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Molecular basis of Diamond-Blackfan anemia: new findings from the Italian registry and a review of the literature

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A B S T R A C T

Background. Diamond-Blackfan anemia (DBA) is a rare, pure red blood cell aplasia of childhood caused by an intrinsic defect in erythropoietic progenitors. Malformations occur in about 40% of patients. More than half of patients respond to steroids; non-responders need chronic transfusions or stem cell transplantation (SCT). Mutations in the gene encoding ribosomal protein S19 are found in 25% of patients, but the link with erythropoiesis is unclear. A second DBA locus has been found on chromosome 8p22-p23; analysis of genes of the region is in progress.

Methods and Information Sources. We present clinical and molecular data from 97 Italian DBA patients and a review of the literature.

Results and State of the Art. We describe five new RPS19 gene mutations: four point mutations and one unbalanced chromosomal translocation. Hematologic findings, malformations and outcome are similar in the RPS19 mutated and the non-mutated groups. No genotype-phenotype correlation has been found so far in RPS19 mutated patients. Our data, however, and a thorough review of literature show a worse outcome (expressed as transfusion dependence) in patients with mutations that completely abolish one allele, i.e. gross chromosomal rearrangements and mutations at the initiation codon. The association of mental retardation with large deletions at the 19q locus points to a contiguous gene syndrome. A recurrent missense mutation (Arg62Trp) is associated with transfusion dependence in eight of the nine reported cases.

Perspectives. Nationwide collaboration and population-based registries recording molecular data are essential for the further dissection of this rare heterogeneous disease and the definition of new therapeutic trials.

Key words: Diamond-Blackfan anemia, erythropoiesis, RPS19, mutation.

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Diamond-Blackfan anemia (DBA, MIM: 205900) is a pure red blood cell aplasia of childhood,[1,2] characterized by normocytic or macrocytic anemia, reticulocytopenia, paucity of bone marrow erythroid precursors and, in more than one-third of patients, somatic abnormalities.[1,2] Although DBA is a rare syndrome, it holds an important place in hematology as a paradigm of an intrinsic genetic disorder of the committed erythroid progenitor. Many of its clinical and pathogenic aspects are still unclear even though more than 500 cases have been reported. Its clinical expression, familial history and therapeutic response are protean, and its molecular background is equally heterogeneous. Mutations in the RPS19 gene, whose link with erythropoiesis remains to be clarified, account for about 25% of cases.[3-5] However, a second locus on human chromosome 8p22-p23 has been identified[6] and further loci are probably present.

Useful insights into clinical presentation, response to treatment and outcome have been recently provided by national DBA Registries.[7-9] Since 1995, we have collected the clinical and biological data of Italian DBA patients through nationwide collaboration on the part of pediatric hematology units belonging to the Italian Association for Pediatric Hematology and Oncology Units (AIEOP) and we now have a panel of 97 patients from 91 families. We report our population data and an update of the literature, to review the features and progression of DBA and closely examine patients carrying RPS19 mutations. We also describe five new RPS19 mutations and suggest a relation between complete allele suppression and poor response to treatment.

Epidemiology and inheritance

The incidence of DBA is estimated to range from 5 to 10 cases per million live births in Europe, with the lowest values in the UK and the highest in Northern Europe;[7,8,10] the incidence in Italy is about 6.5 cases per million live births.[11] The clustering of birth months observed by Ball *et al.* in 1996, which suggested virus-related seasonality,[7] has not been confirmed by subsequent population studies.[8,11] The sex ratio is about 1:1.

The vast majority of cases are sporadic;[12,13] familiarity with either an autosomal dominant or, seldom, a recessive pattern of inheritance is reported in 10%-20% of patients.[12,13] In our series of 97 Italian patients, family inheritance is apparent in 16 families out of 91 (17.5%), with an autosomal dominant pattern in ten and an autosomal recessive pattern, defined as the presence of DBA siblings from unaffected consanguineous parents, in six. The male: female ratio is 1:1.04.

Clinical presentation

Hematologic features

All patients with DBA are, by definition, anemic. Anemia is already evident at birth in 25% of cases; in almost all patients (95% in our series) presentation is in the first year of life.[12,13] Red blood cells are usually macrocytic; reticulocyte counts are decreased or zero. In our own series, hemoglobin (Hb) at diagnosis ranged from 2.3 to 10.8 g/dL (mean; 5.7±2.1 g/dL) and mean cell volume (MCV) ranged from 67.3 to 111 fL (mean: 93±9.9 fL).

