Case Study

A Novel *RPL35A* Mutation Associated with Diamond-Blackfan Anemia: Report of a Case and Review of the Literature

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Abstract: Diamond Blackfan anemia (DBA) is a genetically and clinically heterogeneous disorder characterized by pure red blood cell aplasia, variable congenital anomalies, and a predisposition to cancer. The genes implicated in DBA all encode ribosomal proteins associated with either the small or large ribosome subunits. We report a novel variant of unknown significance in the *RPL35A*gene (p.R76P) in an Asian male presenting with macrocytic anemia, neutropenia, genitourinary malformations, and growth retardation without significant erythroid hypoplasia in bone marrow. We postulate that this variant is likely pathogenic and contributing to this patient's DBA phenotype; the variant has never been reported in the general population, it changes a highly conserved amino acid, and is predicted to be deleterious by *in silico* models, while clinically this patient responded well to steroid treatment.

Keywords: Diamond-Blackfan anemia, ribosomal protein genes, mutation, *RPL35A*

Introduction

First reported in 1936, and formally described and characterized in 1938 [1], Diamond Blackfan anemia (DBA) is also known as Blackfan-Diamond anemia, congenital hypoplastic anemia, and inherited erythroblastopenia [2]. DBA is a heterogeneous disorder characterized by macrocytic anemia, reticulocytopenia, congenital anomalies, and predisposition to cancer. The incidence of classic DBA is about seven per million live births with both genders equally affected [4]. Approximately 40-45% of DBA cases are familial and inherited mostly in an autosomal dominant pattern with incomplete penetrance. The hematological symptoms of DBA typically occur within the first year of life [5]. Congenital malformations are present in approximately half of individuals with DBA, including craniofacial, heart, and genitourinary malformations as well as small or malformed thumbs and other upper-limb malformations. Growth retardation also occurs in about thirty percent of affected individuals [6].

The underlying defect of DBA is hypothesized to be faulty ribosome biogenesis, resulting in pro-apoptotic erythropoiesis and erythroid failure. Mutations are identified in approximately 60% of DBA patients by sequencing fifteen genes, which all encode ribosomal proteins (RP) associated with the small

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or large subunit [7]. To date 15 RP genes have been found mutated in DBA patients, including *RPS7, RPS10, RPS17, RPS19, RPS24, RPS26, RPS27, RPS29, RPL5, RPL11, RPL15, RPL26, RPL27, RPL31, RPL35A* [5]. Ribosomal Protein L35a (RPL35a) is encoded by the *RPL35A* gene in humans, which is located on chromosome 3q29-qter. Pathogenic mutations in *RPL35A* account for approximately 3% of DBA cases [3].

In our study, a novel *RPL35A* variant of unknown significance, p.R76P, was identified in a 4-month-old Asian male who presented with macrocytic anemia, neutropenia, genitourinary malformations, and growth retardation. This variant changes a highly conserved amino acid, and it was not reported in major databases or online literature. This patient's anemia had excellent clinical response to steroid treatment.

Case Report

The patient was a non-identical male twin born at 33 weeks gestation. At 4 months of age, he was first evaluated for groin swelling, predominantly on the right. Physical examination showed that he was small for his age (2nd percentile by WHO growth charts), with multiple genitourinary malformations, including small penile glans, penoscrotal hypospadias, congenital chordee, and hooded foreskin. This patient's complete blood cell count (CBC) showed white blood cell (WBC) count of 3.46 billion/L, hemoglobin (HGB) 7.2 g/dL, hematocrit (HCT) 20.6%, mean corpuscular volume (MCV) 102.1 fL/cell, platelets (PLT) 570 billion/L, and absolute neutrophil count (ANC) 0.2 billion/L. He had no significant family history, and his twin brother was normal in size. His anemia did not improve with 3-month iron supplementation. Bone marrow evaluation revealed mild dyserythropoiesis, relative lymphocytic and megakaryocytic hyperplasia, relative granulocytic hypoplasia, and decreased marrow iron stores [Figure 1]. However, no significant erythroid hypoplasia was identified, and an accurate assessment of the marrow cellularity was not possible due to the subcortical nature of this biopsy. Chromosome studies showed normal male karyotype 46,XY[20] and no clonal abnormality was apparent. Myelodysplastic syndrome (MDS) panel by fluorescence in situ hybridization (FISH) was within normal limits. Erythrocyte adenosine deaminase (eADA) was elevated (2.4 U/g Hb).

