Diamond-Blackfan anemia: a ribosomal puzzle

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doi: 10.3324/haematol.2008.000513

Diamond-Blackfan anemia (DBA; MIM 105650) has long been a puzzle for hematologists. Even the diagnosis has been difficult, because other diseases and syndromes may present a similar phenotype and because there is still no specific biochemical assay available.[1] The identification of *RPS19* (ribosomal protein S19) as the first DBA gene has done little to solve the mystery surrounding the pathophysiology of this syndrome, characterized by defective erythropoiesis, low stature and malformations.[2] Classically, DBA has been included within the inherited bone marrow aplastic syndromes and shares many aspects with other syndromes that may present erythroblastopenia, such as Fanconi anemia, Shwachman-Diamond syndrome (SD), cartilage-hair hypoplasia syndrome and dyskeratosis congenita (DC). Interestingly, the pathogenesis of SD and DC has been connected in some ways to ribosomal failure.[3]

DBA is the first, and so far only known, inherited disease due to a defect in a structural ribosomal protein. The identification of the role of another RP, RPS14, in the pathogenesis of an acquired myelodysplastic disease, the 5q- syndrome has recently attracted great interest.[4] The definition of ribosomapathy to describe DBA has been strengthened by the identification of detrimental mutations in several RP genes other than *RPS19*. The actual involvement of mutated ribosomal components includes 25% *RPS19*,[2] 3% *RPL35A*,[5] 2% *RPS24*,[6] 1% *RPS17*,[7] although mutations have also been frequently found in RPL11 and RPL5[8]. At present, these six genes seem to account for more than 50% of DBA cases. It is apparent that other genes are involved and a systematic survey of all the 80 RP genes has started in the DBA community in the hope of diagnosing all DBA patients.

The paper of Quarello *et al.* in this issue of the journal reports an increased incidence of mutations in *RPS19* compared to that found previously.[9] Since the disease is due to loss-of-function mutations, the Authors decided to evaluate the role of gene deletions in the pathogenesis of DBA. These types of mutations are not screened by methods usually employed for mutation analyses. Since the disease is autosomal dominant, the presence of the wild-type allele masks deletions when a PCR-based methodology, such as Sanger sequencing, is used.

Quarello *et al.* used a technique well-known among geneticists: Multiplex Ligation-dependent Probe Amplification (MLPA) that detects all types of deletions, both those affecting the whole gene and those that affect a single exon. Using MLPA they showed that whole gene deletions are more frequent than expected in *RPS19*. Taking all mutation types into consideration, *RPS19* is mutated in 28% in their panel of 123 Italian patients. They recommend MLPA, or other methods able to detect deletions, such as FISH, as necessary tools for an accurate molecular diagnosis of DBA due to RPS19 deficiency. Such techniques should probably also be implemented for the other frequently mutated DBA genes.

Even though most mutations affecting DBA genes are loss-of-function mutations thought to cause NMD (i.e. deletion, nonsense and frameshift), missense mutations are also well represented. Several papers have addressed the effect of DBA mutations; most of them are focused on RPS19 (recently reviewed in Campagnoli *et al.* 2008;[10] A database reporting mutations and their functional consequences for DBA genes is available at www.dbagenes.unito.it.[11] Analysis of the properties of mutated RPS19 proteins expressed in cultured cells showed that protein stability is more or less decreased according to the different mutations.[12] Moreover, nucleolar localization appears compromised in some cases and, importantly, none of the mutated RPS19 proteins are found assembled into functional ribosomes.[12]

The paper of Crètien *et al.* in this issue of the journal[13] addresses the same aspect, studying the fate of 12 GFP-tagged mutated RPS19 proteins expressed *in vitro* in Cos-7 cells. According to their analysis, the mutated RPS19 proteins fall into two groups: 1) proteins with a slightly decreased stability and normal nucleolar localization; and 2) proteins which show a drastic reduction in stability and an alteration of nucleolar localization. The results match previous studies and are also consistent with the crystal structure of RPS19 from Archea.[14] Mutations of the first class are located in positions which are not predicted to influence the folding of RPS19; mutations of the second class, on the other hand, are supposed to affect the general structure of the protein and therefore its stability. Crètien *et al.* then address the mechanism of degradation of the mutated RPS19 by using proteasome inhibitors. Proteasome is involved in the maturation of ribosome precursors and in the degradation of RPs which are normally produced in excess.[15] By inhibiting proteasomal degradation the researchers obtained an increase in the levels of mutated RPS19 proteins and restored nucleolar localization. Extending the results of previous studies,[12] their findings, therefore, indicate that the fate of the mutated RPS19 is to be degraded by the proteasome.