The other hematologic lineages are not involved as a rule, though slightly abnormal low leukocyte and high platelet counts have been reported at diagnosis.[14,15] Bone marrow aspirates demonstrate isolated erythroblastopenia (erythroblasts <5% of nucleated cells on bone marrow smears) in more than 90% of patients; an alternative uncommon bone marrow pattern is erythroid hyperplasia with maturation arrest; apparently normal numbers and maturation of erythroblasts have been exceptionally described.[13] Bone marrow analysis in our series demonstrated normal cellularity and morphology except for the erythroid line in all cases. Erythroid total aplasia and hypoplasia were found in 93% of patients; three patients presented erythroid maturation arrest with increased numbers of immature precursors and one showed dyserythropoietic morphology. The colony assay for BFU-E demonstrated absent or reduced growth in 83% of patients; addition of stem cell factor (SCF) induced a marked increase of erythroid colonies in all the tested subjects.[11,16]

The activity of erythrocyte adenosine deaminase (eADA), a critical enzyme in the purine salvage pathway, is usually high in DBA patients. In our series, eADA activity was increased in 79% of cases.

Table 1. Diagnostic criteria for DBA (accepted by the DBA working group of the European Society for Paediatric Haematology and Immunology, ESPHI).

- normochromic, often macrocytic anemia developing in the first year of life
- profound reticulocytopenia
- normocellular bone marrow with selective deficiency of erythroid precursors
- normal or slightly reduced leukocyte count
- normal or slightly increased platelet count

Physical abnormalities

Short stature and congenital abnormalities, mainly involving the head, upper limbs, heart and urogenital system, occur in more than one-third of DBA patients.[17] The same dysmorphisms occur in Fanconi's anemia (FA, MIM: 227650); however, FA is characterized by an higher frequency and severity of anomalies and the presence of abnormal cutaneous pigmentation in more than half of patients, which is absent in DBA. Physical abnormalities were detected in 48% of our 97 patients. About 16% of DBA cases displayed growth impairment without dysmorphic features; 32% presented either single or multiple dysmorphisms. The incidence and severity of physical abnormalities were not gender-related in our series, whereas a greater severity of anomalies was found in males by Ball *et al.*[7]

Diagnostic criteria

Diagnosis of DBA is often difficult because incomplete phenotypes and a wide variability of clinical expression are present;[3] requirements for diagnosis are that the major criteria reported in Table 1 are fullfilled and that Parvovirus infection and Fanconi's anemia are ruled out. Nevertheless, some elements not included in these criteria can help, such as the presence of typical malformations, the response to steroids and the chronic course of the anemia. Onset after the age of two years or the absence of isolated bone marrow erythroblastopenia should induce great caution in the diagnosis of DBA. Even so, we included a patient whose bone marrow morphology was referred as normal at diagnosis, because he presented typical DBA malformations, high eADA levels and steroid response, in addition to chronic anemia with reticulocytopenia.

In our experience, the high percentage of patients with increased eADA levels and *in vitro* reduced growth

of erythroid colonies, partially corrected by addition of SCF, make these two parameters, not included in the major diagnostic criteria, useful in the diagnosis of DBA.

Therapy

Steroid treatment

More than 50% of patients respond to standard steroid therapy (prednisone 2 mg/kg/die orally), and some achieve long periods of remission.[12,18] However, many responders become steroid-dependent and may experience steroid-related complications. High dose methylprednisolone (30-100 mg/kg/die) has been proposed as a second-line treatment in non-responders;[19,20,21] four of our ten patients treated with high dose steroids initially responded, but only one had a persistently modified status and subsequently achieved hematologic remission.

Willig *et al.* reported that steroid resistance is not a definitive feature, as some patients recover sensitivity to steroids during the course of the disease;[8] they therefore suggest the repetition of standard doses before high doses are considered.

How glucocorticoids stimulate erythropoiesis is unknown. The steroid response seems not to be mediated by the immunosuppressive activity of these drugs, because most other immunosuppressants are ineffective in DBA. Since steroids are transcription regulators, they are thought to influence the expression of cytokines, growth factors and their receptors.

Other treatments

Therapeutic options in steroid-resistant patients are chronic red cell transfusions or allogeneic stem cell transplantation (SCT).[22-24] Androgens,[13] growth factors (interleukin-3 and erythropoietin)[25,26] and cyclosporine A (CSA)[27,28] have occasionally been used, but eventually discontinued on account of adverse effects or low efficacy. The recent report that metoclopramide may modulate erythropoiesis in DBA by increasing prolactin levels has yet to be validated by larger studies.[29] Thrombopoietin (TPO) stimulates the growth of BFU-E from DBA patients *in vitro*, but its pharmacological effect has not been evaluated.[30]

Three of our patients underwent treatment with CSA, seven with interleukin-3, one with androgens and two with metoclopramide. Only two subjects treated with interleukin-3 achieved an initial response, but had to interrupt the treatment due to side effects. Metoclopramide was ineffective.

