Diamond-Blackfan anemia: genotype-phenotype correlations in Italian patients with *RPL5* and *RPL11* mutations

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ABSTRACT

Background

Diamond-Blackfan anemia is a rare, pure red blood cell aplasia of childhood due to an intrinsic defect in erythropoietic progenitors. About 40% of patients display various malformations. Anemia is corrected by steroid treatment in more than 50% of cases; non-responders need chronic transfusions or stem cell transplantation. Defects in the *RPS19* gene, encoding the ribosomal protein S19, are the main known cause of Diamond-Blackfan anemia and account for more than 25% of cases. Mutations in *RPS24*, *RPS17*, and *RPL35A* described in a minority of patients show that Diamond-Blackfan anemia is a disorder of ribosome biogenesis. Two new genes (*RPL5*, *RPL11*), encoding for ribosomal proteins of the large subunit, have been reported to be involved in a considerable percentage of patients.

Design and Methods

In this genotype-phenotype analysis we screened the coding sequence and intron-exon boundaries of *RPS14*, *RPS16*, *RPS24*, *RPL5*, *RPL11*, and *RPL35A* in 92 Italian patients with Diamond-Blackfan anemia who were negative for *RPS19* mutations.

Results

About 20% of the patients screened had mutations in *RPL5* or *RPL11*, and only 1.6% in *RPS24*. All but three mutations that we report here are new mutations. No mutations were found in *RPS14*, *RPS16*, or *RPL35A*. Remarkably, we observed a higher percentage of somatic malformations in patients with *RPL5* and *RPL11* mutations. A close association was evident between *RPL5* mutations and craniofacial malformations, and between hand malformations and *RPL11* mutations.

Conclusions

Mutations in four ribosomal proteins account for around 50% of all cases of Diamond-Blackfan anemia in Italian patients. Genotype-phenotype data suggest that mutation screening should begin with *RPL5* and *RPL11* in patients with Diamond-Blackfan anemia with malformations.

Key words: red cells, bone marrow failure, anemia, DBA, ribosomal proteins.

Citation: Quarello P, Garelli E, Carando A, Brusco A, Calabrese R, Dufour C, Longoni D, Misuraca A, Vinti L, Aspesi A, Biondini L, Loreni F, Dianzani I, and Ramenghi U. Diamond-Blackfan anemia: genotype-phenotype correlations in Italian patients with RPL5 *and* RPL11 *mutations Haematologica. 2010; 95:206-213. doi:10.3324/haematol.2009.011783*

Funding: this work was supported by grants from Banca del Piemonte to UR, PRIN 2006 Ministero Italiano della Ricerca Scientifica e Tecnologica to UR and ID, Regione Piemonte to UR, Gruppo di Sostegno DBA Italia to UR, DBA Foundation to ID, and Telethon Italia Grant GGP07242 to ID.
We thank the Daniella Maria Arturi Foundation for supporting communication among DBA researchers.

Manuscript received on May 27, 2009. Revised version arrived on July 26, 2009. Manuscript accepted on August 5, 2009.

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Introduction

Diamond-Blackfan anemia (DBA, MIM#105650) is a rare inherited congenital bone-marrow-failure syndrome characterized by normochromic macrocytic anemia typically presenting in infancy or early childhood. The bone marrow is normocellular, but erythroid precursors are absent or their numbers markedly decreased, because their progenitors are unable to differentiate and are prone to apoptosis. Laboratory findings such as increased mean corpuscular volume, high erythrocyte adenosine deaminase activity (eADA), and elevated hemoglobin F after 6 months of age are observed in most patients; an increase in eADA may be the only manifestation.[1] The congenital anomalies, mainly involving the head, upper limbs, heart and urogenital system, found in more than one-third of patients reflect the fact that DBA is a broad disorder of development.[2] The range of severity of such malformations is wide, even within the same family.

The genetic basis of DBA is heterogeneous. Approximately 40% of patients have mutations in one of the genes for ribosomal proteins (RP): *RPS7*, *RPS17*, *RPS19*, *RPS24*, *RPL5*, *RPL11*, or *RPL35A*. These genes encode for RP of either the small or the large ribosomal subunit.[3-7] The identification of the role of another *RP* gene, *RPS14*, in the pathogenesis of an acquired myelodysplastic disease, the 5q- syndrome, has recently attracted great interest, since it extends the spectrum of ribosomal diseases.[8]

A clear genotype-phenotype correlation is not apparent in patients with *RPS19* mutations, and identical mutations have been found in patients with a wide range of clinical presentations, even within the same family. No information of genotype-phenotype correlations is so far available for patients with *RPS24*, *RPS17*, or *RPL35A* mutations, because the number of subjects studied are too small.[3-5] On the other hand a correlation between *RPL11* or *RPL5* mutations and hand malformations and cleft lip and/or palate, respectively, has been reported.[6,7]

Here we report the results of screening for six *RP* genes (*RPS14*, *RPS16*, *RPS24*, *RPL5*, *RPL11*, and *RPL35A*) in 92 unrelated Italian patients who were negative for *RPS19* mutations, and a genotype-phenotype analysis for the *RPL5* and *RPL11* genes.