Molecular tests for Shwachman Diamond syndrome and Fanconi anemia were performed and the results were negative; molecular genetic testing for DBA was then pursued. Genomic DNA was isolated from the patient's blood specimen using standardized methodology and quantified. Nine RP genes were sequenced, including *RPL11, RPL35A, RPL5, RPS10, RPS17, RPS19, RPS24, RPS26, and RPS7*. All the analyzed regions of the genes were amplified through polymerase chain reaction (PCR) and sequence alterations were identified by double-stranded sequencing from sense and anti-sense directions. Sequence analysis revealed a novel c.227G>C (p.R76P) variant, located in coding exon 3 of the *RPL35A* gene, resulting from a G to C substitution at nucleotide position 227 [Figure 2A]. A highly conserved arginine residue at codon 76 was replaced by a proline residue, an amino acid with dissimilar properties (Grantham distance score=103) [Figure 2B]. Evolutionary conservation analysis shows this amino acid position is highly conserved in available vertebrate species [Figure 3]. This variant has not been reported in population based cohorts in the following databases: Database of Single Nucleotide Polymorphisms, 1000 Genomes Project, and the Genome Aggregation Database (gnomAD). In addition, this alteration is predicted to be possibly damaging and deleterious by Polymorphism Phenotyping (PolyPhen) (0.886) [8] and Sorting Intolerant From Tolerant (SIFT) *in silico* analyses (0.000) [9], respectively. No other pathogenic mutations or variants of unknown significance were detected in the genes mentioned above.

Based on this finding and the patient's clinical presentation, a diagnosis of DBA was postulated

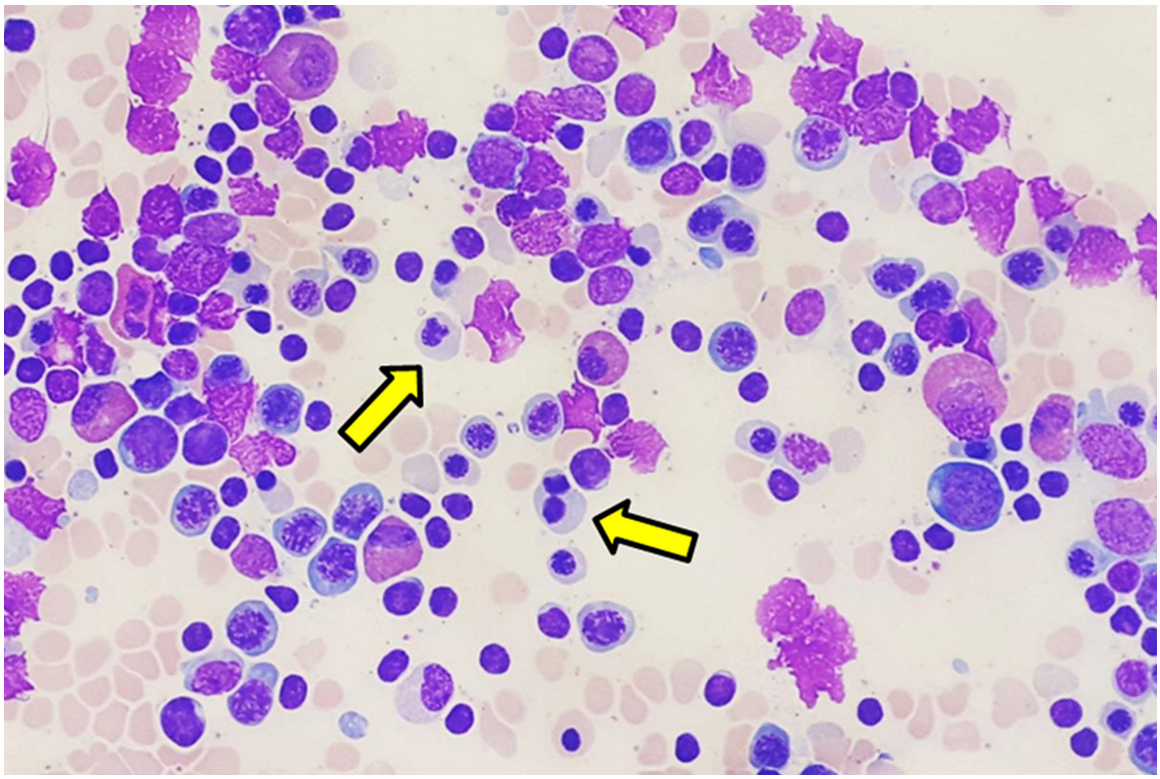

Figure 1: Bone marrow evaluation reveals mild dyserythropoiesis (arrows) without significant erythroid hypoplasia.

and steroid therapy was initiated (prednisolone 1mg/Kg/day, later reduced to 0.8 mg/Kg/day). 17 months later, his anemia had improved and laboratory tests showed WBC 4.35 billion/L, HGB 11.6 g/dL, HCT 33.0%, MCV 98.5 fL/cell, PLT 522 billion/L, and ANC 0.4 billion/L; his growth percentile increased to 7% by WHO growth charts.