Stem cell transplantation (SCT) has been explored as an alternative to chronic transfusions since 1976.[31] More than 70 DBA patients have undergone SCT so far[8,32,33] with an overall survival of about 85% at three years from sibling donors in more recent reports.[8,33] The candidate sibling donor for a DBA patient with mutation in RPS19 should undergo molecular analysis to rule out the presence of the mutation, which could be due to a germinal mosaicism in an unaffected parent. Alternative donor transplantation (MUD, matched unrelated donor) has been reported to be burdened by high mortality, with an actuarial survival of about 15% at 62 months,[33] but this poor outcome was not confirmed in our recent experience, probably due to the improvement of both the conditioning regimen and HLA matching of the donors. Five successful cord blood transplants (CBT) have been reported in DBA patients, although the indications for such transplants for bone marrow failure syndromes are still controversial.[34-38]

SCT was performed in twelve of our patients (5 from a sibling donor, 4 MUD and 3 CBT). No significant difference in the outcome was observed between transplants from a sibling and MUD (*F. Locatelli, personal communication*).

Gene therapy

Identification of RPS19 as the first causal gene for DBA opened the road to gene transfer experiments. Hamaguchi *et al.* have demonstrated that forced expression of RPS19 in $CD34^+$ cells from four RPS19-deficient DBA patients using oncoretroviral vectors significantly enhanced erythroid colony formation *in vitro*, thereby implying that early erythroid development can be improved by gene transfer.[39] However, since erythroid colony formation varies from one patient to another, the effects of RPS19 gene transfer may well be different. Gene therapy for RPS19-deficient DBA seems thus feasible and promising, but further studies are necessary.

Follow-up

Response to treatment and outcome

Ater and Young have summarized long-term survival for more than 500 DBA patients reported in the literature.[13] The median survival age for the patients reported in the past ten years is 54 years, with a significant improvement of survival in steroid-responsive versus transfusion-dependent patients. Of 76 patients followed over a 60-year period in Boston, one-third (n=24) were in remission off treatment and the remaining two-thirds were dependent on either transfusions (n=36) or steroids (n=13); three had been lost to follow-up.[40]

In our series, 85 patients were available for long-term evaluation (mean follow-up: 150±95 months). Their status at the time of the study is reported in Figure 1: 61,2% were undergoing treatment (two-thirds were transfusion-dependent and one-third steroid-dependent) and 30,6% were free from treatment, one-third

after SCT. Seven patients (8.2%) had died, four from SCT-related complications and three from other causes.

A number of patients, though, experience spontaneous remission.[12,40] Hematologic remission was achieved by 21% of our patients (excluding patients who underwent SCT) a median of 14.5 months after diagnosis (range: 7-82 months). Fourteen patients were initially steroid responders and two had never required any treatment due to mild anemia; only two were transfusion-dependent, suggesting that steroid-resistant patients have a very low chance of remission.

Cytopenia and malignancies

Neutropenia and/or thrombocytopenia are not uncommon and occurred in 13 patients (15%) in our series. The pathogenesis of cytopenia is unclear. Several reports suggest that the hematopoietic defect in severe DBA may not be confined to the erythroid lineage. Giri *et al.* reported that long-term culture-initiating cells (LTC-IC) are quantitatively equivalent in DBA patients and normal controls, but the average clonogenic cell output per LTC-IC is significantly lower in DBA patients.[15] Similarly, Santucci *et al.* demonstrated that the ability of the enriched $CD34^+$ bone marrow cell fraction to proliferate and differentiate *in vitro* along the granulo-macrophage pathway is impaired in some DBA patients.[41] Hamaguchi *et al.* reported both significant reduction of the proliferative recruitment of $CD34^+CD38^-$ hematopoietic progenitors in DBA patients and significant improvement of their proliferative capacity in RPS19-deficient DBA patients following enforced expression of RPS19 with lentiviral vectors.[42] Conversely, we have documented the immune pathogenesis of cytopenia in three patients, only one of whom was transfusion-dependent. DBA, like other bone marrow failure syndromes such as dyskeratosis congenita (MIM:305000), may thus predispose to autoimmune processes, even in the absence of heavy exogenous antigenic stimulation, and cytopenia may be due to increased peripheral destruction of blood cells instead of reduced production. The two hypotheses are not mutually exclusive.

Like other marrow failure syndromes, DBA is associated with a slightly increased risk of cancer. Malignancies have been reported in 29 patients:[8,13,23,43-56] 15 hematopoietic neoplasms, primarily acute non-lymphocytic leukemias and myelodysplastic syndromes, 5 osteogenic sarcomas and 9 cases of other solid tumors (hepatocellular carcinoma, gastric and colorectal carcinomas, breast cancer, melanoma and rare sarcomas). No malignancies were observed in our series.