Design and Methods

Patients

One hundred twenty-eight unrelated DBA families were studied. Fourteen had more than one clinically affected individual. The diagnosis of DBA was always based on normochromic, often macrocytic anemia, reticulocytopenia, erythroid bone marrow aplasia or hypoplasia, and, in some patients, congenital malformations and elevated eADA. We excluded short stature because it was difficult to evaluate in the context of severe anemia, iron overload and chronic corticosteroid use.

Informed consent was obtained from all patients and/or their family members participating in the study. *RPS19* mutations were found in 36/128 (28%) unrelated DBA patients using both sequencing and multiplex ligation-dependent probe analysis (MLPA): these data have already been reported.[9,10] Specifically, 33 *RPS19* mutations were identified by sequencing whereas three heterozygous *RPS19* deletions missed by sequencing were found using the MLPA technique.[10]

Molecular analysis of RP genes

Genomic DNA from 92 unrelated Italian DBA probands negative for *RPS19* mutations was isolated from peripheral blood leukocytes using a commercial kit (Gentra Systems, Inc., Minneapolis, MN, USA). We analyzed *RPS24*, *RPL5*, *RPL11* and *RPL35A* because mutations in these genes have been reported in the literature.[3,4,6] We screened *RPS16* because it is involved in binding of initiation factor eIF-2 to the 40S subunit.[11] The *RPS14* gene was screened because it has an important role in erythroid proliferation and maturation.[8] This gene, together with others, is deleted in the 5q- syndrome, an acquired myelodysplastic syndrome which is very different from DBA, but considered a ribosomal disease.[8]

We analyzed these six *RP* genes by direct sequencing of the coding exons and intron-exon boundaries. The primer sequences are available on request. Polymerase chain reaction (PCR) products were purified with the QIAquick purification kit (QIAGEN GmbH, D-40724 Hilden, Germany), and sequenced on both strands with an ABI PRISM BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3100 DNA Sequencer (Applied Biosystems). When sequence changes were found, independent PCR products were sequenced to confirm the mutations.

Subsequently, we sequenced DNA samples from available family members to determine whether the mutation co-segregated with the DBA phenotype within the pedigree. To determine whether these sequence changes were polymorphic variations, we sequenced DNA samples from 100 Italian control individuals, and verified that none was reported in the Single Nucleotide Polymorphism database (dbSNP at *www.ncbi.nlm.nih.gov/SNP*) or in the Ensembl database (*www.ensembl.org*). To verify whether the *RPS24* missense mutation (p.Asn124Ser) was a polymorphism we performed ScrFI enzymatic digestion on samples from 150 Italian control individuals.

The nomenclature used to describe the sequences is in accordance with the Human Genome Variation Society recommendations (*http://www.hgvs.org*).

To ascertain whether the two *RPL5* mutations detected in patient 2 (Table 1) were on the same allele a test based on NlaIII restriction enzyme digestion was set up. The splice site mutations detected in patients 10 and 12 (Table 1) were analyzed with a Splice Site Prediction website (BDGP, Berkeley Drosophila Genome Project, *http://www.fruitfly.org*).

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells (ATCC #CRL-11268) were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2. For transfection, *RPS24* cDNA was reverse transcribed and amplified from total RNA using SuperScript® III (Invitrogen, San Diego, CA, USA). Both variants 1 and 2 (NM_033022 e NM_001026) were obtained and inserted in a pcDNA3.1+ vector (Invitrogen) that contained the neomycin resistance gene and was modified to express a Flag tag at the C-terminus. Site-directed mutagenesis for mutant c.371A>G (p.Asn124Ser) was carried out using PfuTurbo® DNA Polymerase (Stratagene, La Jolla, CA, USA) and the following primers: forward 5'-tgcaaaggccagtgttggtgctg-3', reverse 5'-

cagcaccaacactggcctttgca-3'. A PCR-based strategy was applied to obtain mutant 64_66delCAA, using Platinum®Pfx DNA Polymerase (Invitrogen) and the primers, forward 5'-Phos-atggtcattgatgtccttcaccc-3', reverse 5'-tttcctctgaagtagtcggttggt-3'. All constructs were verified by DNA sequencing. About 2 μg of each plasmid and 20 μL of lipofectamine transfection reagent (Invitrogen, Lipofectamine plus 2000) were used for transfection of HEK293 cells plated at 90% confluence. After 24 h cells were analyzed.