Discussion

DBA is a member of the inherited bone marrow failure syndromes (IBMFS), a heterogeneous group of disorders characterized by bone marrow failure usually in association with one or more somatic abnormalities. Besides DBA, other classical examples in this group include Fanconi anemia, dyskeratosis congenita, Shwachman-Diamond syndrome, congenital amegakaryocytic thrombocytopenia, and severe congenital neutropenia [10]. Heterozygous mutations in genes encoding either the small or large ribosomal subunits have been identified in 60% of DBA patients, and include missense, nonsense, frameshift, and splice site mutations as well as large deletions; consequently, haplo-insufficiency is thought to be responsible for disease [11]. DBA is typically treated with steroids, red blood cell transfusions, and hematopoietic stem cell transplantation.

Diagnosis of DBA is challenging because its clinical features overlap with other IBMFS and often hinges upon exclusion of other IBMFS [5]. According to the 6th Annual Diamond Blackfan Anemia International Consensus Conference held in New York on April 16-18, 2005, DBA is diagnosed using both diagnostic criteria and supportive criteria [4]. The diagnosis of "classic DBA" is made if all of the following diagnostic criteria are met: 1) age <1 year; 2) macrocytic anemia with no other significant cytopenias; 3) reticulocytopenia; 4) normal

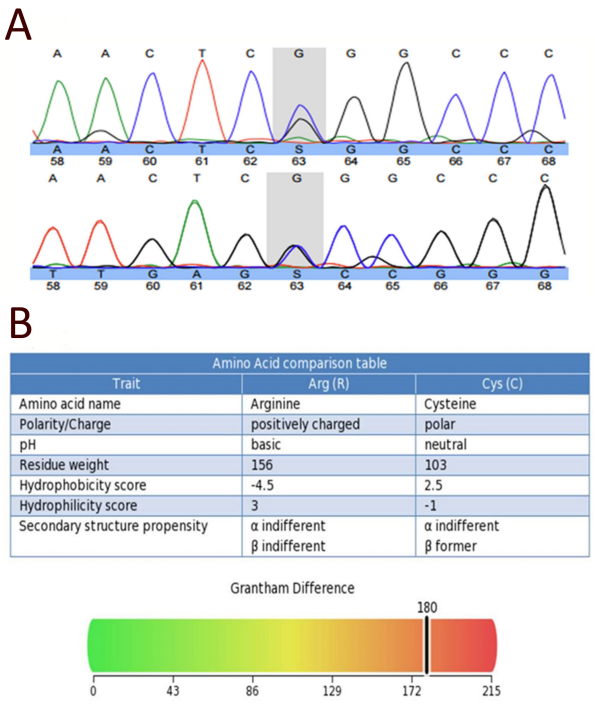

Figure 2: A: Novel c.227G>C (p.R76P) variant identified in exon 3 of *RPL35a* gene. B: Grantham table and difference between arginine and proline.

marrow cellularity with a paucity of erythroid precursors. If all of the above diagnostic criteria cannot be met, supportive criteria, including major and minor findings, can be used to make a "probable diagnosis". The major supporting criteria includes: 1) the presence of a gene mutation associated with DBA; 2) positive family history. The minor supporting criteria includes: 1) elevated eADA activity; 2) congenital anomalies associated with DBA; 3) elevated fetal hemoglobin (Hgb F); 4) no evidence of another inherited bone marrow failure syndrome. In our case, this particular patient had no family history and presented with macrocytic anemia, neutropenia, genitourinary malformations, and growth retardation without significant erythroid hypoplasia in bone marrow. Therefore, he did not meet the criteria and the diagnosis of either "classic DBA" or "probable DBA" cannot be made.

The DBA International Consensus Conference suggests that a diagnosis of "sporadic, non-classic DBA" can be made if a patient has a DBA-associated gene mutation but with insufficient diagnostic criteria. In our case, a novel c.227G>C (p.R76P) variant was identified using DNA sequencing and it has never been reported before. It is a missense variant located in the third exon of the *RPL35A* gene. This variant causes a single nucleotide change from Guanine (G) to Cytosine (C) at position 227. This results in an amino acid change from arginine to proline at codon 76 of the RPL35A protein. Evolutionary conservation diagram shows that this amino acid position is highly conserved in available vertebrate species. Different predicting tools also show that this amino acid alteration is possibly damaging or deleterious. Based on these evidence, we consider that this case is highly suspicious for "non-classic DBA". We also consulted the Diamond Blackfan Anemia Registry and both Dr. Lipton and Dr. Farrar thought that this variant was likely to be disease causing. Therefore, steroid therapy was initiated and this patient had an excellent response.

