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Novel deletion of *RPL15* identified by array-comparative genomic hybridization in Diamond-Blackfan anemia

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Abstract

Diamond-Blackfan anemia (DBA) is an inherited red blood cell aplasia that usually presents during the first year of life. The main features of the disease are normochromic and macrocytic anemia, reticulocytopenia, and nearly absent erythroid progenitors in the bone marrow. The patients also present with growth retardation and craniofacial, upper limb, heart and urinary system congenital malformations in ~30–50% of cases. The disease has been associated with point mutations and large deletions in ten ribosomal protein (RP) genes *RPS19*, *RPS24*, *RPS17*, *RPL35A*, *RPL5*, *RPL11*, *RPS7*, *RPS10*, *RPS26* and *RPL26* and *GATA1* in about 60–65% of patients. Here we report a novel large deletion in *RPL15*, a gene not previously implicated to be causative in DBA. Like *RPL26*, *RPL15* presents the distinctive feature of being required both for 60S subunit formation and for efficient cleavage of the internal transcribed spacer 1 (ITS1). In addition, we detected five deletions in RP genes in which mutations have been previously shown to cause DBA: one each in *RPS19*, *RPS24*, and *RPS26*, and two in *RPS17*. Pre-ribosomal RNA processing was affected in cells established from the patients bearing these deletions, suggesting a possible molecular basis for their pathological effect. These data identify *RPL15* as a new gene involved in DBA and further support the presence of large deletions in RP genes in DBA patients.

Keywords

Diamond-Blackfan anemia; ribosomal protein gene deletion; *RPL15*; ribosomal biogenesis

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Conflict of interest

The authors declare that they have no conflict of interest.

Introduction

Diamond-Blackfan anemia (DBA) (MIM# 105650) is an inherited rare red blood cell aplasia that usually presents during the first year of life. The main features of the disease are normochromic and macrocytic anemia, reticulocytopenia, and nearly absent erythroid progenitors in the bone marrow (Vlachos et al. 2008). These patients have growth retardation and ~30–50% have craniofacial, upper limb, heart and urinary system congenital malformations (Ball et al. 1996; Lipton et al. 2006; Willig et al. 1999). The majority of patients have an increased mean corpuscular volume (MCV), elevated erythrocyte adenosine deaminase activity (eADA), and persistence of hemoglobin F (Glader and Backer 1988; Vlachos et al. 2008; Willig et al. 1999). However, some DBA patients do not have these laboratory findings, adding to the clinical heterogeneity of the disease. Even in the same family, symptoms can vary between affected family members, despite possessing the same genetic mutation. The incidence of DBA is estimated to be 5–7 per million live births, equally distributed between genders (Ball et al. 1996; Campagnoli et al. 2004; Willig et al. 1999). Approximately 45% of patients are familial cases, with disease inherited mostly in an autosomal-dominant pattern; the other 55% of patients are sporadic cases (Orfali et al. 2004).

DBA has been associated with heterozygous mutations in ten ribosomal protein (RP) genes; six small subunit RP genes *RPS7* (MIM# 612563), *RPS10* (MIM# 603632), *RPS17* (MIM# 180472), *RPS19* (MIM# 603474), *RPS24* (MIM# 602412), and *RPS26* (MIM# 603701); and four large subunit RP genes *RPL5* (MIM# 612561), *RPL11* (MIM# 612562), *RPL35A* (MIM# 180468), and *RPL26* (MIM# 603704) (Cmejla et al. 2007; Doherty et al. 2010; Draptchinskaia et al. 1999; Farrar et al. 2008; Gazda et al. 2006; Gazda et al. 2012; Gazda et al. 2008). Mutations in these genes have been reported in ~55% of DBA patients. Mutations in DBA result in haploinsufficiency of the protein, which causes a defect in synthesis of ribosomal subunits and in pre-rRNA maturation that can be detected in patients' RNA (Choesmel et al. 2007; Choesmel et al. 2008; Doherty et al. 2010; Farrar et al. 2008; Flygare et al. 2007; Gazda et al. 2012; Gazda et al. 2008; Idol et al. 2007). These findings strongly suggest that DBA is a disorder of ribosome biogenesis and/or function.