Several reports suggest that iron overload, androgen use and immunosuppression secondary to steroid therapy predispose DBA patients to malignancies.[44,45,49] Furthermore, myeloid malignancies are not unexpected, as DBA primarily involves the intrinsic hematopoietic progenitor. Conversely, the pathogenesis of osteogenic sarcoma is difficult to explain; since this malignancy has been observed in other genetic disorders, such as hereditary retinoblastoma and Li-Fraumeni syndrome, it has been suggested that DBA could be added to the list of syndromes predisposing to osteogenic sarcoma.[56]

Genetics

RPS19 gene

In 1997, identification of a balanced reciprocal translocation t(X;19)(p21;q13) in a sporadic case of DBA suggested the presence of a DBA locus on chromosome 19q13[57] and this suggestion was further supported by linkage studies in multiplex families[58] and by the identification of microdeletions spanning this region in some DBA patients.[59] Subsequent cloning of the 19q13 breakpoint from the DBA patient with t(X;19) revealed that the gene responsible for the disease was RPS19.[60] This gene encodes for the ribosomal protein (rp) S19, associated with the ribosomal 40S subunit, and is espressed ubiquitously in hematopoietic and non-hematopoietic tissues. The involvement of RPS19 in DBA was confirmed by molecular analysis in a larger group of patients:[3,60] mutations in the RPS19 gene were identified in approximately 25% of DBA subjects. Mutations constantly affect only one allele, and, in multiplex families, co-segregate with the DBA phenotype in consecutive generations with an autosomal dominant mode of transmission.

More than sixty RPS19 mutations, scattered over the whole gene, have now been described.[3-5,59,61,62] Missense, nonsense, insertions, deletions and splice site defects have been found (Table 2). Eleven mutations recur in more than one patient; the particularly frequent ones are the nonsense mutation Arg94Stop (6 patients) and the missense mutations Arg62Trp (9 patients) and Arg56Gln (5 patients). A hot spot of missense mutations was identified between codons 52 and 62 (5 mutations in 18/60 overall patients), a highly conserved region likely to have a critical role in RPS19 function.[3]

RPS19 carried a mutation in 24.7% of our families. In the present study, which includes 25 new patients, we describe five new sporadic mutations: a chromosomal translocation with a wide deletion and four point mutations. In the first patient, a female with translocation t(1;19)(p32; q13), microsatellite analysis demonstrated a deletion on 19q13 which included markers CEA and PG1 flanking the RPS19 gene, indicating loss of the paternal RPS19 allele. Because paternal D19S197 and LIPE markers were conserved, the deletion was predicted to span about 1 Mb. The patient presented both DBA

Table 2. Mutations in RPS19 gene.