RPS24 protein analysis

To prepare total extracts, cells were washed twice with phosphate-buffered saline (150 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4 and 1.4 mM KH_2PO_4) and treated with lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [SDS], aprotinin 1 mg/mL, leupeptins 1 mg/mL, pepstatin A 1 mg/mL, phenylmethylsulfonylfluoride 100 mg/mL). After 1 min of incubation on ice, the extract was centrifuged for 10 min at maximum speed in a microcentrifuge at 4°C. For fractionation of the extract, cells were lysed in 300 μL of 10 mM Tris HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.05% NP40 with protease inhibitors and centrifuged for 5 min at 1000 g. To prepare the nuclear fraction, the pellet was resuspended in 1.2 mL of 10 mM Tris HCl (pH 7.9), 10 mM NaCl, 5 mM $MgCl_2$, 0.3 M sucrose and layered onto a 2 mL cushion of 0.6 M sucrose. After 10 min centrifugation at 1,000g at 4°C, the pellet was dissolved in SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer as a nuclear fraction. The supernatant of the first centrifugation at 1000 g was either used as cytoplasmic fraction or further purified. To prepare the ribosomal fraction the cytoplasmic extract was layered onto 1 mL of 15% sucrose, 30 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$ and centrifuged in a Beckman type 70.1 rotor for 90 min at 100,000g. The pellet (ribosomal fraction) was resuspended directly in SDS–PAGE loading buffer. The supernatant (free cytoplasmic proteins) was precipitated with 10% trichloroacetate and the pellet, washed with acetone, was resus-

Table 1. ***RPL5*** **mutations.**

Patient (gender)	Malformation status	eADA	Response to first steroid treatment	Status at last follow-up	Mutation	Exon/ intron	DNA mutation	Predicted effect on protein	Inheritance
1 (F)	None	I	R	SD	Missense	Exon 1	c.3G>C	p.Met1?	n.a.
2 (F)	Atrial septal defect, flat thenar eminence, short stature	I	NR	TD	Nonsense/ missense	Exon 6	c.[678C>A; 680T>G]	p.[Tyr226X; Ile227Arg]	n.a.
3 (F)	Cleft lip, cleft palate, short stature	I	R	SD	Deletion	Exon 3	c.134_138delACACA (*)	p.Asn45ThrfsX66	*De novo* (m,f,s normal sequence)
4 (M)	Myelomeningocele, cleft palate, facial dysmorphism	n.a.	n.a.	FU	Deletion	Exon 3	c.169_172delAACA (*)	p.Asn57GlufsX12	*De novo* (m,f normal sequence)
5 (F)	Flat thenar eminence, grouped carpal bones, short stature	n.a.	n.a.	FU	Deletion	Exon 3	c.169_172delAACA (*)	p.Asn57GlufsX12	Sporadic (f,b normal sequence)
6 (M)	Micrognathia, palpebral ptosis, cleft palate, triphalangeal thumb, brachydactyly, learning difficulties, aortic valve stenosis, short stature	n.a.	n.a.	FU	Deletion	Exon 3	c.183_184delTT	p.Ile61MetfsX51	*De novo* (m,f normal sequence)
7 (F)	Cleft palate, micrognathia, triphalangeal thumb	I	R	REMtrt	Deletion	Exon 5	c.336delG	p.Arg112SerfsX14	*De novo* (m,f,b normal sequence)
8 (M)	Facial dysmorphisms, strabism	I	R	SD	Insertion	Exon 2	c.39_40insT	p.Lys14X	n.a.
9 (F)	Cleft palate, persistent foramen ovale	n.a.	n.a.	n.a.	Insertion	Exon 6	c.692_693insT	p.Thr232AsnfsX50	*De novo* (m,f normal sequence)
10 (M)	Palpebral ptosis	n.a.	NR	TD	Splice site	Intron 1	c.3+3G>C	p.?	Familial (m)
11 (M)	Short stature	n.a.	NR	TD	Splice site	Intron 3	c.189+1G>A	del ex3 fs	n.a.
12 (F)	Cleft palate, micrognathia flat thenar eminence, persistent foramen ovale, facial dysmorphisms	I	no therapy	REMs	Splice site	Intron 4	c.324+5G>T	del ex4 in frame	n.a.

F: female; M: male; I: increased value; n.a.: not available; R: responsive to steroid treatment; NR: not responsive to steroid treatment; SD: steroid-dependent; TD: transfusion-dependent; FU: lost at last follow up; REMtrt: remission after treatment, REMs: spontaneous remission, f: father, m: mother, s: sister, b: brother. (*): mutation previously reported.

pended in SDS–PAGE loading buffer. For western analysis, proteins were separated on a 12% SDS polyacrylamide gel, transferred to a nitrocellulose Protran membrane (Schleicher and Schuell), and incubated with the following primary antibodies and antisera: mouse monoclonal anti-RPS19,[12] mouse anti-Flag (Sigma, F3165), rabbit anti-NPT II (Upstate, 06-747), and mouse anti-GAPDH (Millipore MAB374). Primary antibodies were revealed using horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch) and a chemiluminescence detection system (Ablot Plus, Euroclone). Quantitation analyses were performed using the LAS3000 Image System (Fuji) and ImageQuant software (GE Healthcare).

