2021

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ARTICLE

Genotype-phenotype association and variant characterization in Diamond Blackfan anemia caused by pathogenic variants in *RPL35A*

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Ferrata Storti Foundation

Haematologica 2021
Volume 106(5):1303-1310

ABSTRACT

Diamond Blackfan anemia (DBA) is predominantly an autosomal dominant inherited red cell aplasia primarily caused by pathogenic germline variants in ribosomal protein genes. DBA due to pathogenic *RPL35A* variants has been associated with large 3q29 deletions and phenotypes not common in DBA. We conducted a multi-institutional genotype-phenotype study of 45 patients with DBA associated with pathogenic *RPL35A* germline variants and curated the variant data on 21 additional cases from the literature. Genotype-phenotype analyses were conducted comparing patients with large deletions *versus* all other pathogenic variants in *RPL35A*. Twenty-two of the 45 cases had large deletions in *RPL35A*. After adjusting for multiple tests, a statistically significant association was observed between patients with a large deletion and steroid-resistant anemia, neutropenia, craniofacial abnormalities, chronic gastrointestinal problems, and intellectual disabilities ($P<0.01$) compared with all other pathogenic variants. Non-large deletion pathogenic variants were spread across *RPL35A* with no apparent hot spot and 56% of the individual family variants were observed more than once. In this, the largest known study of DBA patients with pathogenic *RPL35A* variants, we determined that patients with large deletions have a more severe phenotype that is clinically different from those with non-large deletion variants. Genes of interest also deleted in the 3q29 region that could be associated with some of these phenotypic features include *LMLN* and *IQCG*. Management of DBA due to large *RPL35A* deletions may be challenging due to complex problems and require comprehensive assessments by multiple specialists including immunological, gastrointestinal, and developmental evaluations to provide optimal multidisciplinary care.

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Received: January 7, 2020.

Accepted: April 1, 2020.

Pre-published: April 2, 2020.

https://doi.org/10.3324/haematol.2020.246629

Introduction

Diamond Blackfan anemia (DBA) is a rare inherited bone marrow (BM) failure syndrome (IBMFS) with an estimated incidence of 5-10 per million live births.[1-5] It is characterized by failure of red blood cell (RBC) production, congenital malformations, and cancer predisposition. Classic DBA consists of profound anemia diagnosed before one year of age, macrocytosis, reticulocytopenia, and a paucity of erythroid precursors in the BM.[6] However, DBA is a heterogenous disorder with many cases having additional symptoms or no symptoms at all. Congenital abnormalities are variable but include mostly midline craniofacial defects, renal, cardiac, and thumb abnormalities.[1] Malignancies associated with DBA include myeloid neoplasia, colorectal adenocarcinoma, osteogenic sarcoma, and genitourinary cancers.[7,8] Standard treatment for anemia in DBA is long-term corticosteroids. Nearly 40% of people with DBA who respond to initial treatment become steroid-dependent, and those who fail to respond to corticosteroids require chronic RBC transfusions and iron chelation or hematopoietic cell transplant (HCT).[1,9]

DBA is predominately an autosomal dominant disorder caused by pathogenic germline variants in genes encoding ribosomal proteins.[10] Twenty-six ribosomal genes have been linked to DBA etiology as well as two X-linked genes, *TSR2* and *GATA1*, which encode a ribosome chaperone and a hematopoietic transcription factor targeted by altered ribosome levels, respectively.[10,11] Although all known DBA genes are typically included in gene discovery analysis or descriptive cohort studies of DBA, genotype-phenotype studies aimed to find clinical association by gene are currently limited. Only the most frequent ribosomal genes have been examined from a genotype-phenotype perspective (e.g., *RPS19, RPL11, RPL5*) with little to no information available for the majority of disease-causing genes, including *RPL35A*.[10,12] *RPL35A* was first associated with DBA in 2008 and has been reported to cause about 3.5% of DBA cases.[13,14] Typically, data on DBA due to *RPL35A* have been confined to case reports, or as part of a larger DBA study with limited to no phenotypic information.[10,13-20]

RPL35A codes for a large ribosomal subunit protein located at the telomeric end of chromosome 3q (3q29-qter); pathogenic germline variants have been reported as single-nucleotide variants (SNV), small frameshifts, inframe deletions, and large deletions involving the entire *RPL35A* gene with or without multiple contiguous genes deleted in the 3q29 region.[14,15] Patients with DBA caused by *RPL35A* have been reported to have severe anemia as well as additional phenotypes not usually shared by other DBA patients, such as immunodeficiency and autism spectrum disorder.[12,13,15] It is unclear whether these phenotypes are associated with the deletion of *RPL35A* itself or other genes deleted in the region. Some of these phenotypes overlap with 3q29 deletion syndrome, a clinical syndrome in which the contiguous deleted region is near, but does not include, *RPL35A*.[15] The phenotype of 3q29 deletion syndrome is thought to be related to the genes within the deleted region and includes dysmorphic facial features, intellectual disability, musculoskeletal problems, and neuropsychiatric issues.[15,21,22] The deletion in 3q29 deletion syndrome is typically about 1.5 megabase (Mb) in size, and the consistent size is thought to be related to low copy repeat regions at each end of the deletion.[21,23,24]

We conducted a multi-institutional international collaborative study of patients with DBA due to *RPL35A* to determine the clinical consequences of germline large deletions *versus* other pathogenic *RPL35A* variants. We also assembled variant data from additional published cases of *RPL35A*-associated DBA to better characterize pathogenic germline variants in this disease. The phenotypes of these patients were compared with those of the 3q29 deletion syndrome to elucidate the similarities and the differences related to variants in this region of the genome.