Genomic DNA Mutation	*Exon/Intron*	*Expected Protein Alteration*	*Number of families (*)*	*References*
Nonsense mutations				
31 C→T	ex 2	Gln11Stop	2	Willig 1999, Proust 2003
34 C→T	ex 2	Gln12Stop	2	Willig 1999
99 G→A	ex 3	Trp33Stop	2	Willig 1999, Matsson 1999
144 C→A	ex 3	Tyr48Stop	1	Willig 1999
156 G→A	ex 3	Trp52Stop	1	Willig 1999
166 C→T	ex 3	Arg56Stop	2	Willig 1999, Proust 2003
280 C→T	ex 4	Arg94Stop	6	Draptchinskaia 1999, Willig 1999, Matsson 1999, Proust 2003
382 C→T	ex 5	Glun128Stop	1	Proust 2003
Missense mutations				
1 A→G	ex 2	Met1Val protein level unknown	1	Draptchinskaia 1999
3 G→C	ex 2	Met1Ileu protein level unknown	1	Ramenghi 2000
3 G→T	ex 2	Met1Ileu protein level unknown	1	Ramenghi 2000
3 G→A	ex 2	Met1Ileu protein level unknown	1	Proust 2003
43 G→T	ex 2	Val15Phe	1	Willig 1999
53 T→C	ex 2	Leu18Pro	1	Ramenghi 2000
140 C→T	ex 3	Pro47Leu	1	Ramenghi 2000
154 T→C	ex 3	Trp52Arg	1	Draptchinskaia 1999
167 G→A	ex 3	Arg56Gln	5	Willig 1999, Cmejla 2000
182 C→A	ex 4	Ala61Glu	1	Willig 1999
184 C→T	ex 4	Arg62Trp	9	Draptchinskaia 1999, Willig 1999, Ramenghi 2000 and personal data
185 G→A	ex 4	Arg62Gln	2	Cmejla 2000, Proust 2003
302 G→A	ex 4	Arg101His	1	Willig 1999
358 G→C	ex 5	Gly120Arg	1	Willig 1999
380 G→A	ex 5	Gly127Gln	1	Willlig 1999
392 T→C	ex 5	Leu131Pro	1	Proust 2003
Insertions and deletions				
14_15insA	ex 2	Frameshift at codon 5 stop at codon 50	1	Draptchinskaia 1999
19del13bp	ex 2	Frameshift at codon 7 stop at codon 24	1	Proust 2003
24del18bp	ex 2	Deletion of aminoacids 9-14 no frameshift	1	Proust 2003
53_54insAGA	ex 2	Insertion Glu at codon 19 no frameshift	1	Willig 1999
58delG (**)	ex 2	Frameshift at codon 20 stop at codon 28	1	new mutation, personal data
104_105insA	ex 3	Frameshift at codon 35 stop at codon 50	1	Draptchinskaia 1999
TT→AA 157-158 and 160insCT	ex 3	Leu45Gln and frameshift at codon 47	1	Matsson 1999
196_206del11bp	ex 4	Frameshift at codon 66 stop at codon 149	1	Cmejla 2000
222delC	ex 4	Frameshift at codon 74 stop at codon 75	1	Willig 1999
238_239insG	ex 4	Frameshift at codon 80 stop at codon 153	1	Willig 1999
250_251delAG	ex 4	Frameshift at codon 84 stop at codon 153	1	Willig 1999
274_304del31bp	ex 4	Frameshift at codon 92 stop at codon 100	1	Willig 1999
293_294delGT	ex 4	Frameshift at codon 98 stop at codon 152	1	Willig 1999
329delG	ex 4	Frameshift at codon 103 no stop codon	1	Matsson 1999
341delA	ex 4	Frameshift at codon 115 stop at codon 123	1	Willig 1999
384_385delAA data	ex 5	Frameshift at codon 128 stop at codon 152	3	Willig 1999, Ramenghi 2000 and personal data
386_387ins8bp	ex5	Frameshift at codon 131 no stop codon	1	Cmejla 2000
390_391delTC	ex 5	Frameshift at codon 130 stop at codon 151	1	Willig 1999
IVS4_IVS5del	ex 5	Deletion of exon 5 frameshift at codon 120 no stop codon	2	Draptchinskaia 1999, Proust 2003
434del7bp	ex 6	no stop codon	1	Proust 2003
deletion of a complete allele		no protein	2	Gustavsson 1998
t(X;19) (p21;q13)		no protein	1	Gustavsson 1997
46, XX, t(8;19)(q35;q13)		no protein	1	Gustavsson 1998
t(1;19)(p32;q13) (**)		no protein	1	new mutation, personal data
Splice sites defects				
IVS1-1T agATG→atATG	IVS 1	Acceptor splice site defect	1	Draptchinskaia 1999
IVS1-1A agATG→aaATG (**)	IVS 1	Acceptor splice site defect	1	new mutation, personal data
IVS2+1A and IVS4 +169 del 2bp	IVS2/ IVS 4	Donor splice site defect	1	Proust 2003
71del4bp (+3+6) Aagtgagtttggg→AAgtttggg	IVS 2	Donor splice site defect	1	Draptchinskaia 1999
IVS3-2T agCTT→tgCTT	IVS 3	Acceptor splice site defect	1	Willig 1999
del G 173(-1) agCTT→aCTT	IVS 3	Acceptor splice site defect	1	Willig 1999
IVS4+2A ATGGgt→ATGGga	IVS 4	Donor splice site defect	1	Willig 1999
IVS4-1T agCGG→atCGG (**)	IVS 4	Acceptor splice site defect	1	new mutation, personal data
IVS4-1A agCGG→aaCGG (**)	IVS 4	Acceptor splice site defect	1	new mutation, personal data
IVS5+1A CAGgt→CAGat	IVS 5	Donor splice site defect	1	Ramenghi 2000
Promoter region				
(-629_-625)del4bp			3	Willig 1999
(-634_-633)insAGCC			1	Ramenghi 2000

Nucleotide numbering starts from ATG. Alterations in intronic regions are not included because an effect on the protein has not been demonstrated. (*) multiplex families were considered as a single genetic event; conversely, families were considered even when the patient was sporadic; (**) mutations not previously described in the literature.

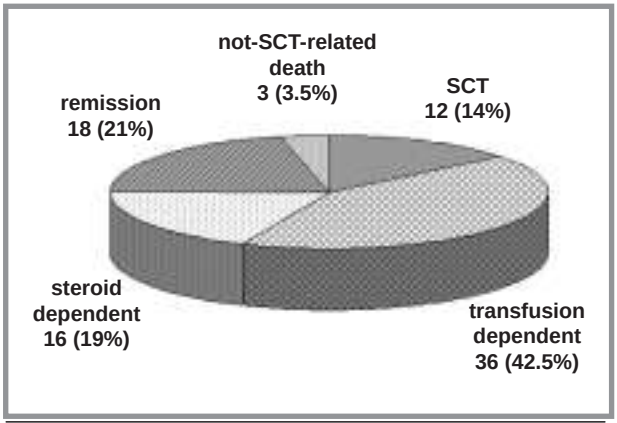

Figure 1. Follow-up of Italian patients. Eighty-five patients were available for long-term evaluation. Seventy-eight patients were alive at the time of the study; 4/12 transplanted patients had died due to SCT-related complications and 3/85 patients had died from other causes (two from infectious diseases, one from trauma).

