software (Bio-Rad). The p53 and GAPDH proteins were detected in lymphoblastoid cell lines from control individuals and *RPL26* mutated patient using rabbit polyclonal anti-p53 and anti-GAPDH antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-rabbit IgG (H+L) HRP conjugated antibodies (Bio-Rad, Hercules, CA). The proteins were visualized as above.

Cell culture and siRNAs

Human cervical carcinoma HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1 mM sodium pyruvate (Invitrogen, Paisley, UK). The 21 nt siRNA duplexes with a 3' dTdT overhang corresponding to L3 mRNA (5'-GACCATGGAGGAGAAGAAA-3'), L19 mRNA (5'-GACCAATGAAATCGCCAAT-3'), L26 mRNA (5'-GTCCAGGTTTACAGGAAGA-3') and L11 mRNA (5'-AAGGTGCGGGAGTATGAGTTA-3'), were purchased from Eurogentec (Seraing, Belgium) and diluted to 100 µM. After washing the cells once in DMEM (without FCS), 10 µL of the siRNA solution were added on ice to 10⁷ HeLa cells in suspension in 200 µL of the same medium. Electro-transformation was performed at 250 V and at 975 µF with a Gene Pulser (BioRad, Hercules, CA) in a cuvette with a 4-mm inter-electrode distance. The cells were diluted in 20 mL DMEM supplemented with FCS and plated in a 140-cm² Petri dish. Control samples were electro-transformed with a scrambled siRNA or without siRNA, which lead to similar results.

Analysis of ribosomes by sucrose density gradient centrifugation

Forty-eight hours after transfection, HeLa cells were treated with 100 µg/ml cycloheximide (Sigma) for 10 min. The cells were successively washed at 4°C in the presence of cycloheximide with DMEM, PBS, and Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl). They were then mechanically disrupted with a Dounce homogenizer in Buffer A containing 0.5 mM DTT. After centrifugation (4°C, 1000×g, 10 min), the upper cytoplasmic fractions were kept. Fractions containing 1 mg proteins were loaded on 10-50% (w/w) sucrose gradients, prepared with a Gradient Master former (BioComp Instruments, Fredericton, NB, Canada). The tubes were centrifuged at 4°C and at 36 000 rpm for 105 min in a SW41 rotor (Optima L100XP ultracentrifuge; Beckman Coulter, Villepinte, France). The gradient fractions were collected at OD_{254 nm} with a Foxy Jr. gradient collector (Teledyne Isco, Lincoln, NE).

Extraction and analysis of pre-rRNAs by Northern blot

Total RNAs were purified from transfected HeLa cells with the Trizol reagent. After alcohol precipitation, RNA pellets were dissolved in formamide, quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and diluted to 1 mg/mL. RNAs (3 µg/well) were separated on a 1% agarose gel prepared with Tri/Tri buffer (30 mM tri-ethanolamine, 30 mM tricine, pH 7.9) containing 1.2% formaldehyde, and run in Tri/Tri buffer at 140 V. Alternatively, small pre-rRNA species were analyzed on 6% denaturing polyacrylamide gels [Gazda et al., 2008]. RNAs were then transferred to a Hybond N+ nylon membrane (GE Healthcare, Orsay, France) and fixed by UV-cross-linking. Membranes were hybridized as described previously [O'Donohue et al., 2010]. The probes used in the present study were 18S (5'-TTTACTTCTCTAGATAGTCAAGTTCGACC-3'), 5'ITS1 (5'-CCTCGCCCTCCGGGCTCCGTTAATGATC-3'), ITS1-721 (5'-GGAGCGGAGTCCGCGGTG-3'), ITS2 (an equal amount of 5'-CTGCGAGGGAACCCCCAGCCGCGCA-3' and 5'-GCGCGACGGCGGACGACACCGCGGCGTC-3'), 5'ITS2 (5'-GGGGCGATTGATCGGCAAGCGACGCTC-3'), 5.8S (5'-CAATGTGTCTTGCAATTAC-3'), 28S (5'-CCCGTTCCCTTGCTGTGGTTTCGCTAGATA-3') and 7SK (an equal amount of 5'-CATGGAGCGGTGAGGGAGGA-3' and 5'-GTGTCTGGAGTCTTGGAAGC-3'). After hybridization, the membranes were washed twice for 10 min at room temperature in 2× SSC, 0.1% SDS, and once in 1× SSC, 0.1% SDS. Labeled RNA signals were acquired with a FLA2000 PhosphorImager (Fuji, Stamford, CT) and quantified with the ImageGauge software.

