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Diamond-Blackfan anemia, ribosome and erythropoiesis

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Abstract

Diamond-Blackfan anemia is a rare inherited bone marrow failure syndrome (5 to 7 cases/million live births) characterized by an are generative, usually macrocytic anemia with an absence or less than 5% of erythroid precursors (erythroblastopenia) in an otherwise normal bone marrow. The platelet and the white cell counts are usually normal but neutropenia, thrombopenia or thrombocytosis have been noted at diagnosis. In 40 to 50% of DBA patients, congenital abnormalities mostly in the cephalic area and in thumbs and upper limbs have been described. Recent analysis did show a phenotype/genotype correlation. Congenital erythroblastopenia of DBA is the first human disease identified to result from defects in ribosomal biogenesis. The first ribosomal gene involved in DBA, ribosomal protein (RP) gene S19 (*RPS19* gene), was identified in 1999. Subsequently, mutations in 12 other RP genes out of a total of 78 RP genes have been identified in DBA. All RP gene mutations described to date are heterozygous and dominant inheritance has been documented in 40 to 45% of affected individuals. As RP mutations are yet to be identified in approximately 50% of DBA cases, it is likely that other yet to be identified genes involved in ribosomal biogenesis or other pathways may be responsible for DBA phenotype.

Keywords

Diamond-Blackfan anemia; erythroblastopenia; ribosome; p53; apoptosis

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CLINICAL PRESENTATION, LABORATORY FINDINGS, AND TREATMENT

Clinical presentation and laboratory findings

Diamond-Blackfan anemia (DBA) (OMIM 105650) is a rare inherited bone marrow failure syndrome (5 to 7/millions live births), diagnosed early in infancy or early childhood with 95% of DBA cases diagnosed between ages of 3 months and 2 years (1–3). DBA is characterized by a moderate to severe are generative usually macrocytic anemia with persistently low reticulocyte counts and an erythroblastopenia (absence or less than 5% of erythroid precursors) in an otherwise normocellular bone marrow. The platelet and the white cell counts are usually normal but moderate thrombocytopenia and/or neutropenia, or thrombocytosis may be present at diagnosis (3). Elevated levels of fetal hemoglobin and expression of i antigen instead of I antigen, both markers of fetal erythropoiesis, are features of red cells in DBA. An increased erythrocyte adenosine deaminase activity (eADA), although nonspecific, is noted in 90% of non-transfused DBA patients (4–5). DBA is characterized by pallor, shortness of breath while suckling, and failure to thrive. Congenital abnormalities are reported in 30 to 50% of DBA patients (3, 6–9). Growth retardation is part of the malformations often worsened by long-term steroid therapy or iron overload (10). The malformation syndrome affects mostly the cephalic area (microcephaly, cleft palate, hypertelorism, high arch palate), thumbs and upper limbs (triphthalangeal thumb, thumb duplication or hypoplasia, radial hypoplasia) but in some cases also the heart, kidneys, urogenital tract and bones. In the absence of identifying a RP gene mutation, diagnosis of DBA is difficult to establish since no specific biologic test exists to confirm the diagnosis. A consensus DBA conference organized by the Daniella Maria Arturi Foundation generated criteria for DBA diagnosis based on major diagnosis criteria and major and minor supporting criteria (Table 1).

Before starting on a work up for diagnosis of DBA, several other diagnoses need to be ruled out: 1) erythroblastopenia following B19 parvovirus infection (IgM and IgG serology and parvovirus B19 DNA isolation by PCR in bone marrow and/or blood); 2) transient erythroblastopenia of childhood (TEC), which spontaneously resolves and 3) some forms of the Fanconi anemia (constitutional karyotype, chromosome breakage test, and western blot analysis for FANCD2) and 4) other diagnostics of acquired erythroblastopenia (for review, (11)).