Methods

Study population

Patients with DBA due to *RPL35A* were identified within the National Cancer Institute (NCI) IBMFS cohort (*clinicaltrials.gov identifier: NCT00027274*),[25] and through collaboration with investigators from the DBA Registry of North America (DBAR: *clinicaltrials.gov identifier: NCT00106015*),[26] DBA registries from Germany, France, Italy, the Czech Republic, and Greece, and through investigators from Alabama Children's Hospital, Arkansas Children's Hospital, and Boston Children's Hospital (Figure 1 and *Online Supplementary Table S1*). All individuals were participants in Institutional Review Board approved protocols and had signed informed consents for participating in research studies.

Additional cases of DBA for *RPL35A* pathogenic variant characterization were identified through a search of PubMed and review of ClinVar[27] and Human Gene Mutation Database (HGMD).[28] All data were extracted as of February 22, 2019. ClinVar variants were restricted to pathogenic or likely pathogenic *RPL35A* DBA variants that met the minimum requirements for data sharing and quality assurance,[29] and HGMD was restricted to *RPL35A* DBA disease-causing mutation. Any case that was a potential duplicate of a collaborator case was excluded (Figure 1).

Clinical data extraction

Data extraction focused on clinical criteria associated with DBA and with 3q29 deletion syndrome.[22,30] A positive finding was counted as present, and a clinical finding marked absent or not stated was considered absent. Standard criteria for defining cytopenia and immunodeficiency were used, and definitions of phenotypes studied are outlined in *Online Supplementary Table S2*.[31]

Pathogenic variant calling and variant annotation

Pathogenic variant locations (i.e., genomic or chromosomal coordinates) were used as provided by collaborators or from publications. Methods used to identify pathogenic variants included targeted sequencing, panel testing, exome sequencing, multiplex ligation-dependent probe amplification, SNP array, or array comparative genomic hybridization (*Online Supplementary Table S3*). Inclusion of a variant in the study required it to be reported as pathogenic, be rare (minor allele frequency [MAF] <1% within any gnomAD ethnic subgroups[32]), missense variants needed to be predicted pathogenic by meta *in silico* predictor programs CADD (>25)[33] and REVEL (>0.5)[34] (*Online Supplementary Table S3*), and the same case could not be included more than once. Any variants of unclear pathogenicity, including untranslated regions (UTR) and duplication, were excluded. A "large deletion" was defined as a deletion of the entire *RPL35A* gene. "Small frameshift" included all insertion and deletions that were not large or inframe. SnpEff was used to annotate missing chromosomal, genomic, or protein positions.[35] ANNOVAR was used to annotate MAF from publicly

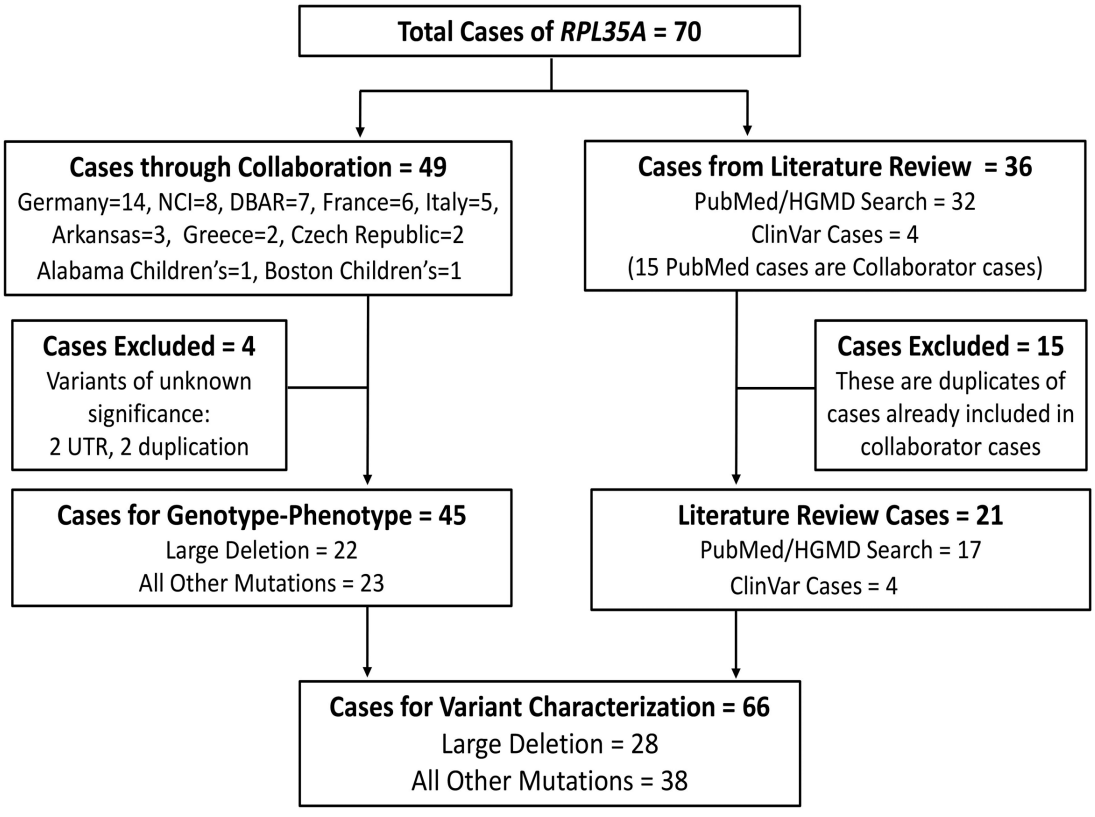

Figure 1. Study population and types of *RPL35A* variants in patients with Diamond-Blackfan anemia (DBA). NCI: National Cancer Institute Inherited Bone Marrow Failure Study; DBAR: DBA registry; HGMD: Human Gene Mutation Database; Alabama Children's: Arkansas Children's Hospital; Boston Children's: Boston Children's Hospital; UTR: untranslated regions; ClinVar: ClinVar public data archive.