Accession Numbers

The GenBank accession number for human RPs are: NM_000987.3 for *RPL26*, NM_000981.3 for *RPL19*, NM_000967.3 for *RPL3* and NM_000984.5 for *RPL23A*. In all genes presented here, nucleotide numbering reflects cDNA numbering with position +1 corresponding to the A of the ATG translation initiation codon. Mutation descriptions on the protein level consider the initiator methionine as codon 1 and have been checked using the Mutalyzer program (<http://www.LOVD.nl/mutalyzer/>). Variants were submitted to an *RPL26* mutation database (<http://www.lovdl.nl/RPL26>).

RESULTS

DNA sequencing analysis

We completed our large scale screen of 79 RP genes in our DBA patient cohort by sequencing 16 RP genes, *RPL14*, *RPL7*, *RPL8*, *RPL35*, *RPL19*, *RPL23A*, *RPL26*, *RPL27*, *RPL17*, *RPS21*, *RPL3*, *RPL10*, *RPL36A*, *RPL39*, *RPS4X* and *RPS4Y1*, on chromosomes 3, 8, 9, 17, 18, 20, 22, X and Y for mutations in 96 DBA probands. We identified a *de novo* heterozygous sequence change in *RPL26* in one proband with severe multiple physical malformations and responsive to steroid treatment (both patient's parents were tested and they are mutation free). The sequence change is the deletion of two nucleotides in exon 2 of the *RPL26* gene (c. 120_121delGA) causing a frameshift at codon 40 and premature termination codon (p.Lys41ValfsX12). We also found a deletion of two nucleotides in exon 6 in the *RPL19* gene (c. 562_563delTT) in one proband who is transfusion-dependant, was born with cleft palate and has a short stature. This sequence change causes a frameshift at codon 188 and a new stop codon 21 amino acids behind the wt stop codon (p.Leu188IlefsX30). We also sequenced DNA samples from this proband's clinically unaffected parents and three siblings (with normal MCV and eADA), and identified the same sequence change in the mother and the brother. In addition, we identified two missense changes in two genes: *RPL3* (c.32A>G) in a steroid-dependent proband, causing His11Arg substitution, and *RPL23A* (c.296T>C) in another steroid-dependent proband and her transfusion-dependent brother, resulting in Ile99Thr substitution. The same sequence changes were found in the first proband's clinically unaffected father and second proband's clinically unaffected mother, respectively.

Case report (the patient with the RPL26 frameshift mutation)

A 31 year-old primigravida presented at 30 weeks gestation for evaluation of an abnormal prenatal scan. Pre-natal imaging showed an absent left kidney and foreshortened arms bilaterally with abnormal angulation of the wrists. Possible diagnoses of Fanconi anemia, VACTERL and thrombocytopenia absent radius were considered. A female infant was delivered via C-section at a local hospital at 37 weeks gestation weighting five pounds five ounces, and was noted to have an isolated bilateral cleft palate, an absent external auditory meatus on the right, and a narrowed meatus on the left. In addition, she had a bicuspid aortic valve and agenesis of the left kidney. On the right side, there was a single bone in the forearm, likely an ulna, and, on the left, synostosis of a shortened radius and ulna. Both thumbs were absent, and she had three digits on each hand. The lower eyelid on the left was incomplete. The baby was anemic at birth with a hemoglobin of 8.5 g/dl and was transfused twice in the perinatal period. A DEB test for Fanconi anemia was negative. The child was discharged home. By seven weeks of age the child was noted to again be anemic and profoundly neutropenic with a hemoglobin of 4.1 g/dl, an absolute neutrophil count (ANC) of 0.2k/ μ l, and an absolute reticulocyte count of 57/ μ l. A bone marrow examination showed a cellularity of 40% with markedly decreased erythropoiesis with only rare erythroblasts and maturing forms with megaloblastoid changes. There were marked granulocytic hypoplasia with left shifted maturation and increased blasts (9%). In light of the significant neutropenia in addition to anemia, the patient received a trial of steroids with good response, and the steroids were weaned as tolerated. The child has not required a transfusion since she was three months of age, and is maintained on a minimal dose of steroids at the age of three and a half years, with occasional neutropenia. The child continues to grow parallel to the standard growth curve for both height and weight, but is just below the fifth percentile for both at age three and a half years. Height is 90 cm (fiftieth percentile is 98 cm) and weight 12 kg (fiftieth percentile is 15 kg). Her weight for height is between the fifth and tenth percentile.