features and mental retardation. She was unresponsive to steroids and is now transfusion-dependent. The four new point mutations were deletion of a guanine at position 58 in exon 2 and three splice site defects: IVS1 –1 G→A, IVS4 –1 G→T and IVS4 –1 G→A. The deletion shifts the reading frame of exon 2 and generates a premature stop codon (PTC) at codon 29. The guanine-to-adenine substitution in the acceptor splice site of intron 1 (IVS1 –1 G→A) is expected to cause skipping of the first translated exon, thus removing the start codon. Both the guanine-to-thymine and the guanine-to-adenine substitutions in the acceptor site of intron 4 (IVS4 –1 G→T and IVS4 –1 G→A) are expected to result in skipping of exon 5 and a frameshift with abolition of the canonic stop codon. The molecular mechanism by which RPS19 mutations cause the DBA phenotype remains unclear. Ribosomal proteins (rp) constitute a major component of cellular proteins, but their functions, except for ribosome assembly and protein synthesis, have not been well characterized.[63] After synthesis in the endoplasmic reticulum, rp are imported into the nucleus where they assemble in the nucleolus, along with the 4 rRNA molecules, to build the ribosome. They are then re-exported into the cytoplasm as ribosomal subunits, where mRNA translation is performed.[64] Although rRNA has been demonstrated to be the most important element in ribosomal function, rp appear to make a major contribution to efficient and accurate protein synthesis, such as regulation of the simultaneous translocation of mRNA and tRNA through the ribosome[65] and of extrusion of the nascent polypeptide.[66]

Mutations in about 50 rp are associated with the *Minute* phenotype of *Drosophila melanogaster*, characterized by delayed larval development, thin bristles and small body size;[67] it has been suggested that the phenotype is the result of insufficient protein synthesis during certain developmental stages, particularly during high cell growth rate stages.

Conversely, non-ribosomal functions of ribosomal proteins have been described in *E. coli*,[68] *Drosophila melanogaster*[69,70] and mammals.[71] As to rpS19, protein dimers have been shown to display chemotactic activity for human monocytes;[72] moreover, free cytoplasmic rpS19 has been shown to interact with fibroblast growth factor 2.[73] The DBA phenotype may thus be caused by failure of a non-ribosomal function of rpS19 that influences the function of hematopoietic cells.

Most RPS19 mutations in DBA patients (nonsense, frameshift, splicing mutations, mutations involving the start codon and wide deletions) are expected to alter the genetic information drastically; only a minority are missense mutations that change a single amino acid of the S19 protein. This pattern of mutations has prompted the inclusion of DBA among the haplo-insufficiency syndromes, even if a dominant negative effect has not been ruled out. Loreni *et al.* have demonstrated that both allelic deletions and mutations resulting in a PTC or lacking of the stop codon cause a decrease in RPS19 mRNA levels (about 50-60% of normal). Usually, these aberrant transcripts are degraded by nonsense mediated decay (NMD) and non-stop decay RNA surveillance systems which prevent the expression of aberrant peptides and require active protein synthesis.[74,75] Since treatment with a translation inhibitor increases RPS19 mRNA levels in the cell lines with PTC or non-stop mutations, Loreni *et al.* suggested that degradation of altered mRNA is due to NMD or non-stop decay (*Loreni, personal coomuniction*). About 50% of the RPS19 mutations described so far could potentially activate NMD or non-stop decay and thus cause haploinsufficency. Moreover, Da Costa *et al.* showed that two missense mutations found in DBA patients, Val15Phe and Gly127Gln, affect the nucleolar localization of rpS19 and determine low protein levels in the cell, probably due to instability of the protein when unable to be targeted to nucleoli.[76] The phenotype associated with some missense mutations is thus also attributable to a dosage-effect.

High levels of RPS19 expression characterize the earlier stages of hematopoiesis and are probably needed for development of erythroid progenitors.[39] RPS19 mRNA and protein expression decrease during terminal erythroid differentiation, consistently with the finding that maturation arrest of erythroid precursors occurs at early stages of erythroid differentiation in DBA.[77]

Genetic heterogeneity

RPS19 mutations account for only 23-25% of cases of DBA and are never involved in the recessive form.[3,11] A second DBA locus, accounting for about 40% of dominant cases, has been identified on human chromosome

Table 3. Table 3. Clinical features of patients carrying rearrangements at 19q locus and RPS19 mutations involving the start codon (see text).