available databases and obtain *in silico* prediction scores.[36] The St. Jude Cloud PeCan tool was used to create lollipop plots.[37] For large deletions, UCSC Table Browser was used to identify all deleted genes in that region.[38]

Genotype-phenotype statistical analysis

The cases for the genotype-phenotype analysis were made up exclusively from the ten collaborating institutions (Figure 1). The cases identified through literature review had limited to no available clinical data and were included in *RPL35A* variant characterization. All statistical analyses used the individual case as the analysis unit. We compared 21 different clinical features between cases with large deletions *versus* all other pathogenic variants using Fisher exact test or Student *t*-test (Stata 15 software[39]). Bonferroni correction for 21 tests was applied. $P<2.4\text{x}10^{-3}$ were considered significant.

Results

Cohort clinical characterization and genotype-phenotype relationship

We compiled a total of 45 cases of DBA caused by *RPL35A* variants from ten different institutions/registries after exclusion of four cases with variants of unknown significance (Figure 1). Table 1 shows the clinical features of the 45 patients from 41 different families. Fifteen of these cases have been published previously (*Online Supplementary Table S3*).[10,14,18,40-42] Nearly half of the patients (n=22) had large deletions involving the entire *RPL35A* along with additional genes deleted in the region. All other pathogenic variants (n=23) were either nonsense, missense, splice site, small frameshift, or inframe deletions. Six of these variants are not present in ClinVar, HGMD, or gnomAD, and are considered novel (*Online Supplementary Table S3*).

Forty-four percent of the cohort was male (male:female 1:1.25; P=0.14) (Table 1). Median age at identification of symptoms was at birth in patients with large deletions *versus* two months of age in those with other pathogenic variants (P=0.04) (Table 1). Age of last follow-up was similar for both groups with a median age of 10 and 12 years, respectively (P=0.37) (*data not shown*). All except two patients were alive at last follow-up. The cause of death was right ventricular outflow obstruction due to thromboembolic plaque in one patient and complications related to treatment for Wilms tumor in the other patient.

Erythrocyte adenosine deaminase (eADA) values were available only for a subset of patients. Six of 11 patients with large deletion and 5 of 13 with all other *RPL35A* variants had elevated eADA with no difference between the groups (P=0.682). Anemia was the most common presenting symptom and was identified in all except one patient. More than 86% of patients in both groups had severe anemia needing treatment (Table 1), but a significant number of patients with *RPL35A* large deletions were steroid-resistant and RBC transfusion-dependent compared with patients with other pathogenic variants in *RPL35A* (P=0.0023) (Table 1). The number of patients who received HCT was similar in both groups.

Neutropenia was significantly associated with large deletions (P=0.0017). In addition, a higher proportion of patients with large deletions with severe neutropenia

Table 1. Clinical phenotypes of cases with *RPL35A* large deletion *versus* all other *RPL35A* pathogenic variants.

	Cases	Cases with *RPL35A* large deletion	Cases with all other *RPL35A* variants	*P*
Demographic information				
Total cases	45	22	23	–
Male/female	20/25	7/15	13/10	0.14
Age of presentation in months, median (range)	–	0 (0-12)	2 (0-60)	0.04*
Hematologic and immune phenotypes[1]				
Anemia	44	22	22	1
Requiring treatment	42	22	20	0.23
HCT	7	4	3	0.70
Steroid-resistant[2]	26	18	8	**0.0023**
Thrombocytopenia	4	1	3	0.61
Neutropenia	33	21	12	**0.0017**
Requiring treatment[3]	10	9	1	0.004
Immunodeficiency	9	8	1	0.01
Recurrent infection	15	11	4	0.03
Congenital abnormalities				
Craniofacial	12	11	1	**0.0006**
Microcephaly[4]	10	7	3	0.17
Skeletal/limb[5]	11	9	2	0.02
Cardiac	10	7	3	0.17
Urogenital	10	5	5	1
Short stature[6]	23	11	12	1
Any congenital abnormality[7]	26	16	10	0.07
≥3 abnormalities	13	11	2	0.003
Other phenotypes				
Chronic GI problem	9	9	0	**0.0006**
Intellectual disability	15	13	2	**0.0004**

All cases meeting criteria for sufficient phenotypic information were included (n=45). Large deletion was defined as entire *RPL35A* gene deleted. All other pathogenic variants included missense, nonsense, small frameshift, splice, and inframe deletions. A positive finding was counted as present, and a clinical finding marked absent or not stated was considered absent. *P*-value based upon Fisher exact test and one was Student *t*-test (*). Statistically significant *P*-values after Bonferroni correction are in bold ($P<2.4x10^{-3}$). Treatment for anemia included steroids, chronic transfusion, erythropoietin, and hematopoietic stem cell transplant (HCT). [1]Standard criteria for defining cytopenia and immunodeficiency were used (see also *Online Supplementary Table S2*):[31] neutropenia: absolute neutrophil count $<1.5x10^9$/L; thrombocytopenia: platelet count $<150x10^9$/L; anemia: hemoglobin <2 standard deviation below the mean for the normal population based on age and sex. [2]Steroid resistant and red blood cell transfusion-dependent. [3]Treatment for neutropenia: eight cases had documentation of granulocyte-colony stimulating factor use; neutropenia treatment was not specified in two cases. [4]Head circumference <5th percentile for age and sex. [5]Abormalities of skeleton, ribs, upper and lower extremity. [6]<10th percentile for height based on age and sex. [7]Includes phenotypes noted in the table plus anal atresia, pyloric stenosis, presacral dimple in one case each and inguinal hernia in two cases. GI: gastrointestinal.