RPL19 protein analysis in lymphoblastoid cell lines from the family with c. 562_563delTT

We performed Western blotting using protein lysates from lymphoblastoid cell lines from the proband with sequence change, c. 562_563delTT, in *RPL19* and his five first-degree relatives (his clinically unaffected mother and brother also carry the same sequence change). This sequence change causes a frameshift at codon 188 and a new stop codon 21 amino acids behind the wt stop codon (p.Leu188IlefsX30). Thus, the aberrant protein (216-amino-acid long with estimated molecular weight ~ 25.6 kDa) is predicted to be 20 amino acids longer than the wt protein (196-amino-acid long with estimated molecular weight ~ 23.47 kDa). As predicted, we found, in addition to the wt RPL19 protein, the mutated protein approximately ~2 kDa larger than the wt protein in all three individuals with the sequence change but not in the rest of the family (Figure 1).

Polysome analysis and pre-rRNA maturation assays in families with mutations in *RPL26*, *RPL19* and *RPL3*

The 18S, 5.8S and 28S rRNAs are the end products of the maturation of the pre-ribosomal RNA (pre-rRNA) through endo- or exonucleolytic processing steps. The order of these processing events may vary, leading to the formation of different intermediates (Figure 2). All of the ribosomal proteins involved in DBA so far have proved essential in ribosome production, and pre-rRNA processing defects can be detected in patient cells.

In order to determine the role of RPL3, RPL19 and RPL26 in pre-rRNA maturation and 60S subunit production, we designed siRNAs to knock down their synthesis in HeLa cells. Using quantitative PCR, we found that the siRNAs effectively reduced the amount of the mRNAs encoding the three proteins by 90%, 94% and 96% respectively. Interfering with the synthesis of any of the three proteins resulted in a strong defect in 60S subunit production: in sucrose gradient analysis of cytoplasmic ribosomes, the amount of free 60S subunits dropped, together with the 80S monosomes and the polysomes (Figure 3A). In addition, shoulders in the polysome peaks indicated the presence of half-mers, which correspond to 40S subunits positioned on start codons that fail to recruit 60S subunits. Pre-rRNA analysis by Northern-blot revealed three different phenotypes (Figure 3B). Upon RPL3 knockdown, we observed a general decrease of the amount of pre-60S specific RNAs and the presence of an unusual precursor migrating between the 32S and the 12S pre-rRNAs. No evidence for this phenotype was found in the *RPL3*^{+/mut} patient (Figure 3C), suggesting that this mutation did not strongly affect RPL3 function in ribosome biogenesis. RPL19 depleted HeLa cells accumulated precursors to the 28S and 5.8S rRNAs, especially the 32S pre-rRNA (Figure 3B). Accordingly, we observed higher levels of 32S pre-rRNA in lymphoblasts from the RPL19^{+/mut} patient (Figure 3C and 3F), whereas the mother and the brother harboring the same mutation in RPL19 did not display this phenotype. This result indicates that DBA is linked to a ribosome biogenesis defect in this patient, but that the mutation of RPL19 is not sufficient to explain this phenotype. Interestingly, the C-terminus of RPL19 extends out of the 60S subunit and is not involved in the interaction of this protein with ribosomal RNA [Ben-Shem et al., 2010] (Supp. Figures S1A and S1B). Finally, depletion of RPL26 with siRNAs in HeLa cells resulted in diminished accumulation of 32S pre-rRNA (Figure 3B, top panel). Interestingly, the pattern of the precursors to the 18S rRNA was also significantly altered, with lower proportions of 30S and 21S pre-rRNAs and a high level of 41S pre-rRNA (Figure 3B, lower panel). In addition, a probe hybridizing to the middle of the ITS1 (ITS1-721 probe) showed an increase in 36S pre-rRNA, as well as another precursor running just below (Figure 3D, arrows). This doublet was also visible with the ITS2 probe (Figure 3B). We interpret this peculiar phenotype as a defect in ITS1 cleavage at site 2, which normally separates the initial 90S pre-ribosomal particle into pre-40S and pre-60S particles. Strikingly, this doublet was found in the RPL26^{+/mut} patient (Figure 3E). The 41S/21S pre-rRNA ratio was also much higher in this patient (Figure 3E and 3F), consistent with