In addition, we recently reported mutations in *GATA1*, a transcription factor required for erythroid differentiation, in three DBA patients (Sankaran et al. 2012).

It was also shown that copy number variation in RP genes may be a cause of DBA in some patients. Previous studies have reported large deletions in *RPS19*, *RPS26*, *RPS17*, *RPL35A*, *RPL11*, and *RPL5* detected by SNP array, quantitative PCR and multiple ligation-dependent probe amplification (MLPA) (Farrar et al. 2011; Kuramitsu et al. 2012; Quarello et al. 2012). In this current study, we aimed to investigate whether deletions or duplications of RP genes and genes potentially involved in ribosomal biogenesis exist in our cohort by performing an array-comparative genomic hybridization (aCGH) analysis on these genes for copy number variation in 87 DBA probands. We found large deletions in six out of 87 probands in five RP genes. Interestingly, one of the deletions was found in *RPL15* (MIM# 604174), a gene not previously implicated to be causative in DBA. In addition to the *RPL15* deletion, we detected five deletions in RP genes in which mutations have been previously associated to cause DBA: one each in *RPS19*, *RPS24*, and *RPS26*, and two in *RPS17*.

Material and Methods

Patients

Eighty-seven unrelated DBA probands participated in this study. The diagnosis of DBA in all probands was based on normochromic, often macrocytic anemia, reticulocytopenia, a low

number or lack of erythroid precursors in bone marrow; and, in some patients, congenital malformations and elevated eADA. All of these samples were previously screened by Sanger sequencing and were negative for mutations in the 10 RP genes known to be mutated in DBA. Informed consent was obtained from all patients and their family members participating in the study under a protocol at Boston Children's Hospital.

Customized aCGH

Genomic DNA was isolated from blood samples using a nucleic acid isolation system QuickGene-610L (Autogen, Holliston, MA) according to the manufacturer's instructions. For aCGH, 136,821 unique probes complementary to ribosomal protein genes and 117 genes shown to be involved in human ribosomal biogenesis or homologous to yeast preribosomal factors (Supplementary Tables 1 and 2) were designed using the UCSC Genome Browser (hg18) database (<http://genome.ucsc.edu/>). Roche Nimblegen Incorporation (Madison, WI) manufactured the aCGH chips. These aCGH chips contained 136,821 probes, whose mean was 30 base pairs and median was 32 base pairs. The tiling design enabled us to detect deletions and duplications larger than 250 base pairs. Genomic DNA samples from DBA probands along with gender-matched controls were independently labeled with Cy3 and Cy5 fluorescent dyes, respectively, using NimbleGen CGH Arrays Protocol. Labeled test and reference DNAs were co-hybridized to NimbleGen 12×136,821 (12 × 136,821 features) CGH arrays using NimbleGen Hybridization System. Following hybridization, the arrays were washed, dried and scanned using Axon GenPix 4200A Scanner. Array CGH data were extracted and analyzed using NimbleScan (Roche Nimblegen) software. We filtered this data with Microsoft Excel and the UCSC Genome Browser (hg18) database using the following criteria: the copy number variant needed to have a log₂ ratio above 0.5 or below -0.5, be larger than 250 base pairs, and contain a protein-coding exon. Copy number variants that met these criteria were visualized using SignalMap (Roche Nimblegen). Deletions and duplications that were detected with fewer than seven probes were removed from our analysis and most likely represented artifacts of the aCGH plates. Lastly, the genomic regions of the deletions and duplications that met our criteria were compared to copy number variants published on DECIPHER (<http://decipher.sanger.ac.uk/>) to rule out deletions and duplications that were polymorphisms.