	Sex	*Age at diagnosis of DBA (mo.)*	*Malformation Status*	*Response at First Steroid Course*	*Status at Last Follow- Up*	*Genomic DNA Mutation Alteration*	*Expected Protein*	*References*
1	M	2	Macrocephaly, mental retardation, short broad bones, extra ribs, malformations of the spine, short stature	PR	TD	Deletion of a complete allele	no protein	Gustavsson 1998
2	NA	NA	Mental retardation, skeletal malformation	NA	NA	Deletion of a complete allele	no protein	Gustavsson 1998
3	F	1	Left kidney aplasia/hypoplasia, short stature	PR	TD	t(X;19) (p21;q13)	no protein	Gustavsson 1997
4	F	2	Mental retardation, short stature	CR	TD	t(8;19)(q35;q13) deletion of a complete allele	no protein	Gustavsson 1998
5	F	infancy	Mental retardation	NR	TD	t(1;19)(p32;q13) deletion of a complete allele	no protein	new mutation, personal data
6	M	0	None	CR	NA	1 A→G	probably no protein	Draptchinskaia 1999
7	M	2	Short stature	NR	TD	3 G→C	probably no protein	Ramenghi 2000
8	M	infancy	None	NR	TD	3 G→T	probably no protein	Ramenghi 2000
9	M	1	None	NR	TD	3 G→A	probably no protein	Proust 2003

NA: not available; CR: complete response; PR: partial response; NR: no response; TD: transfusion-dependent.

8p by linkage analysis, within a 8.1 cM interval on 8p23.3-8p22 (LOD score +3.55).[6] Among genes spanning the region, the transcription factor GATA-4 was considered a candidate for DBA. GATA-4 is a member of the GATA family, which comprises six proteins acting as transcription factors: GATA 1-2-3, predominantly expressed in haematopoietic cells and whose absence causes anemia in mice embryos, and GATA 4-5-6, predominantly expressed in various mesoderm and endoderm derived tissues. GATA-4 is required for ventral morphogenesis and heart tube formation in mice; furthermore, its ectopic expression compensates the erythropoietic defect in GATA-1 deficient embryonic stem cells, thus suggesting a role in erythropoiesis.[78] No mutations in GATA-4 were found in 15 DBA patients.[79]

In some families, including families with recessive transmission, linkage of DBA to either the 8p or 19q critical regions has been ruled out, suggesting the role of further genes.[11,59]

Since the rp S3a, S13, S16 and S24 take part together with rpS19 in binding eukaryotic initiation factor 2 (eIF-2), a key regulator of initiation of mRNA translation and protein synthesis, they have been recently studied as candidate genes for DBA, but no mutations in the coding sequences of RPS3a, S13, S16 and S24 were found in 14 DBA patients.[80]

Genotype-phenotype correlation

Patients with RPS19 mutation

RPS19 alterations have not been correlated with any specific phenotype and are found in patients with very different malformations, hematologic values, eADA levels and responses to treatment.[3] A wide variability of clinical expression of RPS19 mutations is evident in familial cases, with the presence of minor phenotypes, suggesting that some transient anemias (such as transient erythroblastopenia of childhood) may really be misdiagnosed DBA. Although, in some multiplex families, incomplete penetrance has been described,[81] in our experience penetrance was always complete, even if extremely mild phenotypes were observed, for example isolated macrocytosis (Figure 7 in ref. 3). No significant phenotypic difference has been found between the effects of mutations expected to drastically alter genetic information, namely nonsense, frameshift and splice site mutations, and the missense mutations. Nevertheless, a thorough review of the literature together with our own data point to a worse outcome (as defined by transfusion-dependence) in patients with mutations that completely suppress one allele (rearrangements at 19q locus and mutations which affect the start codon), even if the initial response to treatment varies (Table 3).

Table 4. Clinical features of patients carrying the Arg62Trp mutation.

	Sex	Age at Diagnosis of DBA (mo)	Malformation Status	Response at First Steroid Course	Status at Last Follow- Up	Ref.
1	F	2	Triphalangeal thumb, anogenital fistula, interventricular septal defect	NR	TD	Willig 1999
2	M	0	None	CR	Crem	Willig 1999
3	M	2	None	NR	SCT	Willig 1999
4	M	2	Short stature	NR	Dead	Draptchinskaia 1999
5	M	0	Micrognathia, proximal implanted thumbs, short stature	NR	TD	Willig 1999
6	M	0	Bilateral congenital glaucoma, congenital inguinal hernia, right thenar eminence hypoplasia, right ear fistula	CR	TD (SCT)	Ramenghi 2000
7	M	2	None	CR	TD (SCT)	Ramenghi 2000
8	F	<12	None	NR	TD	Draptchinskaia 1999
9	F	0	None	NR	TD	new mutation, personal data

Patients 6 and 7, who underwent SCT, were previously transfusion-dependent. Transfusion-dependence can also be supposed in patients 3 and 4, due to absent response to steroids. CR: complete response; NR: no response; TD: transfusion dependent; Crem: complete remission; SCT: stem cell transplantation.