Genotype-phenotype correlations and statistical analysis

Genotype-phenotype correlations were evaluated in the whole group of patients. Differences between two independent samples were checked with Student's t-test or the Mann-Whitney test as appropriate, whereas the Kruskal-Wallis test was used to assess the differences between more than two groups. Associations between categorical variables were assessed with Fisher's exact test or with odds ratio and 95% confidence interval (95% CI). For categorical variables with more than two categories simple logistic regression was used to calculate the odds ratio and 95% CI.

All tests were two-sided and *P* values less than 0.05 were considered statistically significant. Data were analyzed with the SPSS 16 software (SPSS Inc., Chicago, IL, USA).

Results

RPL5

We identified *RPL5* sequence changes in 12 out of the 92 probands (Table 1). These included seven deletions or insertions of one to five nucleotides causing a frameshift, three donor splice-site mutations, one missense and one nonsense mutation. All mutations but one were new mutations not yet described in the literature and were found in one patient only. The four-nucleotide deletion in exon 3 (c.169_172delAACA) detected in two unrelated patients has already been reported.[6,7] *De novo* sequence changes were identified in six probands; one patient (n. 10, Table 1) inherited the *RPL5* mutation (c.3+3G>C) from his healthy mother, who had normal hemoglobin level, mean corpuscular volume and eADA. Parental DNA was not available for the other five patients.

A five-nucleotide deletion in exon 3 (c.134_138delACACA) was found in a female patient (n. 3, Table 1), who also carried a *RPS24* missense mutation

Table 2. *RPL11* mutations.

Patient (gender)	Malformation status	eADA	Response to first steroid treatment	Status at last follow-up	Mutation	Exon/intron	DNA mutation	Predicted effects on protein	Inheritance
1 (F)	Cleft palate, flat thenar eminence	n.a.	NR	TD	Deletion	Exon 2	c.60_61delCT (*)	p.Cys21SerfsX33	n.a.
2 (F)	Short stature	n.a.	R	TD	Deletion	Exon 2	c.60_61delCT (*)	p.Cys21SerfsX33	Familial (m)
3 (M)	Hypospadias, renal ectopy, café au lait spots	n.a.	R	REM HSCT	Deletion	Exon 2	c.74delG	p.Cys25LeufsX9	*De novo* (m,f,s,b normal sequence)
4 (M)	None	n.a.	NR	TD	Deletion	Exon 2	c.95_96delGA	p.Arg32ThrfsX22	n.a.
5 (F)	Hypoplastic thumb, ventricular septal defect	I	n.a.	n.a.	Deletion	Exon 2/Intron 2	c.143_157+32del	del ex 2 fs	n.a.
6 (F)	Bilateral triphalangeal thumbs, persistent foramen ovale	n.a.	R	REM HSCT	Deletion	Intron 2	c.157+1_157+16del	del ex 2 fs	*De novo* (m,f,s normal normal sequence)
7 (F)	Cathie's facies, flat thenar eminence, short stature	I	R	SD	Deletion	Exon 3	c.198_202delAAAGA	p.Glu66AspfsX26	Sporadic (m normal sequence)
8 (F)	n.a.	n.a.	n.a.	FU	Deletion	Exon 4	c.291_302del	p.Asn98_Asp101del	*De novo* (m,f,b normal sequence)
9 (M)	Flat thenar eminence, tetralogy of Fallot, short stature	I	R	TD	Deletion	Exon 4	c.314_315delTT (*)	p.Phe105TrpfsX15	*De novo* (m,f normal sequence)
10 (F)	Septal atrial defect, flat thenar eminence, short staure	I	NR	TD	Deletion	Exon 5	c.465_466delCA ,	p.His155GlnfsX16	*De novo* (m,f sequence)
11 (M)	Bilateral triphalangeal thumbs, cleft palate, micrognathia, short staure	I	R	Exitus	Deletion	Exon 5	c.469delA	p.Ile157SerfsX37	*De novo* (m,f,s normal sequence)
12 (F)	Short stature	n.a.	NR	TD	Splice site	Intron 5	c.508-2A>G	p.?	Familial (f)

F: female; M: male; I: increased value; n.a.: not available; R: responsive to steroid treatment; NR: not responsive to steroid treatment; SD: steroid dependent; TD: transfusion dependent; FU: lost at last follow-up; REM HSCT: remission after hematopoietic stem cell transplantation, f: father, m: mother, s: sister, b: brother. (*): mutation previously reported.

in exon 4 causing a substitution of an Asparagine with a Serine at codon 124 (c.371A>G; p.Asn124Ser). This patient is steroid-dependent, has a cleft palate, short stature and increased eADA. The *RPL5* mutation was *de novo*, while the *RPS24* mutation was found in her healthy father and unaffected sister (Figure 1; Table 1, n. 3).

Patient n. 2 (Table 1) had two mutations on the same allele. The first was a nonsense mutation causing a premature stop codon (p.Tyr226X). The second was a missense mutation involving the adjacent codon 227 (p.Ile227Arg), and caused the substitution of an Isoleucine with an Arginine. Parental DNA was not available for molecular analysis.