Although, as decribed above, several studies suggest that DBA is a ribosomapathy, the link between the gene dosage defect for an RP and the defective erythropoiesis remains unsolved. A defect in the processing of ribosomal RNA (rRNA) has been identified both in $CD34^+$ cells from DBA patients and in erythroid cells down-regulated for RPS19.[16] Similar alterations in the rRNA maturation pathway have also been shown in the case of deficiency (experimentally induced or due

to mutations) of the other DBA-associated RPs: RPS24, RPS17, RPL35A, RPL5 and RPL11.[5,17,18] The processing step affected is different among the various RPs (summarized in Figure 1) but results, in all cases, in a ribosome biogenesis defect. This observation supports the hypothesis of a defect in general protein synthesis to explain the pathogenesis of DBA: reduced capacity of protein production would affect proliferation mostly evident in tissues with a high turnover such as the erythron and the embryo. Indeed, decreased protein synthesis has been shown in lymphocytes from DBA patients.[19] This hypothesis, though, is not completely convincing and alternative views have been proposed to explain the tissue specificity. The amount of RP expression could be tissue-specific, as such certain RPs would be a limiting factor in a definite tissue.[20]

Another hypothesis focuses on a non-ribosomal function for RPs, that would be decreased by haploinsufficiency. The identification of the RPS19 interactome has increased speculation about other possible roles for this protein, but has not solved the DBA issue.[21]

The paper by Rey *et al.* in this issue of the journal[22] presents another suggestive hypothesis based on the observation of increased abnormally spliced forms of FLVCR1 in DBA immature erythroid cells. This has been obtained in cells from patients that did not carry RPS19 mutations, but the same scenario was shown by K562 cells down-regulated for RPS19. The FLVCR1 protein is a heme exporter and the receptor for the subgroup C leukemia retrovirus. This virus causes anemia in infected cats because its Env protein blocks the heme exporter activity. A phenotype similar to DBA (i.e. anemia and craniofacial and limb deformities) is shown by *Flvcr1* null mice.[23] Although *Flvcr1* is not mutated in DBA patients, the similarity among the two phenotypes is striking. The paper by Rey *et al.* now suggests a possible functional link between the two syndromes that could explain the similar phenotype. The abnormally spliced forms of *FLVCR1*, that could not produce a normal protein, could play a role in the defective erythropoiesis of DBA patients. The authors suggest that a disruption of heme transport leads to heme toxicity and apoptosis of early erythroid progenitors. Actually, a reduction of CFUe has been found in *Flvcr1* null mice.[23] Interestingly, human erythroid precursors down-regulated for FLVCR1 exhibit features of defective erythropoiesis, but normal myelopoiesis: an exact recapitulation of DBA pathophysiology.

The definite role of FLVCR1 in regulating erythropoiesis is still unknown. Neither is it known how exactly a defect in RPS19 or other DBA genes can influence *FLVCR1* splicing. The identification of splicing factors among RPS19 interactors[21] can suggest interesting speculation about a role for RPs in controlling the spliceosome. More experiments are needed to explain the abnormal *FLVCR1* splicing in DBA, but the observation is intriguing.

A further interesting hypothesis to explain the pathophysiology of DBA proposes that the impairment of ribosome biogenesis induced by the decrease of an RP (*nucleolar or ribosomal stress*) might cause p53 activation