the pattern observed in RPL26 depleted cells (Figure 3D). This unique phenotype, not observed in the other patients or in controls, indicates that RPL26 function in ribosome biogenesis is affected.

DISCUSSION

Sequence changes in four RP genes, *RPL26*, *RPL19*, *RPL3*, and *RPL23A*, were identified in four DBA probands. In the case of *RPL19*, *RPL3*, and *RPL23A*, the mutations were found in clinically unaffected family members as well. The *RPL26* gene is located on chromosome 17 and contains four exons with the start codon in exon 2. *RPL26* encodes a 145-amino-acid long RPL26 protein, a component of the 60S ribosomal subunit. The *de novo* frameshift mutation (c. 120_121delGA) identified here is associated with severe multiple physical abnormalities and is predicted to create a 51-amino-acid long truncated protein which is unlikely to associate with the nascent ribosomal subunits. RPL26 is essential for the synthesis of the large ribosomal subunits: knockdown of RPL26 expression in HeLa cells resulted in low levels of free 60S subunit, formation of half-mers in the polysomes and a decrease in the amount of the precursors to large subunit rRNAs. Interestingly, depletion of RPL26 also has a significant impact on the 18S rRNA maturation pathway: pre-rRNA processing analysis by Northern blot strongly suggests a defect in cleavage of the ITS1 at site 2 that is not observed upon depletion of other large subunit ribosomal proteins mutated in DBA [Choesmel et al., 2008; Farrar et al., 2008]. This unique defect in the maturation of ribosomal RNA was unambiguously detected in the patient mutated in *RPL26*, while it was not observed in cells from the patient's unaffected father. These findings indicate that the identified mutation in *RPL26* is most likely a pathogenic mutation and causes DBA in the patient. This frameshift mutation in *RPL26* presumably results in haploinsufficiency of the protein. First, the premature termination codon due to frame-shift is expected to induce nonsense mediated decay and prevent translation. Second, any synthesized protein would be missing around 90 amino-acids that ensure multiple contacts with the rRNA [Ben-Shem et al., 2010] (Supp. Figure S1C) and should be essential for RPL26 incorporation into the nascent ribosomal particles.

Remarkably, RPL26 was described as a regulator of p53 activity [Chen and Kastan, 2010; Ofir-Rosenfeld et al., 2008; Takagi et al., 2005; Zhang et al., 2010]. After RPL5, RPL11 and RPS7 [Gazda et al., 2008], RPL26 is yet another ribosomal protein involved in p53 regulation that is mutated in DBA. RPL26 binds the 5'-UTR of p53 mRNA and stimulates its translation [Chen and Kastan, 2010; Ofir-Rosenfeld et al., 2008; Takagi et al., 2005]. More recently, it was also proposed to be a negative regulator of Hdm2 [Zhang et al., 2010], the E3 ubiquitin ligase that promotes p53 degradation, as shown before for RPL5, RPL11 and RPS7. It is of note that this mutation of *RPL26* is associated with severe skeletal defects, while patients with mutations in *RPL5* and *RPL11* also display developmental anomalies, especially cleft palates and triphalangeal thumbs. It is currently thought that the activation of p53 in response to a ribosomal stress caused by ribosomal protein haploinsufficiency is central to DBA physiopathological mechanisms. Indeed, we verified that p53 levels are higher in RPL26 mutated cells than in control cells (Supp. Figure S2). How loss of function of positive regulators of p53 fits with this hypothesis still calls for an answer. Redundant signaling pathways may be involved, as illustrated by the different modes of p53 regulation involving RPL11, which regulates p53 stability, and RPL26, which modulates both p53 synthesis and stability.