Multiplex PCR

To confirm the presence of the deletions in the probands, multiplex PCR (mPCR) assays were designed and performed. mPCR assays and mPCR-based approaches have been previously utilized to detect exonal deletions (Beggs et al. 1990; Ceulemans et al.). Primers (Supplementary Table 3) were designed with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify the coding exons and intron/exon boundaries of *RPL15*, *RPS19*, *RPS24*, *RPS26*, and *RPS17*. We compared these RP gene fragments against exons 8 or 55 of *FBN1* as a control reference. We reasoned that pathogenic mutations of *FBN1* leading to Marfan syndrome were unlikely to be present in DBA populations and therefore we chose *FBN1* as a control gene. In total, we designed and utilized 12 different mPCR assays (Supplementary Table 4). For each assay, DNA from three controls and the proband were used. mPCR products were run on a 1.3% agarose gel to visualize the bands and these products were also run and analyzed on the QIAxcel system (Qiagen, Valencia, CA) to quantitate the intensity of the band. The RP intensities were normalized to the *FBN1* band intensity and averaged for the proband and controls. We repeated these assays three times and ran a Student's T-test to determine if the proband's ratio was statistically significant compared to the controls. However, we did not have an available DNA sample to complete our studies for the *RPS19* mPCR assays.

Breakpoint detection

Ultimately, to confirm the presence of the deletion in the proband, we attempted to find the exact breakpoints of these RP deletions. Using the start and end points of the deletions detected by aCGH as a guide, primers pairs were designed where the forward primer would be several hundred bases upstream of the start of the deletion and the reverse primer would be several hundred bases downstream of the end of the deletion. If a PCR product were formed from these primer pairs in only the proband and not in the control samples, it would suggest the presence of a deletion in the proband. Once a unique PCR product formed, it was treated with the reagent ExoSAP-IT (USB, Santa Clara, CA) and sequenced with an Applied Biosystems 3730 DNA Analyzer. The chromatograms were analyzed with the Sequencher 4.8 program (Gene Codes, Ann Arbor, MI).

Copy number assay

The copy number assay was carried out in duplicate using Taqman Copy Number Reference Assay RNase P (Invitrogen, Grand Island, NY) according to manufacturer protocol, using 15 ng of DNA from P5, P6, 3 controls, and the unaffected parents of P5. Three of the five exons of RPS17 (1, 3, and 5) were amplified. A 7300 Real Time PCR System (Applied Biosystems, Grand Island, NY) was used to perform the reaction under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The sequences of the RPS17 primers used were exon 1: forward 5' - TTGCCTGTGCTTCCTGTT-3', reverse 5' -GGCCTCGAGCCAAAACAC-3'; exon 3: forward 5' -GGAGGAGAGAGAAAGGAGAGACAAT-3', reverse 5' - GATTAGCCAGAAGCCCAAATATCCA-3'; and exon 5: forward 5' - TCCATTTTCTCGTTCCAGGACTTC-3', reverse 5' - GCGTTTGTAAATTCATCCCAACTGT-3'. Data obtained using the 7300 Real Time PCR System software were then analyzed using Microsoft Excel to calculate the copy number relative to controls.

Tissue culture and knockdown of *RPL15* with small interfering RNAs

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. To knock down RPL15, 10 µl of a 100 µM siRNA solution (Eurogentec, Seraing, Belgium) was added to the cell suspension (10⁷ cells in serum-free medium) and electro-transformation was performed with a Gene Pulser at 250 V and 950 µF in a 4 mm cuvette (Bio-Rad, Hercules, CA). Two different 21-mer siRNAs were used to knock down expression of RPL15: siRNA *rpl15-2* (5' -UGGUGUUAACCAGCUAAAGdTdT-3') and siRNA *rpl15-3* (5' -UCCAGGAGCUAUGGAGAAAdTdT-3'). Experiments on transfected cells were performed 48 h after transfection of the siRNAs. Control cells were electro-transformed with a scramble siRNA (siRNA-negative control duplex; Eurogentec). Knockdown of the RPL15 mRNA was assessed by qRT-PCR using primers 5' - ACATCCAGGAGCTATGGAGAAA-3' and 5' -CGTAAGTTGCACCCTTAGGAAC-3'.