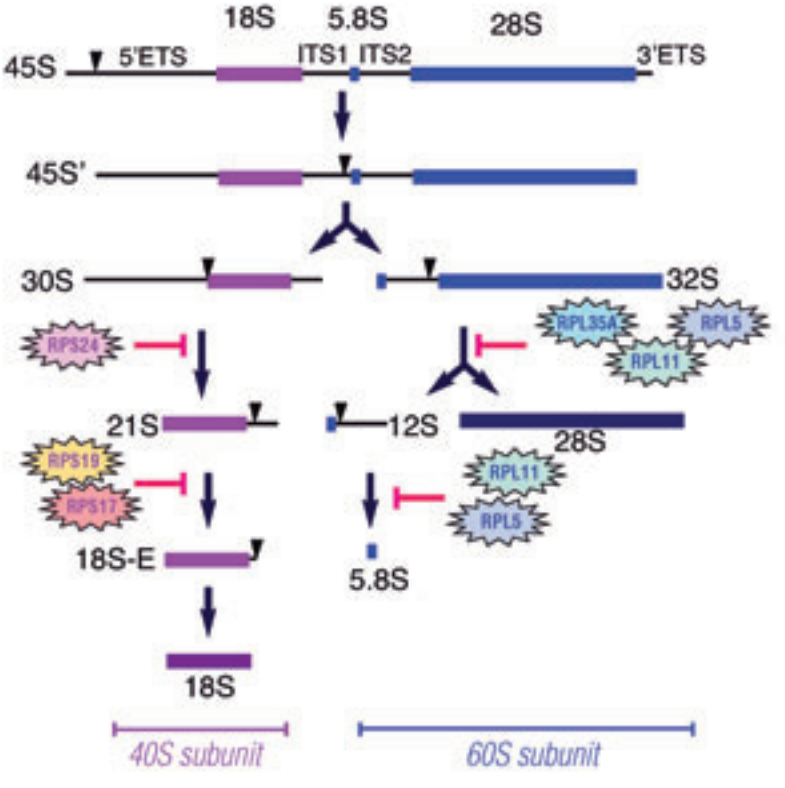

Figure 1. Simplified scheme of rRNA processing in human cells. Only the major pathway and cleavage sites are shown. The name of precursors is indicated on the side of each molecule. The mature rRNA forms (18S, 5.8S, 28S), and the external (5' ETS, 3' ETS) and internal (ITS1, ITS2) spacers are indicated on the first precursor (45S). The principal cleavage sites are indicated by the black triangles. The processing steps possibly affected in DBA cells with mutations in RPS17,[18] RPS19,[16] RPS24,[17] RPL35A,[5] RPL5,[18] RPL11[18] (Gleizes PE, Gazda HT, personal communication), are indicated by red lines.

with a consequent cell growth arrest and/or apoptosis, as schematized in Figure 2.[3] Recently, vertebrate models for DBA have been produced that support this hypothesis. Two studies published in Human Molecular Genetics[24] and Blood[25] propose zebrafish as a model system for studying the molecular mechanism underlying DBA. In both cases the researchers induced RPS19 deficiency by injecting antisense oligonucleotide analogs (morpholinos) into one-cell-stage embryos. Uechi *et al.*[24] report that the decrease of RPS19 causes a dramatic reduction in blood cells and deformities in the head and tail region of the injected embryos. Importantly, the phenotype can be rescued by the injection of *rps19* mRNA, but not if it contains mutations found in DBA patients. The authors of the study then induced deficiency for other 20 RPs and grouped the mutants according to the severity of the phenotype. They found that the knock-down of three other RPs causes blood cell defects similar to rps19 mutants. Interestingly, among them is *rpl35a* which has been recently found mutated in DBA patients.[5] In the second study, Danilova *et al.*,[25] after finding that rps19 deficiency results in hematopoietic and developmental abnormalities similar to DBA, address the molecular mechanism of the defects. They found that the decrease of rps19 in the embryos injected with morpholinos induces changes in the transcription of p53 family members. Particularly evident is the increase of deltaNp63 in red blood cell progenitors purified from embryos. Similar alterations of expression of the p53 family were observed in zebrafish with mutations in *rps8*, *rps11* and *rps18*. Therefore, the Authors propose that ribosomal stress caused by rps19 deficiency in zebrafish results in the upregulation of the p53 family with a consequent imbalance between the network components. This would lead to the observed alterations in development and differentiation. Consistent with their model, the authors could alleviate the phenotype of rps19 mutants by treatment with an inhibitor of p53 function, so suggesting the possibility of a novel drug treatment for DBA by targeting p53.

In addition to these zebrafish models, a new mouse recapitulating DBA has been produced.[26] This mouse, obtained by random chemical mutagenesis, presents a R32L mutation in Rps19 and a different phenotype as compared to the *RPS19* knock-out mouse produced a few years ago.[27]

Indeed both mice, Rps19 knock-out and the R32L mutant, are homozygous lethal in the embryo. By contrast, the heterozygous KO mouse is identical to the WT, whereas the heterozygous R32L mouse shows a slightly reduced erythrocyte level, increased apoptosis of bone marrow erythroid precursors, reduced body weight and, most strikingly, dark spots in the limb and tail (*dark skin*). This phenotype is reminiscent of DBA, that is characterized by defective erythropoiesis, apoptosis of erythroid precursors and reduced body weight. However, this mouse does not carry physical malformations. Moreover, discromy is not normally present in DBA, although sometimes it has been reported in DBA and in Fanconi patients. McGowan *et al.* have made great efforts to explain the mice discromy. Epitheliocytes from mice defective in *rps6* show an increased expression of p53 targets and, especially, Mdm2 and Kitl. This scenario supports the hypothesis that stabilization of p53 is due to the increased availability of free