(absolute neutrophil count $<0.5x10^9$/L) required treatment for neutropenia compared with patients with other pathogenic variants (n=9 *vs.* n=1; *P*=0.004). Treatment of neutropenia included eight cases with documented use of granulocyte-colony stimulating factor (GCSF) and treatment not specified in two cases. Bone marrow findings were available only in nine patients with large deletions and three with other pathogenic variants. Patients with large deletion and severe neutropenia (n=7) appeared to have both myeloid and erythroid hypoplasia while those with no neutropenia had mainly erythroid hypoplasia (5 of 7 *vs.* 0 of 3; *P*=0.06).

Cases with large deletions involving *RPL35A* were more likely to have an immune system abnormality compared with other pathogenic variants, including an immunodeficiency diagnosis (n=8 *vs.* n=1; *P*=0.01) with low immunoglobulins, low numbers of lymphocyte subsets, or poor response to childhood vaccination, and treatment with intravenous immunoglobulins. Recurrent severe infections requiring hospitalization and treatment with systemic antimicrobials were also reported more commonly in cases with large deletions (n=11 *vs.* n=4; *P*=0.03). It is noteworthy that all cases with large deletion with recurrent infections or immune system abnormalities (12 unique cases) were also neutropenic.

There were six cases with loss of function (LOF) variants (splice site=3, nonsense=1, frameshift=2) among patients with other pathogenic variants in *RPL35A*. Univariate analysis comparing these six cases with the 19 cases with missense or inframe deletions showed an earlier age for the diagnosis of anemia in cases with LOF variants (*P*=0.02) but no association with neutropenia or immunodeficiency. Only one patient (frameshift variant) had severe neutropenia that was managed with GCSF, and one (splice site defect) had neutropenia and immunodeficiency treated with immunoglobulins.

Fifty-eight percent (n=26) of the cohort had at least one congenital abnormality involving craniofacial, skeletal and/or limb, cardiac, renal, genital abnormalities, or microcephaly (Table 1). The abnormalities described in each individual case are listed in *Online Supplementary Table S4*. Craniofacial abnormalities were significantly more frequent in cases with *RPL35A* large deletions compared with other pathogenic variants (n=11 *vs.* n=1; *P*=0.0006). Cases with large deletions were also more likely to have skeletal/limb abnormalities (n=9 *vs.* n=2; *P*=0.02) as well as three or more abnormalities (n=11 *vs.* n=2; *P*=0.003). Short stature, a common feature of DBA, was identified in approximately 50% of cases in each group and there was no difference in the use of steroids in

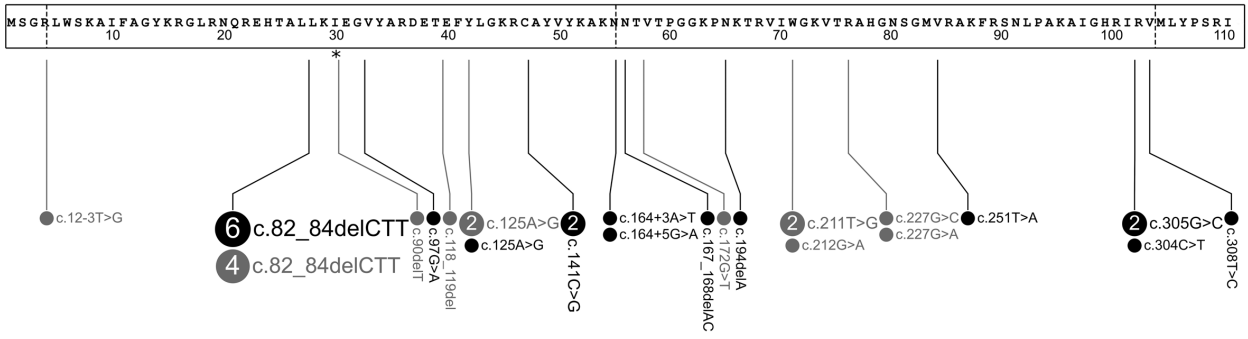

Figure 2. Distribution of *RPL35A* pathogenic variants reported in patients with Diamond-Blackfan anemia (DBA). All pathogenic single nucleotide and small indel variants are represented once per family. cDNA position based on transcript NM_000996.3. Numbers and letters indicate amino acids. Dotted lines indicate exons. Gray circles: literature, HGMD, and ClinVar cases. Black circles: collaborator cases. Cluster, defined as >5 unrelated cases by family in one area, is located between codon 28 to 33 (*). The numbers within the circles indicate the number of unique families with that specific pathogenic variant.

Table 2. Pathogenic germline *RPL35A* variant type.

Variant type	N.	Percent of families
Large deletion	28	45%
Inframe deletion	10	16%
Missense	14	23%
Small frameshift	4	6%
Splice	4	6%
Nonsense	2	3%

Pathogenic variants were only counted once per family. Percent is based upon a total of 62 unique unrelated families in the study. Pathogenic variants must have been reported by collaborators as pathogenic and have minor allele frequency <1% in gnomAD. Variants of unknown significance including two UTR and two duplication variants were excluded. Large deletion was defined as entire *RPL35A* deleted, small frameshift included insertion/deletion that was not large or inframe, and no large insertions were identified.

either group. Delayed development and intellectual disability including learning problems requiring an individualized education program in school were significant among cases with *RPL35A* large deletions compared with other pathogenic variants (n=13 *vs.* n=2; *P*=0.0004) (Table 1); one case also had a diagnosis of autism spectrum disorder. Chronic gastrointestinal (GI) problems, including chronic diarrhea, gastrostomy feeding tube-dependence, and chronic enterocolitis, were also significantly associated with large deletions compared with all other pathogenic variants (n=9 *vs.* n=0; *P*=0.0006) (Table 1).