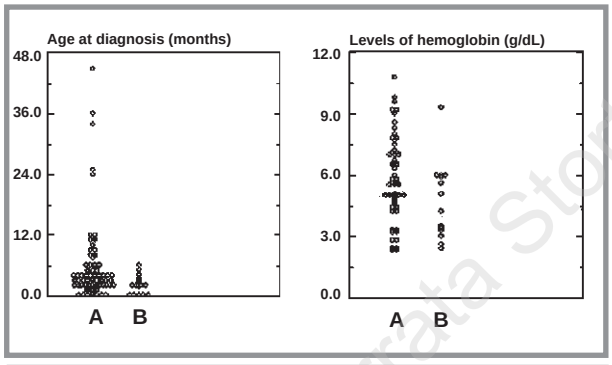

Figure 2. Age at presentation and hemoglobin levels in RPS-mutated versus non mutated DBA patients. Mean age at diagnosis in non-mutated (A): 5.93±8.35 months; mutated (B): 2.33±2.1 months. Mean hemoglobin level at diagnosis in non-mutated (A): 5.7±1.99 g/dL; mutated (B): 4.7±1.89g/dL.

Reported rearrangements include a balanced translocation t(X;19),[57] two unbalanced translocations t(1;19) and t(8;19) and two wide deletions.[3,59] A further patient carrying a deletion at the RPS19 locus and reported by us in a previous paper[11] is not included in the series, since his deletion seems not to include the RPS19 coding sequence. Sequencing of the intronic regions, in fact, showed heterozygosity for 5 SNP (*personal data*).

It is noteworthy that wide deletions at 19q13 are always associated with mental retardation: this is the case of our patient harboring t(1;19) and the three patients reported in the literature.[60] Conversely, the patient with balanced translocation t(X;19) which interrupts RPS19, without loss of other genes, has normal mental development. A contiguous gene syndrome has been hypothesized for patients with wide deletions at the locus.[82]

Mutations of the translation-initiator ATG (Met1) are associated with transfusion-dependence in the three patients whose present clinical status is known (*Proust 2003 and personal data*). Loss of the normal initiation codon is supposed to drastically alter the expression of the allele and determine initiation of the mutated mRNA at the first AUG available in the sequence;[83] the first available AUG in RPS19 is that at nt96, which would produce a sequence not in frame and a PTC.

The genotype-phenotype correlation suggested above does not reach statistic significance due to the low number of cases: significance will be evaluable when a wider panel of rearrangements at the locus and start codon mutations is phenotypically described. Curiously, we have also observed that the recurrent Arg62WTrp mutation is prevalently associated with transfusion-dependence: this is apparent in eight out of nine subjects (Table 4), including five from our series and four from the literature.[3,60] This mutation does not alter the nucleolar localization of the protein and thus is expected to impair a different function.[76] The study cited, however, does not address the question of whether the mutated protein is actually included in the mature ribosome.

Patients with or without RPS19 mutation

Willig *et al.* reported a lower prevalence of RPS19 mutations among initially steroid responding patients than among initially steroid-resistant patients, sug-

gesting a poorer response to steroid treatment in mutated patients. However, mutation frequency was comparable in transfusion-dependent individuals , in long-term steroid-dependent patients and in patients free from treatment on long-term follow-up.[3]

In our series, the groups with and without mutated RPS19 did not differ significantly for any of the variables evaluated. The frequencies of physical abnormalities and high eADA levels were similar. There were no differences in the development of other cytopenias, initial response to steroids and long-term outcome, including steroid or transfusion dependence, remission rate and mortality. Age and Hb levels at diagnosis were lower, but not significantly so, in the mutated group (Figure 2: p=0.06 and p =0.1 respectively). Greater dispersion of age at diagnosis in the non-mutated group is probably attributable to genetic heterogeneity.

Conclusions

In conclusion, our data show the importance of registries for rare genetically heterogeneous diseases in order to dissect clinical phenotypes, devise genetic studies, define prognostic factors and delineate new therapeutic trials. Nationwide collaboration is essential for these purposes. Lastly, comparative analysis of the DBA registries, including molecular data, is desirable in order to achieve more precise characterization of this intriguing disease.

ID and UR conceived and supervised the study. MFC wrote the paper with PQ. EG performed ADA evaluation and molecular analysis of RPS19 with AC and PQ. VP performed karyotype analysis in the patient with t(1;19); BN, SV, DL, MZ and CD followed the patients, collected data, helped in the data evaluation and critically discussed the paper. The authors indicated no potential conflicts of interest.

The AIEOP Centers of Bari (D DeMattia), Bologna (A Pession), Bolzano (L Battisti), Brescia (L Notarangelo), Catania (G Russo), Ferrara (C Borgna), Firenze (A Lippi), Genova (AC Molinari), Modena (M Cellini), Monza (G Masera), Napoli (B Nobili), Napoli Pausilipon (G Loffredo), Padova (L Zanesco), Palermo (M Aricò), Parma (GC Izzi), Pavia (F Locatelli), Pesaro(F Felici), Perugia (A Amici), Pisa (C Favre), Roma (D DelPrincipe), Torino (U Ramenghi), Trieste (P Tamaro) and Vicenza (G Castaman) followed the patients. Their collaboration is gratefully acknowledged.

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