The splice site mutations detected in patients n. 10 (c.3+3G>C; Table 1) and n. 12 (c.324+5G>T; Table 1) modified the splice site strength score from 0.97 and 0.98 to 0.32 and 0.23, respectively (BDGP, *http://www.fruitfly.org*). These data suggested splice site suppression in both mutants. We could not perform expression analysis because fresh blood samples from these patients were not available.

RPL11

We identified sequence changes in 12 patients (Table 2). Most mutations (11/12, 92%) were 1-47 nucleotide deletions. An acceptor splice-site mutation was also observed. Nine mutations are described here for the first time; two, including one present in two unrelated probands, had been reported by Gazda *et al.*[6] Parental DNA was available for nine probands; seven had *de novo* mutations. Two probands (n. 2 and n. 12, Table 2) inherited the mutation from their apparently healthy mother and affected father, respectively. This father reported mild anemia during infancy associated with short stature, increased mean corpuscular volume and high eADA; he has never needed any treatment, whereas his daughter is transfusion-dependent, and does not respond to steroids.

RPS24

We found two new heterozygous changes in *RPS24* (2/92). A deletion of three nucleotides in exon 2 (c.64_66delCAA), resulting in the loss of the highly conserved glutamine 22, was identified in a patient without somatic malformations and in clinical remission at last follow-up. This mutation was not found in other family members, nor has it been reported as a polymorphism (*www.ncbi.nlm.nih.gov/SNP*; *www.ensembl.org*).

A missense mutation (p.Asn124Ser) in exon 4 was found in a patient who also carried a five-nucleotide deletion (c.134_138delACACA) in *RPL5* as described above (Figure 1; Table 1 n. 3). This missense mutation was carried by two healthy family members (Figure 1; Table 1, n. 3), but has never been reported as a polymorphism nor was it found in 300 Italian control chromosomes.

Neither *RPS24* mutation predicted a dramatic alteration of the gene product, differently from *RPL5* and *RPL11* mutations. Thus, we decided to study the properties of RPS24 proteins encoded by the mutated genes, using an approach similar to that which we previously used to study *RPS19* missense mutations.[12] We prepared cDNA constructs tagged with the Flag epitope at the C terminus and inserted them into mammalian expression vectors

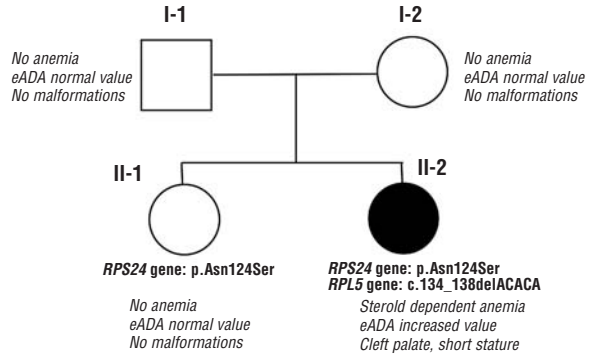

Figure 1. Pedigree of a DBA patient carrying mutations in both the *RPL5* and *RPS24* genes.

(pcDNA3.1). *RPS24* pre-mRNA is alternatively spliced into three different mRNA isoforms that encode proteins with a slightly different C terminus (one or three additional amino acids).[3] We prepared cDNA encoding the two major *RPS24* variants (1 and 2) but, since in a preliminary analysis they showed identical behaviors (*data not shown*), we focused our attention on one protein variant (variant 1). DNA constructs encoding for: (i) wild type RPS24 (WT), (ii) RPS24 with codon 22 deletion (M1), and (iii) RPS24 with the p.Asn124Ser mutation (M2) were used in transient transfection experiments into HEK293 cells. After transfection, cell extracts were analyzed by western blotting to measure protein levels or further purified to investigate the subcellular localization and the capacity to assemble into the ribosome. The levels of WT, M1 and M2 RPS24 were normalized for the amount of neomycin phosphotransferase II expressed by the pcDNA3.1 vector. Assuming that the different RPS24 cDNA have the same transcriptional and translational activity, the results can be considered proportional to the stability of the different proteins. As shown in Figure 2A the levels of both M1 and M2 are clearly lower than that of WT RPS24. This indicates that both mutations affect protein stability. Consistent with the instability of the mutated RPS24, a possible degradation product appeared sometimes on the western blot as a faster-migrating band of variable intensity. Next, we separated nuclear and cytoplasmic fractions from the extract of the transfected cells. Western blot analysis, illustrated in Figure 2B, showed that mutated RPS24 (M1 and M2) accumulated into the nucleus more evidently than did the WT RPS24. Finally, to verify the capacity of the mutated RPS24 to be incorporated into the ribosome, we further fractionated cytoplasmic extracts through ultracentrifugation on a sucrose cushion. After 2 h of centrifugation all ribosomes and ribosomal subunits were found in the pellet whereas free cytoplasmic proteins could be recovered from the supernatant. As an additional control for this experiment, we transfected a plasmid expressing an RPS19 construct (RPS19flag) with the Flag epitope at the N terminus. The RPS19flag fusion protein was previously shown to assemble poorly into the ribosome,[12] possibly as a consequence of the position of the Flag epitope (N terminus).