In an attempt to evaluate the phenotypes of cases with non-large deletion pathogenic variants based on their genomic location, we noted that the variants were spread across the *RPL35A* gene with one area of clustering from codon 28 to 33 (Figure 2). A cluster was defined as >5 unrelated cases in one area. There were nine cases from seven different families from collaborators in this cluster. The variants in six of the seven families were the same inframe deletion (c.82-84delCTT NM_000996.3). We did not find any difference in the hematologic or physical phenotype between the cases in the cluster and those spread across the *RPL35A* gene (*data not shown*).

RPL35A pathogenic variant characterization

In addition to the 45 cases obtained from collaboration with DBA registries/investigators used in the above genotype/phenotype analysis, we identified 21 other cases of DBA due to *RPL35A* pathogenic variants reported in the literature. We characterized the pathogenic variants in all 66 cases from 62 unrelated families. Two families had three affected family members each sharing a variant c.305 G>C or c.82_84CTT (NM_000996.3), respectively.

Twenty-eight of the 62 pathogenic *RPL35A* variants from unrelated families were large deletions (45%) (Table 2). Genomic co-ordinates were not available for six cases with large deletions. The size of the large deletions in 22 cases with available data ranged from 5Kb to 13.42 Mb (*Online Supplementary Table S5*). The number of genes deleted in these cases ranged from two to 153. Large deletions extending upstream and downstream of the 3q29 deletion syndrome region were observed in 13 cases, four cases included part but not all of the region, and five did not include the region at all (Figure 3 and *Online Supplementary Table S5*).

Thirty-four of the 62 pathogenic variants were non-large deletions from 19 unique collaborator families and 15 unique families from literature cases. Fifty-six percent (19 of 34) of the unique family variants were observed more than once (*Online Supplementary Table S6*). The inframe deletion c.82_84delCTT was the most common pathogenic variant (n=10) (*Online Supplementary Table S6*), followed by missense c.125A>G (n=3); three other variants were reported in two families each. All other variants were spread across the gene without clear mutational hotspots and no additional clusters were identified using the pathogenic variants from the cases in the literature (Figure 2). Most of the variants were located in the middle and 3' end of the gene with only one pathogenic variant located near the beginning of the gene.

Discussion

We have compiled the largest cohort of patients with DBA due to pathogenic germline *RPL35A* variants. This international multi-institutional collaboration allowed us to combine well-curated genomic information with extensive clinical data to better characterize the genotype and DBA phenotype associated with variants involving *RPL35A*. We conclude that cases with *RPL35A* large deletions are phenotypically different from cases with other variant types in *RPL35A* and have a more complex clinical phenotype.

Large deletions in *RPL35A* were frequent, comprising 45% of the pathogenic variants in this cohort. The fre-

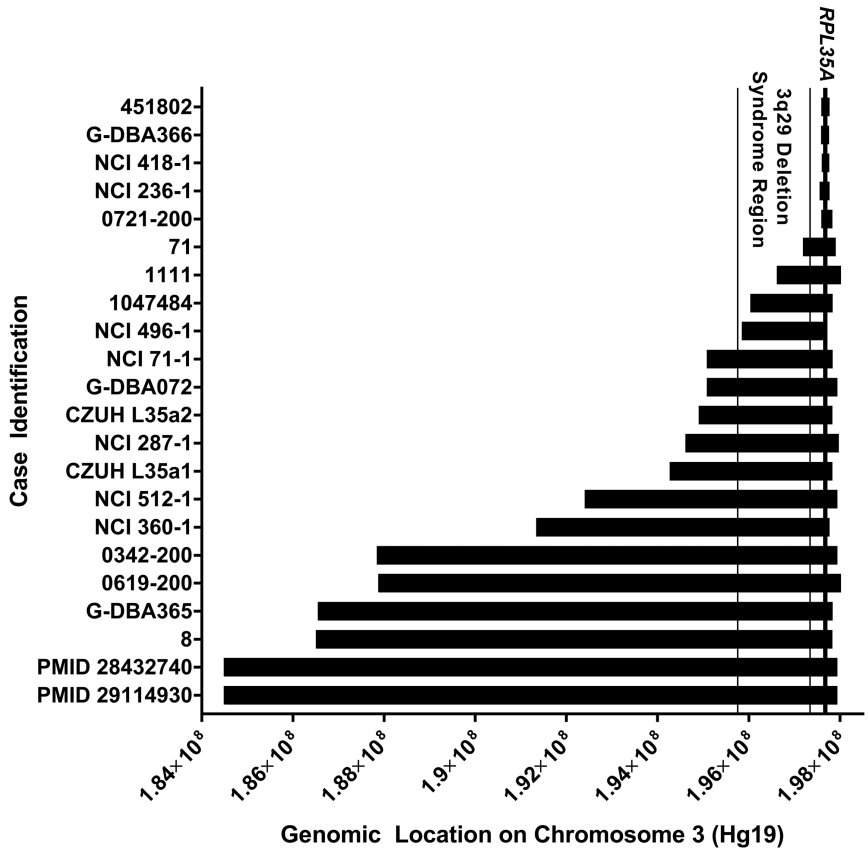

Figure 3. Cases of *RPL35A* large deletions ordered by size in megabases (mB). Each horizontal bar represents the starting and stopping genomic co-ordinates of a *RPL35A* large deletion case. For literature cases without a case identification (ID) in article, the PubMed ID (PMID) was used as case ID (*Online Supplementary Table* S3). Thin black vertical lines indicate genomic starting and stopping co-ordinates of 3q29 deletion syndrome; thick black vertical line is the estimated location of *RPL35A*. See *Online Supplementary Table S5* for exact genes deleted per case.

quency of large deletions in published cases of DBA caused by pathogenic variants in *RPL35A* is unknown due to the rarity of the disease. However, our findings are consistent with what is reported in HGMD where 40% (8 of 21) of DBA cases caused by *RPL35A* are reported as large/gross deletions.[28] Fifty-six percent of non-large deletion pathogenic variants were comprised of five variants. Domain information is not well described for this gene, but we did identify apparent clustering of cases between codon 28 to 33 that may be indicative of a potentially important domain (Figure 2). A sufficient number of variants were not available for a variant cluster-phenotype analysis.