Analysis of pre-ribosomal RNA processing

Analysis of ribosomes on sucrose gradients and detection of pre-rRNAs by Northern blot were performed as previously described (Gazda et al. 2012; Preti et al. 2013). The probes used in the present study were 18S (5' - TTTACTTCCTCTAGATAGTCAAGTTCGACC-3'), 28S (5' - CCCGTTCCCTTGCTGTGGTTTCGCTAGATA-3'), 5 ITS1 (5' - CCTCGCCCTCCGGCTCCGTTAATGATC-3'), ITS1-721 (5' - GGAGCGGAGTCCGCGGTG-3') and ITS2 (an equal amount of 5' - CTGCGAGGGAACCCCCAGCCGCGCA-3' and 5' - GCGCGACGGCGGACGACACCGCGGCGTC-3').

Results

Our aCGH analysis detected six deletions in RP genes in six out of 87 probands (Table 1). Interestingly, our analysis detected a deletion in region 23935136–23937131 of chromosome 3 in proband 1 (P1) (Fig. 1A) (all genomic coordinates are relative to hg18). This deletion contains exon 4 of *RPL15*. In addition to detecting the *RPL15* deletion, we were able to find deletions in other probands in previously described DBA-associated RP genes, *RPS19*, *RPS24*, *RPS26*, and *RPS17* (Cmejla et al. 2007; Doherty et al. 2010; Draptchinskaia et al. 1999; Gazda et al. 2006) (Table 1). In proband 2 (P2), we found a deletion in region 47056452–47061521 of chromosome 19. This deleted region contains exon 2 and 3 of *RPS19* (Fig. 1B). In proband 3 (P3), our aCGH analysis found a deletion in region 79463528–79465261 of chromosome 10 (Fig. 1C). This deleted region corresponds to exons 1, 2, and partially 3 of *RPS24*. Also, we identified a deletion in region 54721956–54724062 of chromosome 12 in proband 4 (P4) (Fig. 1D). This deleted region encompasses all four exons of *RPS26*. Interestingly, we found two deletions in *RPS17* in our cohort: chromosome 15, 81002660–81005580 in proband 5 (P5) (Fig. 1E); and chromosome 15, 81002660–81005546 in proband 6 (P6) (Fig. 1F). These coordinates contain exons 3, 4, and 5 of *RPS17*.

We validated the deletions we found by performing mPCR assays and showed that the band intensities of PCR products of deleted exons are reduced in the probands (Table 2). For P1, we assessed all four exons of *RPL15* and found only the amount of PCR product of exon 4 is significantly decreased, while the ratios of the remaining exons are similar to our controls. For P3, we tested five exons of *RPS24* and showed that the amount of PCR product is mildly reduced for exon 1, significantly reduced for exons 2 and 3, and not reduced for the remaining exons when compared to the controls. For P4, we examined all four exons of *RPS26* and found the amounts of PCR product for all four exons to be significantly decreased. For P5 and P6, we investigated all five exons of *RPS17*. For P5, we detected that the amounts of PCR product for exons 1, 2, 4, and 5 were significantly reduced, while mildly reduced for exon 3 when compared to controls. For P6, we found that the band intensities of PCR products of exons 2, 3, and 5 are significantly reduced, while those of exons 1 and 4 are mildly reduced compared to controls. Subsequently, we performed mPCRs on the deleted genes on available family members of the probands to determine if their RP gene deletion was inherited from a parent or was a sporadic deletion. For P1, P2, P3, P4, and P5, all family members' ratios appeared to be more similar to the controls than to their affected related proband (Table 3 and Supplementary Table 5). These results indicate that probands P3, P4, and P5 have *de novo* deletions, as their parents' DNA analysis did not show deletions of the *RPS24*, *RPS26* and *RPS17* genes, respectively. DNA from both parents was not available for probands P1, P2 and P6.