Treatment

DBA affected individuals receive one or several transfusions following diagnosis to manage the anemia. 60% of DBA patients respond to steroid therapy for yet to be defined reasons and steroid usage is recommended only after one year of age in order to facilitate growth during the first year of life (11). The initial dose for conventional oral steroid therapy is usually 2 mg/kg/day of prednisone or equivalent. A rise in reticulocyte count occurs within 10–15 days if the patient is a good responder. If this rise occurs, the daily dosage should be cautiously and gradually decreased to a dose 0.5 mg/kg/day, which can be prescribed on alternate days. Patients who require doses above this threshold should not be maintained on long-term steroid therapy, due to its deleterious side effects. DBA patients can switch from steroid responsiveness to resistance, or vice versa (3). Such unpredictability leads many physicians to test again the steroid effect in formerly resistant patients, especially before taking crucial decisions, such as bone marrow transplantation. Patients who are not steroid responders enter into a long term transfusion program in conjunction with an efficient iron chelation (deferrioxamine, or deferiprone, or deferasirox (12)) when the serum ferritin level raises above 1000 ng/ml in the absence of overt inflammation. Iron overload should be carefully managed by liver and heart (T2* sequence) MRI follow-up (13).

Some patients can be completely weaned off all treatment. Such treatment independence, which often occurs in teenagers, but also in young children, can be lifelong or interrupted by relapse, which entails treatment as needed. Treatment independence can in no case be considered as complete remission or cure: in most patients erythropoiesis continues to display anomalies such as persistent moderate anemia, macrocytosis, and high eADA levels. Occurrence and duration of treatment-free periods are both unpredictable.

Bone marrow transplantation (BMT) is the only curative treatment. However, only DBA patients affected by severe complications such as leukemia, myelodysplasia, aplastic anemia are candidates for transplantation with bone marrow from HLA-identical donor sibling. The donor should be carefully screened in order to avoid a donor with a minor form of the disease (silent phenotype) exhibiting a macrocytosis, an elevation of the eADA activity and/or a mutation in a RP gene without anemia. Unrelated matched donor should be avoided except in cases with major hematological complications in the DBA patient and no HLA-identical donor in the family.

Metoclopramide, a prolactin inducer showed early promise for treatment of DBA but a well designed therapeutic trial in a cohort of transfusion-dependent patients resulted in disappointing results, with less than 10% of partial responses (14–16). However, since the deleterious side effects of this drug are mild or absent, it can be assessed in some transfusion dependent DBA patients and can provide an increase in the time interval between transfusions. In some patients a synergy between metoclopramide and low doses of steroids has also been reported as successful (16).

Other treatments have been explored and shown to be effective in only few patients or in individual case reports: interleukine-3 (IL-3), cyclosporine, valproic acid, rituximab, and more recently leucine (17–22). Gene therapy approaches for replacement of the impaired *RPS19* gene are also being explored (23–25).

Clinical Evolution

Clinical evolution in DBA is unpredictable. Severe complications are in most cases treatment-related, due to the side effects of long-term steroid use, iron overload, blood borne infections and adverse effects of BMT. However, over the last decade increasing attention is being paid to the deleterious effects of long-term steroid therapy, and a awareness for the importance for prevention of iron overload. A higher risk for malignancies including mainly, solid tumors (osteosarcoma), and other various cancers (breast, liver, gastric or other digestive carcinomas) and hematological malignancies (acute myeloid and lymphoblastic leukemia, lymphoma, myelodysplasia) of around 2 to 4% have been reported in DBA affected patients (3, 8, 26). Aplasia is a rare complication of DBA. With the longer life span of DBA patients due to the progresses in the DBA management, pregnancies in DBA affected women become more frequent. Pregnancies in DBA present a higher risk of complications for both the mother and the fetus (*in utero* growth retardation, fetal loss, prematurity, pre-eclampsia) independent of the DBA status or the presence or absence of a *RPS19* gene mutation (27). As a result, pregnancy in women with DBA should be carefully monitored and managed with close cooperation between obstetricians, hematologists and neonatologists.

GENETICS

Dominant inheritance is a feature of familial DBA cases (45% of cases), while it is sporadic in other 55% of cases. In 1999, from study of a 7 y-old Swedish girl, carrying a balanced translocation t(X;19)(p21;q13), the first gene involved in DBA was identified (28). Unexpectedly, it was the ribosomal protein (RP) S19 (*RPS19*) gene (29). 25% of DBA