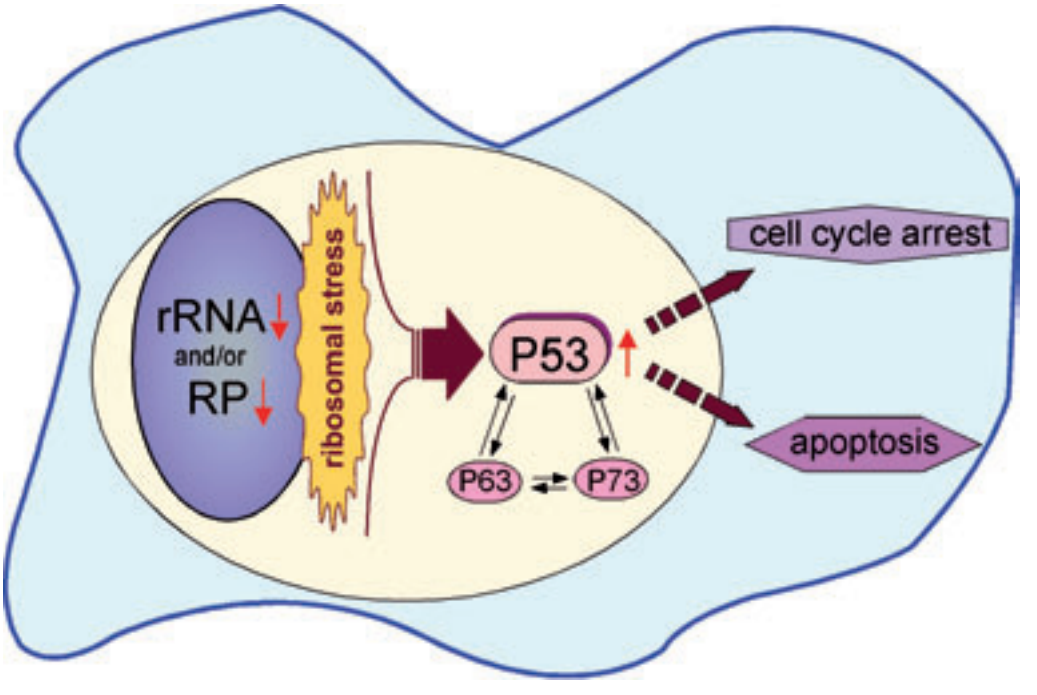

Figure 2. Ribosomal stress hypothesis. A decrease in the synthesis of an RP and/or rRNA triggers stabilization and/or activation of p53 and, possibly, unbalance with the other p53 family members. According to the susceptibility of the cell type to this alteration the induced response could be apoptosis or cell cycle arrest. This in turn can generate different phenotypical alterations: anemia, hyperpigmentation, growth retardation, malformations.

RPs, not used to assemble ribosomal subunits. Some RPs, such as RPL5 and RPL11, were shown to interact with MDM2 and prevent MDM2-dependent p53 ubiquitination and degradation. Kitl, a p53 target, is known to regulate melanocyte migration from the neural crest and their function. Interestingly, the increased number and activity of melanocytes in the epidermis of dark skin mice is due to a p53 effect, because the mouse *Trp53*$^{-/-}$ Rps19 R32L$^{-/+}$ shows a phenotype reversal and no dark spots. Authors state that an increased *Kitl* cannot be involved in the defective erythropoiesis, because mice defective in *Kitl* (the so-called Steel mice) are anemic and their anemia is not cured by bone marrow transplantation, unlike DBA patients. It is interesting to note that erythroid progenitors from DBA patients show a dramatic response *in vitro* by the addition of KITL to EPO and IL-3.[28] Moreover, the heterozygous defect in the KIT gene is the cause of piebaldism (MIM 172800) in humans, a condition characterized by discromy, but not by anemia.

In conclusion, many strides have been made towards solving DBA mystery. The most striking successes have been obtained in genetics, diagnostics and clinical treatment. In this regard, for the sake of brevity, we refer to the consensus document generated by the DBA community.[1]

The pathophysiology of DBA has been addressed by a number of elegant and systematic approaches, including the production of cell and animal models. These strategies have shed light on several aspects of DBA and proposed several not mutually exclusive hypotheses, but have failed to obtain a comprehensive picture. Therefore, DBA still remains a ribosomal puzzle.

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