Ultimately, to confirm the presence of the deletion in the proband, we attempted to find the exact breakpoints of these RP deletions. Using the PCR method we were able to find the exact breakpoints of the *RPL15*, *RPS24*, and *RPS26* deletion (Table 4). For P1, the *RPL15* deletion was 2,393 base pairs in size and encompassed the region 23,935,161–23,937,553 on chromosome 3. For P3, the *RPS24* deletion was 5,134 base pairs in size and spanned the region 79,460,166–79,465,299 on chromosome 10. For P4, the *RPS26* deletion was 20,456 base pairs in size and located in region 54,711,095–54,731,551 on chromosome 12. Interestingly, by finding the exact breakpoints, we showed that all three deletions were larger than those found by aCGH. The *RPL15* deletion was larger by 397 bps, *RPS24* by 3,401 bps, and *RPS26* by 18,349 bps (Tables 1 and 4).

Since mPCR of all five exons of *RPS17* in probands P5 and P6 showed reduction compared to control samples (by aCGH only exons 3, 4 and 5 were deleted), and because there are 4

functional copies of *RPS17*, we performed a copy number assay on exons 1, 3 and 5 of this gene on DNA samples from P5, P6, three control samples and the unaffected parents of P5. The copy number assay revealed a 50% reduction of *RPS17* DNA in both probands compared to control samples and samples from the parents of P5 (Fig. 2). These results indicate the deletion of 2 out of 4 copies of *RPS17* in both probands, P5 and P6.

Interestingly, the proband P1 (*RPL15* deletion) was diagnosed with anemia at birth, responded to corticosteroid treatment and for the last six years has been in remission. She had a ventricular septal defect and triphalangeal thumbs. The proband P5 (*RPS17* deletion) required red blood cell transfusions every several months between age 6 weeks and 6 years, then entered remission. This proband was also diagnosed with developmental delay. Other probands were either steroid-dependent (P2 (*RPS19* deletion) and P3 (*RPS24* deletion)) or transfusion-dependent (P4 (*RPS26* deletion) and P6 (*RPS17* deletion)). Proband P2 has a webbed neck.

The pathogenic effect of mutations in ribosomal protein genes has been associated with defects in pre-ribosomal RNA processing (Choesmel et al. 2007; Choesmel et al. 2008; Doherty et al. 2010; Farrar et al. 2008; Flygare et al. 2007; Gazda et al. 2012; Idol et al. 2007). Hence, we have previously shown that loss of function of *RPS17*, *RPS19*, *RPS24*, or *RPS26* affects maturation of the 18S rRNA (Choesmel et al. 2007; Choesmel et al. 2008; Doherty et al. 2010; Gregory et al. 2007; O'Donohue et al. 2010). These four proteins are essential for formation of the small ribosomal subunit. Likewise, all ribosomal proteins linked to DBA so far are strictly required for synthesis of one subunit or the other. To assess whether this was also true for *RPL15*, we knocked down *RPL15* in HeLa cells and fractionated the cytoplasmic ribosomes on a sucrose gradient. In parallel, we analyzed the pre-rRNAs by Northern blot. *RPL15* knockdown induced a drop in free 60S subunits and appearance of half-mers in the polysome profile, a phenotype characteristic of defective 60S subunit production (Fig. 3B). This loss of 60S subunits was associated with decreased levels of 32S and 12S pre-rRNA, the precursors to the 5.8S and 28S rRNAs (Fig. 3C, central panel). In addition, we observed accumulation of the 41S, 30S and 18S-E pre-rRNAs relative to the 21S pre-rRNA, suggesting a defect in ITS1 cleavage at site 2 (Fig. 3C, left panel). Moreover, two species migrating above the 30S and the 32S pre-rRNA were detected with probes ITS1-721 and ITS2 (Fig. 3C, middle and right panel). We previously observed these precursors upon depletion of *RPL26*, whose mutation was recently detected in a DBA patient (Gazda et al. 2012), and identified them as the 36S and 36S-C pre-rRNAs (Fig. 3A), the latter being an exonucleolytic processing product of the former (Preti et al. 2013).