patients carry a mutation in *RPS19* gene (30–34). All mutations identified to date in various registries are heterozygous, and homozygosity is in all likelihood lethal (29–32, 34–35). All types of mutations (missense, non sense, deletions, insertions, splice site defects) affecting each of the six exons in the *RPS19* gene have been identified. A “hot spot” for mutations between codons 52 to 62 has been noted (34). Recently, the importance of 4 single nucleotide polymorphism (SNP) haplotype in association with a rare ethnic polymorphism has been described (36). The *RPS19* gene is a 11 Kb housekeeping and highly conserved gene. It contains two promoters, 6 exons, the first one being non-coding and it encodes for a 144 amino-acid protein of 16 kDa. RPS19 protein binding partners identified to date include the fibroblast growth factor 2 (FGF-2), Pim-1, a serine threonine kinase and proto-oncogene involved in the Stat 5 pathway, which phosphorylates RPS19, and a protein similar to RPS19 (37–40). RPS19 is one of the 78 RPs of the ribosome and is part of the small ribosome subunit 40S. Reinforcing its role in ribosome biogenesis, RPS19 localizes predominantly to the nucleoli of cells (41). Some of the RPS19 mutations identified in DBA patients have been experimentally shown to result in cellular mislocalization and to decreased expression levels of the protein (41). The mutations associated with slightly decreased RPS19 expression levels and normal nucleolar localization are all accessible residues clustered within or around alpha helix 3 located in the central position in the three-dimensional structure of RPS19, and thereby impairing the function of the protein without altering its overall fold (class II mutations- all the “hot spot” mutations) (42). In contrast, mutations, which exhibit a dramatic decrease in RPS19 expression levels alter structural residues affecting the folding of the protein and hence its stability (class I mutations).

Recently, other ribosomal genes involved in DBA have been identified by Hanna Gazda et al: RPS24 (2.4%), RPL5 (6.6%) and RPL11 (4.8%), RPS7 (<1%), RPS15 (<1%), RPS27a (<1%), RPL9 (1%), RPL36 (1%), RPS10 (2.6%), RPS26 (6.4%); Cmejla et al.: RPS17 (<1%); and Farrar et al.: RPL35a (3%) (43–47). Four variants of unknown significance (RPS15, RPS27a, RPL9, and RPL36) have also been identified. RP genes involved in DBA encode proteins that are components of both the small ribosomal subunit and the large ribosomal subunit. As with RPS19, all the mutations identified in various RP genes are present in the heterozygous state. In the 25 patients, for which all the RP genes have been sequenced, none of them carry mutations in more than one RP gene (45). Only one report described the association of a mutation in *RPS24* and *RPL5* genes but the variant in *RPS24* gene is of unknown functional significance (48). No phenotype/genotype correlation could be defined in the case of *RPS19* gene mutations. By contrast, an association between *RPL5* gene mutation and cleft palate has been reported by several groups (44, 48–49). None of DBA patients with cleft palate carry mutation in *RPS19* gene. Thumb anomalies have been found to be more frequently associated with *RPL11* gene mutation (48). As only 53% of DBA patients carry a mutation in one of the RP genes in spite of extensive sequencing of various RP genes, it is likely that other genes involved in ribosome biogenesis are likely to be responsible for DBA phenotype.

DBA AS THE FIRST RIBOSOMOPATHY

DBA is the first human ribosomopathy identified, heterogeneous in both phenotype and genotype. The ribosome is assembled with two ribosomal subunits, a small unit of 40S and a large one of 60S. Ribosome is assembled in a well coordinated fashion in time and space from the small and the large subunits and 4 ribosomal RNA (rRNA): 18S rRNA in the small subunit and the 5.8S, 28S and 5S rRNA in the large subunit. The 18S, 5.8S and 28S rRNAs are produced from the maturation of the large precursor rRNA 90S, which is cleaved in two pre-particles, the pre-40S and pre-60S (Figure 1A). It has been well established first in yeast cellular model, then in primary human cells from DBA patients (fibroblasts and EBV lymphoblastoid infected lymphocytes) that DBA is related to a defect in ribosome

biogenèses (50, 51). Indeed, following deletion of one of the two *RPS19* genes or after generation of *RPS19* gene mutation reproducing human mutation in yeast, as well as from studies of various *RPS19* gene mutations in cells from DBA patients, the rRNA18S maturation has been found to be abnormal with a defect identified at the cleavage site A2 of the internal transcribed spacer 1 (ITS1) in the 3' UTR of 18S rRNA (Figure 1B) (50, 51). It results in a decrease in 18S-E and an accumulation of the 21S leading to an abnormal pre-particle 40S, which is unable to be exported into the cytoplasm due to binding impairment of some cofactors (50). This process results in a defect in ribosome assembly. Strikingly, the rRNA maturation defect varies with the genetic anomalies identified in DBA. Indeed, mutations in genes other than *RPS19* can lead to other ribosomal rRNA maturation defects or to the same 18S rRNA defect but at another stage in the rRNA maturation process. For example, *RPS24* deficiency leads to an impairment in the ETS (external transcribed spacer) of 18S rRNA (52), *RPS10* and *RPS26* deficiency to an accumulation of the 18S-E rRNA (45). Thus, different RP gene mutations either from the small or the large ribosome subunits lead to different rRNA defects but as a phenotypic result to the same erythroid precursor defect, in association or without congenital abnormalities (Figure 2) (53).