In our genotype-phenotype analysis comparing *RPL35A* large deletions with all other pathogenic variants in *RPL35A*, patients with large deletions exhibited more severe phenotypes across multiple phenotypic domains, including severity of anemia (steroid-resistance and/or RBC transfusion-dependence), neutropenia, craniofacial abnormalities, chronic GI problems, and intellectual disabilities. This suggests that a complex phenotype may be associated with the entire *RPL35A* gene deletion itself or due to contiguous genes deleted in the 3q29 region. Although not well described in DBA cases caused by *RPL35A* variants, similar phenotypes in DBA patients with large deletions in other ribosomal genes have been reported, including multiple congenital malformations, steroid resistant anemia, and neurodevelopmental abnormalities (e.g., *RPL11, RPS17, RPS19, RPS26*).[18,43] Studies comparing cases with *RPL35A* large deletion with DBA due to large deletions involving other ribosomal protein genes may elucidate genotype-phenotype relationships and influences of contiguous gene deletions on DBA phenotypes. Notably, immunodeficiency and recurrent infections were both common features in cases with *RPL35A* large deletions compared with all other pathogenic variants in *RPL35A* (P=0.01 and 0.03, respectively). Although the number of cases is small, neutropenia appears to be contributing to immunodeficiency and recurrent infections, since all these cases were also severely neutropenic and received GCSF treatment. It is unclear from our study whether the severe neutropenia and immunodeficiency seen here is driven by *RPL35A* gene deletion with or without the contribution of contiguous genes deleted in the region or by other LOF variants within *RPL35A* itself, since severe neutropenia (accompanied by immunodeficiency in one case) was also seen in two of the six cases with other LOF variants.

Some of the clinical phenotypes of patients with DBA due to large deletions involving *RPL35A* are similar to those observed in 3q29 deletion syndrome, including congenital abnormalities and intellectual disability, and may be influenced by additional genes deleted in the 3q29 region.[22,23] Previous speculations suggested genes of importance in 3q29 deletion syndrome studies include *PAK2, DLG1*, and *IL1RAP* in intellectual disabilities and *TP63* and *OSTN* in bone defects.[24,44-46] However, immunodeficiency and steroid-resistant anemia are not features found in 3q29 deletion syndrome,[22,23] making it less likely that a gene deleted in the 3q29 deletion syndrome region is the etiology of the immune and severe hematologic phenotype seen in our cohort. A case report of DBA caused by a *RPL35A* large deletion and immune deficiency speculated that *RNF168*, an autosomal recessive gene located in the 3q29 deletion syndrome region that underlies the immune deficiency Riddle Syndrome, could be a possible candidate

leading to immunodeficiency in that case.[15] However, only 12 cases in our cohort with available clinical information and a large deletion also had deletion of *RNF168*. Furthermore, cases of large deletions in our study that did not overlap with 3q29 co-ordinates (n=4) also had evidence of steroid-resistance and/or RBC transfusion-dependence (4 of 4 cases) and neutropenia requiring treatment (3 of 4 cases).

It is possible that genes in close proximity to *RPL35A* but not in the 3q29 deletion syndrome region may contribute to the DBA phenotypes described here. Possible candidate genes could include the closest neighbors and most frequently deleted genes *LMLN* and *IQCG* along with *RPL35A*. These genes were deleted in 15 and 17 of 17 large deletion cases with available co-ordinates, respectively. *LMLN* is a zinc-metalloprotease, and its protein product is invadolysin.[47,48] Antibodies to invadolysin have been shown to concentrate in the edge of macrophage migration, and Drosophila mutants lacking this gene show abnormal cell migration during development.[47,48] *IQCG* has been observed in a somatic fusion with *NUP98* in T-cell acute lymphoblastic leukemia and acute myeloid leukemia. This fusion protein has been hypothesized to block differentiation of hematopoietic stem cells.[49-51] IQCG knockout zebrafish had severely impaired levels of neutrophils, monocytes, macrophages, and lymphocytes.[49] Further studies with functional data are warranted to determine the effect of other gene deletions on the phenotype characteristics of patients with large deletions which include *RPL35A*.

In summary, we compiled the largest cohort of DBA cases with *RPL35A* pathogenic variants to date. Our study is limited by small sample size, retrospective, and non-uniformity of assessment, phenotype coded as absent if not specifically mentioned in patient records, and limited family data. Since our cohort is made up of European and North American cases, the genotype-phenotype relationships may have limited generalizability. We could not examine the impact of a familial component on variation in phenotypes since we only had two families with >1 case per family. We were also limited to the 45 cases from our multi-institutional collaboration for genotype-phenotype analysis, excluding all variants found in the literature due to insufficient phenotypic data. Future studies evaluating genotype-phenotype relationships in rare diseases would benefit from detailed clinical information (as in *Online Supplementary Table S4*) in publications. Moreover, 30 cases included here have never been published, underscoring the importance of reporting all available cases of rare diseases to better characterize the genotype, phenotype, and potential etiology of these diseases. It is also important to include deletion analysis in molecular characterization of patients with DBA, since large deletions may not be identified on standard IBMFS gene panel testing or exome sequencing analysis.