We next analyzed RNA from lymphoblastoid cell lines (LCL) established from the DBA patients harboring the large deletions mentioned above. RNA from the patient with the *RPL15* deletion (P1) showed a pre-rRNA pattern close to control cells, including for the 32S and 12S precursors (Figs 3D and 3E). The 41S and the 21S pre-rRNAs were slightly more abundant, suggesting a delay in ITS1 cleavage at site 2, while more 18S-E pre-rRNA was detected, a pattern consistent with *RPL15* knockdown in HeLa cells. But most interestingly, we observed the weak but reproducible presence of a band migrating at the same position as the 36S-C precursors, as shown on the profile of the pattern observed with the ITS1-721 probe (Fig. 3D, right). These data strongly suggest that *RPL15* depletion affects pre-rRNA processing in the patient cells. RNA from the patients mutated in *RPS17* (P5) and *RPS26* (P4) showed higher levels of 21S and 18S-E pre-rRNA, which matches the pattern observed upon knockdown of these proteins (Figs 3D and 3E). In contrast, the deletion in *RPS24* resulted in higher levels of 30S pre-rRNA (Figs 3D and 3E), as expected for i-RPSs (Choesmel et al. 2008; O'Donohue et al. 2010). Therefore, these results support a direct impact of the four deletions on pre-rRNA processing and provide a molecular basis for their pathological effect.

Discussion

We found six deletions of RP genes in six probands from our aCGH analysis of RP genes in our cohort of 87 DBA patients. In contrast, we did not find any copy number variations in the 117 genes related to ribosomal biogenesis that we screened. In total, 7% of our cohort of 87 probands without known RP point mutations contained RP deletions in *RPL15*, *RPS19*, *RPS24*, *RPS26*, and *RPS17*. Since ~50% of DBA probands have point mutations in RP genes, large deletions in our cohort are present in ~3.5% of all DBA patients. This percentage is lower than previously published reports (Farrar et al. 2011; Kuramitsu et al. 2012; Quarello et al.). Strikingly, we found a deletion in the *RPL15* gene for the first time in a DBA patient. The *RPL15* gene is 3,694 base pairs long and located on chromosome 3. *RPL15* is known to have many alternatively spliced transcripts, but the gene typically consists of four exons varying in size. The largest of these alternative transcripts is 2,600 base pairs long and encodes a 204-amino-acid-long *RPL15* protein, a component of the large ribosomal subunit. Usually, the start codon is in exon 2, and the stop codon is in exon 4. The *RPL15* deletion we detected includes intron 3 and exon 4. As a result, any transcript of this *RPL15* variant, despite how it is alternatively spliced, would not have a stop codon. Additionally, the largest alternatively spliced *RPL15* transcript's exon 4 is 1,600 base pairs long and encodes 102 amino acids of the *RPL15* protein. Without exon 4, the *RPL15* transcript is significantly smaller than the wild type *RPL15* transcript; therefore this *RPL15* transcript is most likely prematurely degraded and the *RPL15* deletion is DBA causal in P1.

Depletion of *RPL15* affects pre-rRNA processing in a fashion similar to knockdown of *RPL26*: both proteins are required early in the 60S subunit synthesis pathway, and both are necessary for efficient cleavage of the ITS1 at site 2. Accumulation of the 36S and 36S-C precursors has only been reported for a small subset of proteins so far, which suggests that the two proteins are functionally related. They are both located at close positions in the 60S subunit where they make contact with both the 28S and the 5.8S rRNAs (Supplementary Fig.1). They are likely to associate with large 90S pre-ribosomal particles before cleavage of the ITS1. Interestingly, ITS1 processing is affected in most DBA patients bearing a mutation in a ribosomal protein gene, since it also involves *RPS19*, *RPS26*, *RPS10* and *RPS17*. It is therefore tempting to speculate that a pathological mechanism is linked to this pre-rRNA maturation step in DBA, the mechanism of which remains to be defined.