In addition to DBA, other bone marrow failure syndromes, namely cartilage hair hypoplasia, Shwachman-Diamond syndrome, and dyskeratosis congenita have been recently shown to be related to rRNA maturation impairment at different levels (54–55). All these diseases involve genes implicated in rRNA maturation, encoding for nucleolar proteins, reinforcing their role in ribosome biogenesis and are associated with a risk of malignancies. DBA thus belongs to this new class of diseases, “ribosomopathies”. All of these “ribosomopathies” are constitutional but in 2008, an acquired hematological syndrome, the minus 5q syndrome has been shown to be originate from the deletion of a RP gene, the *RPS14* gene (55). The Minus 5q syndrome is an acquired myelodysplastic syndrome characterized by an anemia, usually macrocytic, thrombocytosis and signs of myelodysplasia in the bone marrow, specifically on megakaryocytes lineage (presence of micromegakaryocytes and hypoplastic megakaryocytes). Thus yet another RP gene is involved in various bone marrow failure syndromes due to a mechanism that have yet to be fully defined.

DBA PATHOPHYSIOLOGY

In spite of significant progress made in identifying mutations in various ribosomal genes in DBA, mechanistic understanding of how these mutations account for DBA pathophysiology including erythroblastopenia remains to be resolved. An important unanswered question is why in DBA is there a tropism for an erythroid specific defect in the face of defective ribosomal assembly, which should affect the protein synthesis process in all cells and tissues? While it is generally accepted that DBA is an intrinsic defect of erythroid progenitors that are unable to complete normal differentiation process, this intrinsic defect is incompletely elucidated (56–57).

Blockade of erythroid differentiation and proliferation in DBA has been shown to affect immature progenitor cells or erythroid-Burst-Forming Unit (BFU-e). Downstream, CFU-e are also subsequently affected (57–58). A study using a two-phase culture system has localized the defect at the interphase between erythropoietin (EPO)-independent and EPO-dependent cells, just after the CFU-e transition (59). Other experimental studies have shown, using long-term culture-initiating cells (LTC-IC), that differentiation and proliferation can also be impaired in other cell lineages: an early maturation defect with an impact on granulocytic and megakaryocytic as well as erythroid lineages has been identified (60–64). It is noteworthy that the cloning efficiency of CFU-GM cells decreases with aging in DBA patients (60, 63). Such experimental data are consistent with the progression of some patients towards pancytopenia and bone marrow hypoplasia. Circulating EPO levels are

increased in DBA patients, indicating the unresponsiveness of the bone marrow to anemia related EPO stimulation (65–66). An increased propensity of erythroid progenitors to apoptosis during *in vitro* EPO deprivation and in RPS19 deficiency has also been reported (67–69).

Animal models are likely to be useful in deciphering the mechanistic understanding of DBA pathophysiology. In the fruit fly, *Drosophila melanogaster*, malformations such as delay in larval development, anomalies of bristles, wings or abdominal segments, short stature or infertility have been extensively described as the Minute syndrome (70–72). Minute syndromes have been shown to be related to mutations in RP genes. The mode of inheritance is dominant and homozygosity is lethal. Haploinsufficiency has been demonstrated, as the expression level of the RP mRNA and is related to extent of phenotypic alterations (lower the mRNA level larger are the phenotypic changes). However, there is no spontaneous Minute syndrome for fruit fly *RPS19* gene, which is located on the X chromosome. Manipulations of the *RPS19* gene promoter region with a P-element insertion, however, resulted in a Minute-like syndrome (J. Fixler, N. Mohandas, unpublished data). A knock-out mouse model of *RPS19* gene did not provide any new insights since the homozygous *RPS19*^{-/-} mice died at the pre-implantation stage of the blastocysts and the heterozygous *RPS19*^{+/-} mice exhibited no phenotype (normal Hemoglobin level, normal BFU-e and CFU-e proliferation) (73). Recently reported animal models appear to be more promising: morpholinos of *RPS19* and *RPL11* in zebrafish, Dark skin *Dsk3*^{+/-} mice, which corresponds to a amino acid change in *RPS19* gene and a transgenic mice model, which reproduces the *RPS19* “hot spot” mutation, Arg62Trp (74–77). This latter model exhibits growth retardation and anemia associated with decreased erythroid progenitors in the liver and decreased BFU-e and CFU-e proliferation. Strikingly, this model suggests a dominant negative mechanism in DBA pathophysiology. DBA may be related in some cases to haploinsufficiency mechanism while in others it is the result of a dominant negative mechanism.