In contrast, sequence changes in *RPL19*, *RPL3*, and *RPL23A* are present in the probands and in their clinically unaffected family members. The *RPL19* gene is located on chromosome 17 and contains six exons with the start codon in exon 1. It encodes a 196-amino-acid long RPL19 protein, a component of the 60S ribosomal subunit. The frameshift

mutation (c. 562_563delTT) was identified in one proband and in his clinically unaffected mother and brother. This sequence change causes a frameshift at codon 188 and a new stop codon 21 amino acids behind the wt stop codon (p.Leu188IlefsX30). The mutation is predicted to result in the creation of a mutated protein 20 amino-acids longer than the wt protein. Accordingly, we detected a ~2kD larger aberrant protein in the proband, as well as in his clinically unaffected mother and brother who carry the mutation, but not in his father and two sisters who are mutation free. The wild-type and mutated forms were detected in similar amounts, which indicates that they are equally stable. This strongly suggests that the mutated form is incorporated into ribosomal subunits like wild-type RPL19 and might be functional. Interestingly, pre-rRNA processing of the large subunit is affected in the proband, but not in his mother and his brother carrying the *RPL19* mutation. The abnormal pre-rRNA processing in the proband resembles the pattern of the rRNA processing in RPL19 depleted HeLa cells, but is also similar to the defects in DBA cells with *RPL5* and *RPL11* mutations [Gazda et al., 2008]. This patient's DNA was screened by direct sequencing for mutations in all 79 RP genes, and no mutation was found in any of these genes including *RPL5* and *RPL11*, although a large deletion in one of these genes in the proband cannot be excluded as it would not be detected by direct sequencing. Silent mutations in relatives of DBA patients were reported before in the case of RPS19 [Willig et al., 1999a]. However, these mutated, but unaffected, individuals displayed high levels of eADA, while the mother and the brother carrying the mutation in *RPL19* display normal levels of ADA and a normal MCV. Therefore, we conclude that the frameshift mutation (c. 562_563delTT) in *RPL19* is most likely a rare variant of unknown significance (not seen in 200 control individuals) that does not strongly affect RPL19 function. It is possible however that RPL19 mutation contributes to the phenotype in combination with a second mutation still to be found in the proband, or that a modifier gene suppresses *RPL19* mutation pathogenic effect in the two other family members with the *RPL19* mutation. We identified a missense mutation in the *RPL3* gene (c.32A>G) in one proband and his clinically unaffected father causing His11Arg substitution. The *RPL3* gene is located on chromosome 22 and comprises two isoforms, which contain ten exons. In both isoforms the start codon is located in exon 1. Isoform a encodes a 403-amino-acid, and isoform b, a 354-amino-acid long RPL3 protein, a component of the large ribosomal subunit. While RPL3 depletion in HeLa cells strongly affected the processing and the stability of the large subunit precursors, we did not detect similar defects in cells from the patient and his father, which indicates that the mutation does not affect RPL3 function in the ribosome assembly process. This suggests that the identified mutation in *RPL3* is most likely a rare variant of unknown significance. We also found a missense mutation in the *RPL23A* gene (c.296T>C) resulting in Ile99Thr substitution in one proband and in her clinically unaffected mother. The *RPL23A* gene is located on chromosome 17 and contains 5 exons with the start codon in exon 1. *RPL23A* encodes a 156 amino-acid long protein, a component of the large ribosomal subunit. This missense change in *RPL23A* was not identified in ~200 control samples, and the family has one more child with DBA who has the same missense change, therefore this sequence change may be a pathogenic mutation present in the patient, her brother and her clinically unaffected mother. However, analysis of the *RPL23A* missense mutation (c.296T>C) was predicted to be benign when analyzed using PolyPhen software (<http://genetics.bwh.harvard.edu/pph/>).