The other deletions we detected occur in genes previously associated with DBA: *RPS19*, *RPS24*, *RPS26*, and *RPS17*. However, previous studies have found deletions in all of these RP genes except for *RPS24* (Farrar et al. 2011; Kuramitsu et al. 2012; Quarello et al.). Here we show large-scale deletions can also occur in *RPS24*. Interestingly, we detected two large-scale deletions in *RPS17*. Previous studies have also found large deletions in *RPS17* in 10 patients out of 150 studied without RP gene point mutations (7%) (Farrar et al. 2011; Kuramitsu et al. 2012; Quarello et al.). However, there have only been three point mutations described in *RPS17* (Cmejla et al. 2007; Gazda et al. 2008; Song et al. 2010). Large-scale deletions occur often on chromosome 15, where two copies of the *RPS17* gene are situated.

Our data reinforces the presence of large-scale RP deletions in DBA patients. However, about 35–40% of DBA patients are still negative for RP mutations, both on the nucleotide and copy number level. We believe there are still genes yet to be discovered that are mutated in DBA patients. Recently, we identified *GATA1* mutations in two DBA families (Sankaran et al. 2012). *GATA1* is an important transcription factor required for erythroid differentiation. Hence, other genes, such as those encoding transcription factors needed for erythroid differentiation, may be mutated in DBA patients and could elucidate the disease mechanisms behind DBA.

Our current study found six deletions in six DBA probands. This study further supports the need to screen copy number variation in RP genes in DBA patients who are negative for RP gene point mutations. Although many of the RP deletions found before this study have been in DBA-associated RP genes, we believe other RP genes may be deleted in DBA patients as seen by our detection of the *RPL15* deletion. Because of this finding, we propose a total examination of all 80 RP genes for copy number changes in DBA patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Ribosomal protein gene deletions detected by aCGH

Visual images of entire RP genes containing a deletion detected by CGH analysis were made using Nimblegen's SignalMap v1.9 software. A) *RPL15* deletion in P1, B) *RPS19* deletion in P2, C) *RPS24* deletion in P3, D) *RPS26* deletion in P4, E) *RPS17* deletion in P5, F) *RPS17* deletion in P6.

Fig. 2. *RPS17* copy number measured by copy number assay

Copy number at exons 1, 3 and 5 of *RPS17* was measured after normalization to *RNase P* in three control individuals, parents of P5, and in both DBA probands P5 and P6. Probands P5 and P6 showed ~50% reduced copy number at exons 1, 3 and 5 of *RPS17* as compared to 3 control individuals and unaffected parents of P5.

Fig. 3. Analysis of pre-rRNA processing in cells derived from DBA patients

(A) Schematic representation of the human pre-rRNA primary transcript and its maturation intermediates. The endonucleolytic cleavage sites are indicated by arrowheads. The colored bars show the positions of the probes used in this work. The main precursors detected in the rest of the figure are schematized. (B) Polysome analysis on sucrose gradient shows decreased levels of free 60S subunits upon knockdown of RPL15, induced here with two different siRNAs. Half-mers are concomitantly detected in the polysomes (arrows), consistent with a defect in 60S subunit synthesis. Reduction of the *RPL15* mRNA level was more efficient with the with the *rp15-2* siRNA (13% of the level observed with the scramble siRNA) than with the *rp15-3* siRNA (40%), as assessed by qRT-PCR, (C) Northern blot analysis of pre-rRNA processing in RPL15-depleted cells with the 5 ITS1, ITS2 and ITS1-721 probes. The ITS2 and ITS1-721 probes reveal the accumulation of 36S and 36S-C pre-rRNAs. The mature 18S and 28S rRNAs were detected with specific probes.