Interestingly, both *RPS19* morpholino in zebrafish and *Dsk3*^{+/-} animal model have implicated the activation of p53 pathway in *RPS19* deficiency, which may be one important clue for the mechanistic understanding of DBA pathophysiology (76, 78). In the zebrafish model, the specific *RPS19* dose-dependant morpholino leads to a decrease in erythroid differentiation with a defect in hemoglobinization associated or not to abnormalities such as growth retardation, reduced forebrain, microphthalmia, defective left-right patterning, defect in cartilage formation, and oedema (74). p53 and some other partners of the p53 family have been found to be stabilized and activated, suggesting a link between a decrease in *RPS19* mRNA level and p53 pathway activation. The *RPL11* morphants displayed brain abnormalities, leading to embryonic lethality 6–7 days after the fertilization, increased apoptosis, and activation of p53 pathway (75). In the *Dsk3*^{+/-}, a single point mutation in *RPS19* is responsible for the melanocyte accumulation in the epidermis via kit ligand activation, growth retardation, very mild anemia and increased apoptosis. In this study, the authors documented that some RP genes (*RPS19* or *RPS20* or *RPS6* genes) lead to stabilization and activation of p53, which could be responsible for growth retardation, increased apoptosis and hypoplastic anemia. A similar mechanism may be involved in DBA. *RPL5* and *RPL11* are related to p53. In case of a nucleolar stress or ribosomal stress, *RPL5*, *RPL11*, and/or *RPL23* expression level in the nucleoplasm is increased and inhibit MDM-2, a E3 ubiquitin ligase targeting p53 (Figure 3) (79–87). p53 linked to MDM-2 is ubiquitinated and addressed to the proteasome for degradation. After RP gene mutation or rRNA anomaly, free *RPL5*, *RPL11* or *RPL23* are able to bind MDM-2. p53 free of MDM-2 translocates to the nucleus, arrests the cell cycle and induces apoptosis. This mechanism may explain the erythroblastopenia as suggested by our recent results after depletion of *RPL5* or *RPL11* or *RPS19* in CD34⁺ from cord blood (88).

However, if the relation between a mutation in a RP gene and the erythroblastopenia is the result of p53 activation and downstream increased apoptosis and/or cell cycle arrest, the erythroid specific tropism is still to be further defined. The translation level of some proteins, which may be important for erythroid progenitor and precursor proliferation and differentiation, may be more affected by a defect ribosome biogenesis than other important proteins in other types of cells. Deciphering the mechanistic understandings of DBA pathophysiology is an important challenge for the near future as well as finding other candidate genes involved in DBA for the 50% of DBA patients in whom mutation in a *RP* gene have not yet been identified.

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Figure 1.
 Figure 1A: normal rRNA maturation
 Figure 1B: rRNA maturation defect in DBA.

Figure 2. Different rRNA maturation defects due to various RP gene mutations identified in DBA

From Aguisa-Touré, et al [53].

Figure 3. Increased RPL11 expression level and p53 activation after a rRNA maturation defect due to a mutation in a ribosomal protein
D'apres Fumagalli, et al [79].

Table 1
Diagnosis criteria of Diamond-Blackfan anemia

From Vlachos et al [11].

Diagnosis criteria

Age less than 1 year

Macrocytic anaemia with no other significant cytopenias

Reticulocytopenia

Normal marrow cellularity with a paucity of erythroid precursors

Supporting criteria

Major

Gene mutation described in “classical” DBA

Positive family history

Minor

Elevated erythrocyte adenosine deaminase activity

Congenital anomalies described in “classical” DBA

Elevated HbF

No evidence of another inherited bone marrow failure syndrome