Patients with DBA due to large deletions in *RPL35A* have a complex, multi-system disease phenotype with a high frequency of hematologic as well as non-hematologic problems that is clinically different from DBA associated with other pathogenic variants in *RPL35A*. Distinction of this subtype of DBA with *RPL35A* haploinsufficiency due to large deletion is important for patient management, and evaluations should include thorough investigation for immunodeficiency, GI problems, developmental delays and intellectual disabilities including neuropsychiatric problems. Identification of patients with large deletions in *RPL35A* should trigger early comprehensive assessment by pediatric specialists to provide optimal multidisciplinary care.

Disclosures

DP and JV are supported by the Czech Ministry of Health Grant 16-32105A. AK is consultant of CELGENE and Novartis and and Vifor Pharma and receives Honoraria from CELGENE, Novartis and ApoPharma; CN is a consultant and is on the advisory committee for Celgene; all other authors have no conflicts of interest.

Contributions

DMG and NG wrote the manuscript and performed data analyses; NG, BPA and SAS supervised the study; all authors provided data on the patients in their studies and are responsible for the reported research and have participated in the concept and design, analysis and interpretation of data, and revising the manuscript, and approved the submitted manuscript.

Acknowledgments

We are indebted to the participating families, whose generosity and co-operation have made this study possible.

Funding

This research was supported by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics of the National Cancer Institute; the Czech DBA registry is supported by the Czech Ministry of Health Grant 16-32105A: we acknowledge the contributions made by Ann Carr MS, CGC, Lisa Leathwood, RN, and Maureen Risch, RN provided through contract HHSN261201100018C with Westat Inc. (Rockville, MD, USA); this work utilized the computational resources of the NIH High Performance Computing Biowulf cluster.

References

1. Vlachos A, Ball S, Dahl N, et al. Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. Br J Haematol. 2008;142(6):859-876.
2. Campagnoli MF, Garelli E, Quarello P, et al. Molecular basis of Diamond-Blackfan anemia: new findings from the Italian registry and a review of the literature. Haematologica. 2004;89(4):480-489.
3. Ball SE, McGuckin CP, Jenkins G, et al. Diamond-Blackfan anaemia in the U.K.: analysis of 80 cases from a 20-year birth cohort. Br J Haematol. 1996;94(4):645-653.
4. Willig TN, Niemeyer CM, Leblanc T, et al. Identification of new prognosis factors from the clinical and epidemiologic analysis of a registry of 229 Diamond-Blackfan anemia patients. DBA group of Societe d'Hematologie et d'Immunologie Pediatrique (SHIP), Gesellshaft fur Padiatrische Onkologie und Hamatologie (GPOH), and the European Society for Pediatric Hematology and Immunology (ESPHI). Pediatr Res. 1999;46(5):553-561.
5. Willig TN, Ball SE, Tchernia G. Current concepts and issues in Diamond-Blackfan anemia. Curr Opin Hematol. 1998;5(2):109-115.
6. Diamond LK, Wang WC, Alter BP. Congenital hypoplastic anemia. Adv Pediatr. 1976;22:349-378.
7. Vlachos A, Rosenberg PS, Atsidaftos E, et al. Incidence of neoplasia in Diamond Blackfan anemia: a report from the Diamond Blackfan Anemia Registry. Blood. 2012;119(16):3815-3819.
8. Vlachos A, Rosenberg PS, Atsidaftos E, et al. Increased risk of colon cancer and osteogenic sarcoma in Diamond-Blackfan anemia. Blood. 2018;132(20):2205-2208.
9. Narla A, Vlachos A, Nathan DG. Diamond Blackfan anemia treatment: past, present,