Western blot analysis, presented in Figure 2C, confirmed that RPS19flag was mostly in the free cytoplasmic fraction. In contrast, both the WT and the mutated RPS24 (M1 and M2) appeared to be mainly associated with the ribosome. This result indicates that, although the mutated RPS24 are less stable and accumulate into the nucleus, a small fraction of them is incorporated into the ribosome and exported into the cytoplasm. The conclusion of our analyses is that the two mutations here analyzed alter some properties of RPS24 in a similar way. However, a fraction of the mutated RPS24 was able to associate with the ribosome.

RPS14, RPS16 *and* RPL35A

No mutations were found in *RPS14*, *RPS16*, or *RPL35A*.

Genotype-phenotype correlations

The clinical features of patients with *RPL5* and *RPL11* mutations are summarized in Tables 1 and 2. The results of the genotype-phenotype statistical analysis performed by logistic regression are presented in Figure 3.

Most of the patients with *RPL5* (83%) and *RPL11* (73%) mutations had physical malformations. Patients with *RPS19* mutations and patients without mutations in any of these three genes showed lower percentages of malformations (43% and 29%). Specifically, the risk of malformation was 12-fold higher in *RPL5*-mutated patients than in patients with no mutations in *RPL5*, *RPL11* or *RPS19* and 7-fold higher in *RPL5*-mutated patients than in *RPS19*-mutated patients. Similarly, *RPL11*-mutated patients had a 6-fold higher and 3.5-fold higher risk of somatic anomalies compared to *RPL5*, *RPL11* and *RPS19* non-mutated and *RPS19*-mutated patients. Moreover, patients with either *RPL5* or *RPL11* mutations more frequently had multiple malformations than did *RPL5*, *RPL11* and *RPS19* non-mutated and *RPS19*-mutated patients.

We also found that craniofacial abnormalities were closely associated with *RPL5* mutations: *RPL5*-mutated patients had a 37-fold and an 8-fold higher risk of craniofacial malformations than *RPL5*, *RPL11* and *RPS19* non-mutated and *RPS19*-mutated patients, respectively. *RPL11*-mutated probands had a higher risk of craniofacial malformation than *RPL5*, *RPL11* and *RPS19* non-mutated patients only. Interestingly, cleft lip and/or palate was observed in 8/111 clinically evaluable patients. None of these eight had a mutation in *RPS19*, whereas all had mutations in *RPL5* or *RPL11*.

A significant association between hand malformations and *RPL11* mutations was also observed: *RPL11*-mutated patients had a 13-fold and a 7-fold higher risk of hand malformation compared to *RPL5*, *RPL11* and *RPS19* non-mutated and *RPS19*-mutated patients, respectively. A similar, though weaker, association was found in *RPL5*-mutated patients compared to *RPL5*, *RPL11* and *RPS19* non-mutated patients; no difference was observed between *RPS19* and *RPL5*-mutated patients.

The risk of cardiac malformations was 5-fold and 6-fold higher in *RPL5* and *RPL11*-mutated patients compared to *RPL5*, *RPL11* and *RPS19* non-mutated patients only. We did not find a correlation between mutational status and response to first steroid treatment or status at last follow-up.

Discussion

For about 10 years, *RPS19* seemed to be the only gene involved in the pathogenesis of DBA and mutations in this gene accounted for 25% of cases.[9] In the last 3 years, however, heterozygous mutations in several genes encoding ribosomal proteins of either the small or the large riboso-