(D) Pre-rRNA processing in LCL cells established from DBA patients was analyzed with the same probes as in C. A faint band corresponding to the 36S-C pre-rRNA is detected with the ITS1-721 probe in the RPL15 +/-mut cells (P1), while 36S pre-rRNA is detected in the RPS17 +/-mut sample (P5) (arrowheads). This is best seen on the intensity profiles of the upper part of the lanes shown on the left panel. The experiment was reproduced twice with similar results. (E) The Northern blot signals with the 5 ITS1 and ITS2 probes were measured by phosphorimaging, quantified using MultiGauge software and normalized to the 18S rRNA. For each species, the value of the mean of the two control samples was arbitrarily set to 1.

Table 1

Ribosomal Protein Deletions Detected by aCGH

Proband's ID (Gender)	Chromosomal region	Gene	Size (bp)	Deleted exons
P1 (F)	Chr 3: 23935136 - 23937131	<i>RPL15</i>	1996	4
P2 (M)	Chr 19: 47056452- 47061521	<i>RPS19</i>	5070	2 and 3
P3 (M)	Chr 10: 79463528 - 79465261	<i>RPS24</i>	1733	1, 2 and partially 3
P4 (F)	Chr 12: 54721956 - 54724062	<i>RPS26</i>	2107	1, 2, 3 and 4
P5 (M)	Chr 15: 81002660 - 81005580	<i>RPS17</i>	2921	3, 4 and 5
P6 (M)	Chr 15: 81002660 - 81005546	<i>RPS17</i>	2887	3, 4 and 5

Table 2

mPCR Assay Ratios Confirming aCGH Results

Proband's ID	Gene	Exon	Proband's Ratio	Control Ratios	P-value
P1	<i>RPL15</i>	1	1.130±0.014	1.073±0.226	0.681
		2	0.983±0.255	1.214±0.552	0.547
		3	1.176±0.006	1.082±0.099	0.177
		4	0.518±0.088	1.402±0.532	0.047
P3	<i>RPS24</i>	1	0.746±0.309	1.774±0.663	0.072
		2	0.447±0.120	1.054±0.242	0.018
		3	1.207±0.195	2.435±0.210	0.002
		4	1.112±0.250	1.087±0.260	0.910
P4	<i>RPS26</i>	5a	1.449±0.268	1.526±0.220	0.720
		1	0.365±0.131	1.608±0.025	9×10 ⁻⁵
		2	0.426±0.043	1.690±0.060	8×10 ⁻⁶
		3	0.212±0.063	0.620±0.038	7×10 ⁻⁴
		4	0.248±0.039	1.522±0.335	0.003
P5	<i>RPS17</i>	1	1.766±0.416	4.157±0.539	0.004
		2	0.278±0.016	0.630±0.079	0.002
		3	1.339±0.450	2.489±0.793	0.094
		4	1.230±0.389	2.891±0.837	0.036
		5	0.551±0.020	1.181±0.061	7×10 ⁻⁵
P6	<i>RPS17</i>	1	1.572±0.348	2.189±0.389	0.110
		2	1.313±0.088	2.399±0.442	0.014
		3	1.286±0.102	2.357±0.461	0.017
		4	1.005±0.304	1.687±0.467	0.101
		5	0.734±0.205	1.234±0.145	0.026

Table 3

Family Members of Probands P1–P5 with Performed mPCR

RP Gene	Proband	Family Member
<i>RPL15</i>	P1	<i>m</i>
		<i>s</i>
<i>RPS24</i>	P3	<i>f</i>
		<i>m</i>
<i>RPS19</i>	P2	<i>f</i>
<i>RPS26</i>	P4	<i>f</i>
		<i>m</i>
<i>RPS17</i>	P5	<i>f</i>
		<i>m</i>

m, mother; s, sister; f, father

Table 4

Ribosomal Gene Deletions Confirmed by Breakpoint Primer Analysis

Proband's ID	Chromosomal Region	Gene	Size (bp)
P1	Chr 3: 23935161–23937553	<i>RPL15</i>	2,393
P3	Chr 10: 79460166–79465299	<i>RPS24</i>	5,134
P4	Chr 12: 54711095–54731551	<i>RPS26</i>	20,456