Pediatric MDS is a group of rare clonal hematopoietic stem cell disorders characterized by varying degree of cytopenias, ineffective and dysplastic hematopoiesis, and the risk of leukemic transformation. The clinical, laboratory, and histologic presentation of pediatric MDS shares significant overlap with other inherited and acquired bone marrow failure disorders [12]. Therefore, pediatric MDS should be ruled out as a possibility when an IBMFS is suspected. Careful physical examination for congenital anomalies, past medical history, family history, specific laboratory tests and genetic studies can provide evidence to distinguish pediatric MDS from IBMFS. In the current case, the patient's age (4 months old), congenital malformations, growth retardation, laboratory and genetic tests results, and negative results of cytogenetic and MDS FISH studies disfavor pediatric MDS.

In conclusion, we have identified a single missense variant in the *RPL35A* gene. We postulate that this variant is likely pathogenic and contributing

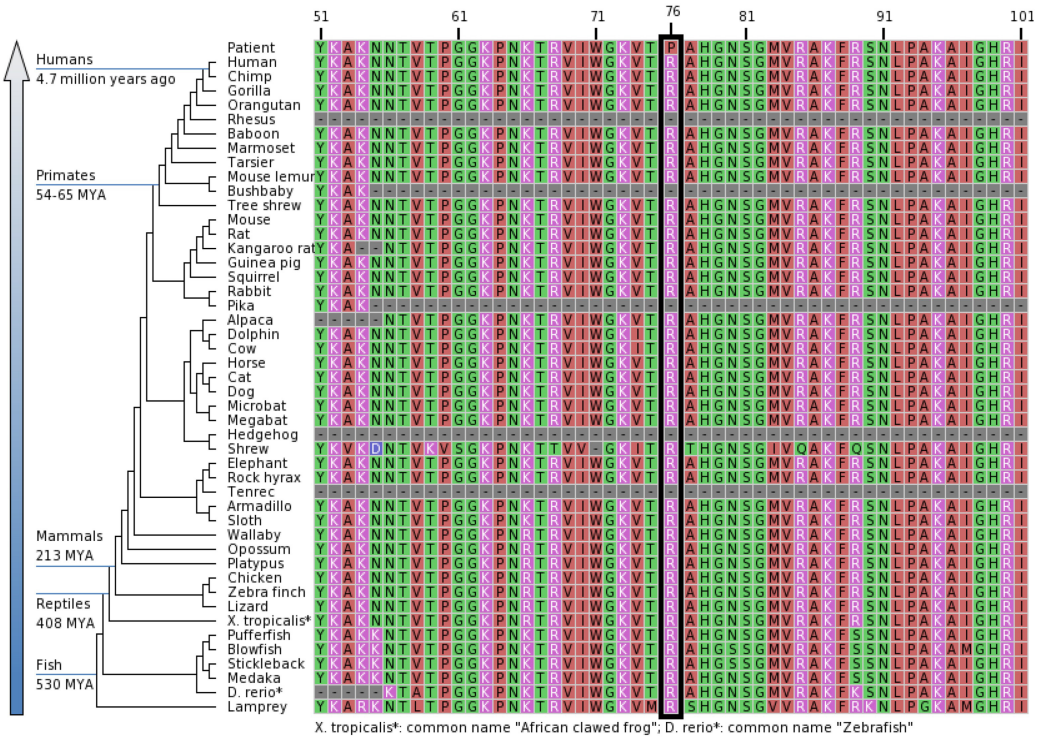

Figure 3: Evolutionary conservation diagram showing amino acid alignment of the *RPL35A* protein in different vertebrate species. The boxed amino acid is poorly conserved among vertebrate species analyzed.

to the patient's DBA phenotype based on its rarity, change of a highly conserved amino acid, prediction of pathogenicity by *in silico* analyses, and the excellent clinical response of the patient's anemia to steroid treatment. Genetic testing of this patient's alternative samples such as buccal swabs and skin biopsy, as well as testing other family members including the patient's parents and sibling, can provide important information on whether this *RPL35A* variant is germline or somatic, inherited or *de novo* and provide further evidence of its pathogenicity.

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