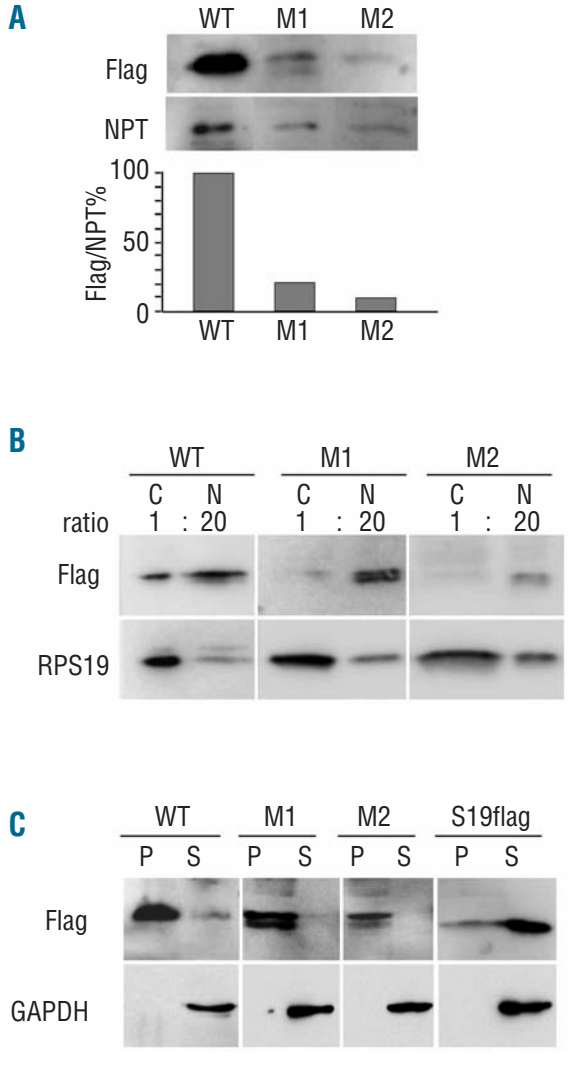

Figure 2. Transient transfection of RPS24 constructs. (A) Total extracts from HEK293 cells transfected with plasmids coding for RPS24 WT (WT) and mutated forms of the protein (M1, M2) were separated by SDS-PAGE and transferred on nitrocellulose membrane. The blots were decorated with anti-Flag (Flag) and anti-neomycin phosphotransferase II (NPT) antibodies. Quantitation of the signals is reported in the lower part as the ratio of Flag/NPT; (B) Total extracts from transfected cells (as in A) were separated into nuclear (N) and cytoplasmic (C) fractions. The loading ratio between cytoplasmic and nuclear extracts was 1:20 (number of cells). Western blotting was performed as in A with anti-Flag (Flag) and anti-RPS19 (RPS19). (C) Cytoplasmic extracts from HEK293 cells transfected as in A were fractionated by ultracentrifugation on a sucrose cushion as indicated in the Design and Methods section. Pellets, which include ribosome and ribosomal subunits (P) and trichloroacetate-precipitated supernatants, which include free cytoplasmic proteins (S) were analyzed by western blot with anti-Flag (Flag) and anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibodies.

mal subunit have been reported.[3-7] Specifically, Gazda *et al.* found mutations in *RPL5* and *RPL11* in about 7% and 5%, respectively, of DBA patients,[6] while Cmejla *et al.* showed a higher frequency of *RPL5* (21%) and *RPL11* (7%) mutations among Czech patients.[7]

We report here the results of our screening of *RPS24*, *RPL5* and *RPL11* in 92 Italian patients who were negative for *RPS19* mutations. No mutations were found in *RPS14*, *RPS16*, and *RPL35A*.

Twenty-eight percent of our patients (36/128) showed a mutation in *RPS19*,[9,10] 9.3% (12/128) in *RPL5*, 9.3% (12/128) in *RPL11*, and only 1.6% (2/128) in *RPS24*. This frequency of *RPL11* mutations was higher than that found in other screening studies. As far as concern *RPL5* mutations, the frequency in our study was similar to that found by Gazda *et al.* but lower than in Czech patients.[6,7] These differences may be due to the populations studied. All but three mutations found in these genes had never been previously described. Interestingly, we found a double heterozygote for two *RP* genes. This patient, a steroid-dependent female with cleft palate, had a *de novo* five-nucleotide deletion in *RPL5* exon 3 (c.134_138delACACA) and a substitution in *RPS24* (p.Asn124Ser). The latter was inherited from her healthy father, and also carried by a healthy sister (Figure 1). Several points support the hypothesis that the *RPL5* mutation is pathogenic: it is a *de novo* frameshift mutation and the malformation phenotype of this patient is typical of that of patients with a *RPL5* mutation. There is, however, controversy on the role of the missense *RPS24* p.Asn124Ser: this mutation is carried by healthy relatives and Asparagine 124 is a non-conserved amino acid. However, a silent phenotype has already been described in DBA patients with *RPS24* mutations.[3] p.Asn124Ser was not found in 300 Italian normal chromosomes and has never been reported as a single nucleotide polymorphism.

To ascertain whether p.Asn124Ser is a mutation or a silent polymorphism we checked whether this mutant was able to reach the nucleolus and be included in active ribosomes. Our studies show that though the mutant is rather unstable as compared with the wild type form, it is able to reach the nucleolus and a small fraction is even able to associate with the ribosome. Altogether, our studies support the following model: the bulk of the DBA phenotype is most probably due to the *RPL5* frameshift mutation, whereas the *RPS24* missense mutation is a phenotype modifier.

Interestingly, similar functional data were found for the in frame *RPS24* mutation: however, in this case, the fact that this is a *de novo* mutation is a strong indication that this deletion has a role in the pathogenesis of the disease.

Overall, our data suggest that all DBA patients, even those with a recognized mutation in *RP* genes, should be screened for other DBA genes to ascertain whether a second mutation is present. A possible digenic effect may explain the variable expressivity and incomplete penetrance in members of the same family.