In summary, we completed large-scale sequencing of 79 ribosomal protein genes and identified a frameshift *de novo* mutation in *RPL26* in one proband. This report brings the total number of known DBA associated RP genes to ten: *RPS19* (~25% of the patients), *RPS24* (~2%), *RPS17* (~1%), *RPL35A* (~3.5%), *RPL5* (~6.6%), *RPL11* (~4.8%), *RPS7* (~1%), *RPS10* (~6.4%), *RPS26* (~2.6%), and *RPL26* (~1%) [Doherty et al., 2010]. In total, these mutations account for approximately 53.9% of DBA patients. Characteristic defects in pre-rRNA maturation observed in cells from patients indicate that mutations in these genes

have a direct effect on the protein function in ribosome biogenesis. We also found seven rare variants in *RPS15*, *RPS27A*, *RPL3*, *RPL9*, *RPL19*, *RPL23A* and *RPL36* [Doherty et al., 2010] which could not be convincingly linked to pre-rRNA maturation defects. For example, as described above, pre-rRNA processing was normal in the family members with mutations in *RPL3* and *RPL19*. Thus, without an alternative molecular phenotype to evaluate the loss-of-function of a ribosomal protein, these sequence changes can only be classified as rare variants of unknown significance. Further work is needed to understand whether these rare variants contribute in any way to the pathological mechanism of DBA, possibly in combination with other gene modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Marie Arturi for stimulating DBA research collaboration; and all of the physicians and DBA patients for participating in the study.

Grant Sponsor This work was supported by grants from the Diamond-Blackfan anemia Foundation (HTG), The Manton Center for Orphan Disease Research (HTG), NIH R01HL107558 (HTG), NIH R01AR044345 (AHB), NIH R01HL079571 (AV, EA, JML), the Centers for Disease Control and Prevention (AV), the Pediatric Cancer Foundation (JML) and the Agence Nationale de la Recherche (ANR-RIBOCRASH program) (PEG). DNA sequencing was performed by the Children's Hospital Boston Program in Genomics and the Molecular Genetics Core Facility supported by the Developmental Disabilities Research Center (NIH P30 HD18655) and the Harvard Neuromuscular Disease Project (NIH P50 NS040828).

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Figure 1. Detection of RPL19 mutant form by Western blot

Immunoblot shows RPL19 protein in all family members (with and without deletion (c. 562_563delTT) in the *RPL19* gene). RPL19 mutant protein was detected in the probands and his clinically unaffected mother and brother with *RPL19* deletion. Mut, mutation; Pt, patient; M*, mother; B*, brother; F, father; Sa, sister a; Sb, sister b.

Figure 2. Pre-rRNA processing in human cells

The early 47S precursor is processed along different pathways depending on the order of the cleavages. Given the higher abundance of 30S pre-rRNA relative to the 41S species, cleavage of the ITS1 at site 2 appears to occur most frequently before processing of the 5'-ETS. Arrowheads indicate the pre-rRNA endonucleolytic cleavage sites, although some processing steps involve exonucleases rather than endonucleases. Dashed lines indicate the hypothetical pathway induced by RPL26 depletion.

Figure 3. Pre-rRNA processing in DBA patients

(A) Sucrose gradient analysis of cytoplasmic ribosomes extracted from HeLa cells treated with siRNAs against RPL3, RPL19 and RPL26. The decrease of the free 60S ribosomal subunit and the formation of half-mers (arrows) indicate a strong defect in 60S subunit production. (B) Northern blot analysis of pre-rRNAs in HeLa cells upon depletion of RPL3, RPL19 and RPL26. siRNAs targeting RPL11, another 60S subunit protein involved in DBA, were used as a comparison [Doherty et al., 2010]. Precursors to the 18S rRNA are detected with the 5'-ITS1 probe, while the ITS2 probe reveals precursors to the 60S subunit RNAs. Arrowheads indicate the 36S pre-rRNA and a shorter precursor detected in RPL26 depleted cells (see panel D). (C) Northern blot analysis of pre-rRNAs in DBA patients and their relatives. RNAs were extracted from lymphoblastoid cells. Controls correspond to non-DBA individuals. RNAs from a DBA harboring a mutation in RPL11 were included as a comparison. These results were reproduced on three independent northern blots. (D) A probe hybridizing to the core of the ITS1 reveals accumulation of 36S pre-rRNA together with a shorter precursor (arrowheads). (E) A similar doublet is detected in cells from the *RPL26* mutated patient. Similar results were obtained on two independent northern blots. (F) Quantification of the Northern blot data by phosphorimager. For each ratio, the values were divided by the means of the values obtained for the controls. Measurements were performed three times on independent Northern blots with similar results, except for the 36S/18S ratio which was only measured on one blot. This ratio takes into account both the 36S pre-rRNA and the band migrating right below (see panel E).