Incomplete penetrance was shown for *RPL5* and *RPL11* mutations in two families of our cohort (n. 10, Table 1; n. 2, Table 2), as previously described for *RPS19*.[13] All *RPS19* mutations described have been found in heterozygosis, suggesting haploinsufficiency due to a loss-of-function

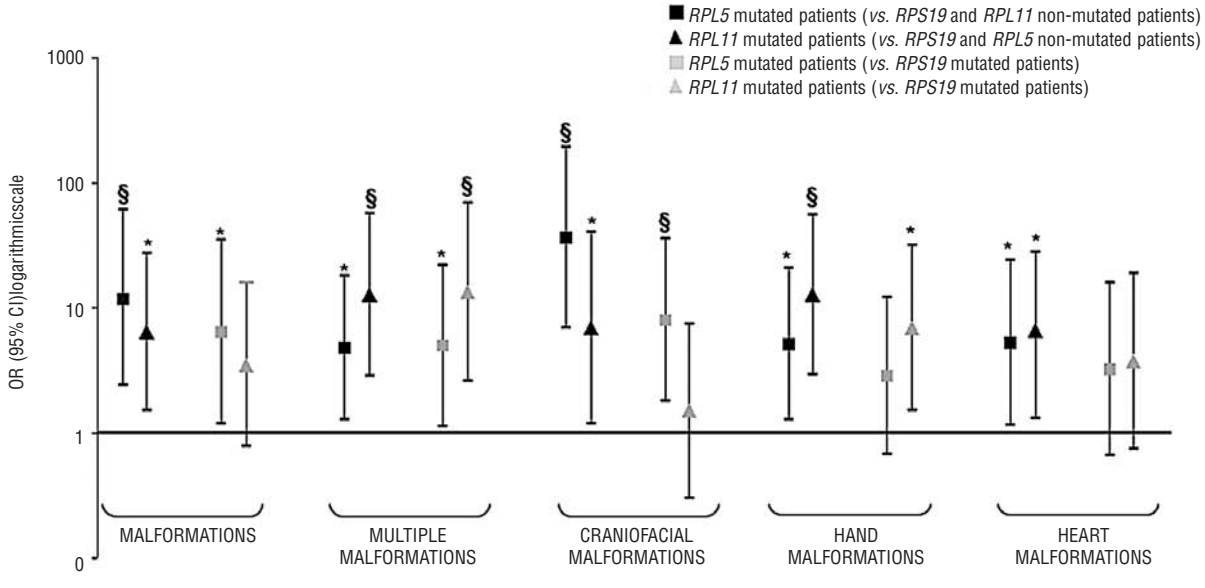

Figure 3. Malformation status of patients with *RPL5* and *RPL11* mutations. Associations between malformation status and *RP* gene mutations were assessed with odds ratio (OR) and 95% CI calculated from logistic regression; OR are drawn on a logarithmic scale. *RPL5* and *RPL11*-mutated patients were compared to both *RPS19*-mutated patients and non-mutated patients. The Wald test was used to test the statistical significance of each association, the *P* value is indicated as (*) *P*<0.05; (§) *P*<0.01.

effect.[9] Similarly, most *RPL5* and *RPL11* mutations cause a premature termination, suggesting that haploinsufficiency is the cause of the disease.

Gazda *et al.* reported that *RPL5* and *RPL11* mutations are more frequently associated with physical malformations than are *RPS19* mutations. *RPL5* mutations seem to cause a more severe malformation status, including craniofacial, thumb, and heart anomalies, compared to mutations in *RPS19*.[6] Remarkably, Gazda *et al.* reported a close association between *RPL5* and cleft lip and/or palate. Mutations in *RPL11* were predominantly associated with thumb abnormalities.[6] Similarly, Cmejla *et al.* observed that all ten Czech DBA patients with either an *RPL5* or an *RPL11* mutation had one or more physical malformations; specifically, hand anomalies were always present and most patients were born small for gestational age.[7] Cleft lip and/or palate was not observed in *RPL5*-mutated and *RPL11*-mutated patients.

We observed a high percentage of multiple somatic malformations in patients with *RPL5* and *RPL11* mutations. Close associations between *RPL5* mutations and craniofacial malformations and between hand malformations and *RPL11* mutations were also evident. However, unlike Gazda *et al.*, we observed cleft lip and/or palate in both *RPL5*- and *RPL11*-mutated DBA patients, and can thus rule out a unique association between *RPL5* mutations and this type of malformation.

In conclusion, we report a high frequency of *RPL5* (9.3%) and *RPL11* (9.3%) mutations in our DBA cohort. Mutations in four ribosomal proteins account for around 50% of all cases of DBA in Italian patients. Genotype-phenotype data suggest that mutation screening should begin with *RPL5* and *RPL11* in DBA patients with a malformation, and specifically in those who have craniofacial aberrations, such as cleft palate, or hand abnormalities.

Authorship and Disclosures

PQ performed genotype-phenotype correlation analyses and drafted the manuscript with UR and ID, who were involved in the conception of the study. UR is the author taking primary responsibility for the paper. EG and AC performed sequencing analyses. AB critically revised the paper and was responsible for important intellectual content. RC was responsible for the statistical analysis. CD, DL, AM and LV contributed clinical data. AA performed cloning. LB and FL performed RPS24 functional experiments. All authors contributed to the revision of the manuscript.

The authors declare no potential conflicts of interest.

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