and future. Semin Hematol. 2011;48(2):117-123.
10. Ulirsch JC, Verboon JM, Kazerounian S, et al. The genetic landscape of Diamond-Blackfan anemia. Am J Hum Genet. 2018;103(6):930-947.
11. Khajuria RK, Munschauer M, Ulirsch JC, et al. Ribosome levels selectively regulate translation and lineage commitment in human hematopoiesis. Cell. 2018;173(1):90-103.e119.
12. Arbiv OA, Cuvelier G, Klaassen RJ, et al. Molecular analysis and genotype-phenotype correlation of Diamond-Blackfan anemia. Clin Genet. 2018;93(2):320-328.
13. Kuramitsu M, Sato-Otsubo A, Morio T, et al. Extensive gene deletions in Japanese patients with Diamond-Blackfan anemia. Blood. 2012;119(10):2376-2384.
14. Farrar JE, Nater M, Caywood E, et al. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. Blood. 2008;112(5):1582-1592.
15. Alkhunaizi E, Schrewe B, Alizadehfar R, et al. Novel 3q27.2-qter deletion in a patient with Diamond-Blackfan anemia and immunodeficiency: case report and review of literature. Am J Med Genet A. 2017; 173(6):1514-1520.
16. Smetanina NS, Mersiyanova IV, Kurnikova MA, et al. Clinical and genomic heterogeneity of Diamond Blackfan anemia in the Russian Federation. Pediatr Blood Cancer. 2015;62(9):1597-1600.
17. Waespe N, Dhanraj S, Wahala M, et al. The clinical impact of copy number variants in inherited bone marrow failure syndromes. NPJ Genom Med. 2017;2:18.
18. Quarello P, Garelli E, Brusco A, et al. High frequency of ribosomal protein gene deletions in Italian Diamond-Blackfan anemia patients detected by multiplex ligation-dependent probe amplification assay. Haematologica. 2012;97(12):1813-1817.
19. Wang R, Yoshida K, Toki T, et al. Loss of function mutations in RPL27 and RPS27 identified by whole-exome sequencing in Diamond-Blackfan anaemia. Br J Haematol. 2015;168(6):854-864.
20. Wan Y, Chen X, An W, et al. Clinical features, mutations and treatment of 104 patients of Diamond-Blackfan anemia in China: a single-center retrospective study. Int J Hematol. 2016;104(4):430-439.
21. Glassford MR, Rosenfeld JA, Freedman AA, et al. Novel features of 3q29 deletion syndrome: results from the 3q29 registry. Am J Med Genet A. 2016;170a(4):999-1006.
22. Murphy MM, Lindsey Burrell T, Cubells JF, et al. Study protocol for The Emory 3q29 Project: evaluation of neurodevelopmental, psychiatric, and medical symptoms in 3q29 deletion syndrome. BMC Psychiatry. 2018;18(1):183.
23. Digilio MC, Bernardini L, Mingarelli R, et al. 3q29 microdeletion: a mental retardation disorder unassociated with a recognizable phenotype in two mother-daughter pairs. Am J Med Genet A. 2009;149a(8):1777-1781.
24. Willatt L, Cox J, Barber J, et al. 3q29 microdeletion syndrome: clinical and molecular characterization of a new syndrome. Am J Hum Genet. 2005;77(1):154-160.
25. Alter BP, Giri N, Savage SA, et al. Cancer in the National Cancer Institute inherited bone marrow failure syndrome cohort after fifteen years of follow-up. Haematologica. 2018;103(1):30-39.
26. Lipton JM, Atsidaftos E, Zyskind I, et al. Improving clinical care and elucidating the pathophysiology of Diamond Blackfan anemia: an update from the Diamond Blackfan Anemia Registry. Pediatr Blood Cancer. 2006;46(5):558-564.
27. Landrum MJ, Lee JM, Benson M, et al. ClinVar: improving access to variant interpretations and supporting evidence. Nucleic Acids Res. 2018;46(D1):D1062-D1067.
28. Stenson PD, Mort M, Ball EV, et al. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Hum Genet. 2014;133(1):1-9.
29. ClinGen. Clinical laboratories meeting minimum requirements for data sharing to support quality assurance. https://clinicalgenome.org/tools/clinical-lab-data-sharing-list/Last accessed 22 Feb 2019.
30. Da Costa L, Narla A, Mohandas N. An update on the pathogenesis and diagnosis of Diamond-Blackfan anemia. 2018;7:F1000 Faculty Rev-1350..
31. Bessler M, Mason P, Link D, et al. Nathan and Oski's Hematology of Infancy and Childhood E-Book: Expert Consult: Online and Print. In: Orkin SH, ed. Inherited Bone Marrow Failure Syndromes. 7th ed: Elsevier Health Sciences; 2009:351-360.
32. Karczewski KJ, Francioli LC, Tiao G, et al. Variation across 141,456 human exomes and genomes reveals the spectrum of loss-of-function intolerance across human protein-coding genes. bioRxiv. 2019:531210.
33. Rentzsch P, Witten D, Cooper GM, et al. CADD: predicting the deleteriousness of variants throughout the human genome. Nucleic Acids Res. 2019;47(D1):D886-D894.
34. Ioannidis NM, Rothstein JH, Pejaver V, et al. REVEL: an ensemble method for predicting the pathogenicity of rare missense variants. Am J Hum Genet. 2016;99(4):877-885.
35. Cingolani P, Platts A, Wang LL, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly. 2012;6(2):80-92.
36. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010;38(16):e164.
37. Zhou X, Edmonson MN, Wilkinson MR, et al. Exploring genomic alteration in pediatric cancer using ProteinPaint. Nat Genet. 2016;48(1):4-6.
38. Karolchik D, Hinrichs AS, Furey TS, et al. The UCSC Table Browser data retrieval tool. Nucleic Acids Res. 2004;32(Database issue):D493-D496.
39. StataCorp. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC. 2017.
40. Mirabello L, Khincha PP, Ellis SR, et al. Novel and known ribosomal causes of Diamond-Blackfan anaemia identified through comprehensive genomic characterisation. J Med Genet. 2017;54(6):417-425.
41. Delaporta P, Sofocleous C, Stiakaki E, et al. Clinical phenotype and genetic analysis of RPS19, RPL5, and RPL11 genes in Greek patients with Diamond Blackfan Anemia. Pediatr Blood Cancer. 2014;61(12):2249-2255.
42. Quarello P, Garelli E, Carando A, et al. Ribosomal RNA analysis in the diagnosis of Diamond-Blackfan anaemia. Br J Haematol. 2016;172(5):782-785.
43. Farrar JE, Dahl N. Untangling the phenotypic heterogeneity of Diamond Blackfan anemia. Semin Hematol. 2011;48(2):124-135.
44. Li F, Lisi EC, Wohler ES, et al. 3q29 interstitial microdeletion syndrome: an inherited case associated with cardiac defect and normal cognition. Eur J Med Genet. 2009; 52(5):349-352.
45. Pollazzon M, Grosso S, Papa FT, et al. A 9.3 Mb microdeletion of 3q27.3q29 associated with psychomotor and growth delay, tricuspid valve dysplasia and bifid thumb. Eur J Med Genet. 2009;52(2-3):131-133.
46. Quintero-Rivera F, Sharifi-Hannauer P, Martinez-Agosto JA. Autistic and psychiatric findings associated with the 3q29 microdeletion syndrome: case report and review. Am J Med Genet A. 2010; 152a(10):2459-2467.
47. McHugh B, Krause SA, Yu B, et al. Invadolysin: a novel, conserved metalloprotease links mitotic structural rearrangements with cell migration. J Cell Biol. 2004; 167(4):673-686.
48. Cobbe N, Marshall KM, Gururaja Rao S, et al. The conserved metalloprotease invadolysin localizes to the surface of lipid droplets. J Cell Sci. 2009;122(Pt 18):3414-3423.
49. Chen LT, Liang WX, Chen S, et al. Functional and molecular features of the calmodulin-interacting protein IQCG required for haematopoiesis in zebrafish. Nat Commun. 2014;5:3811.
50. Pan M, Zhang Q, Liu P, et al. Inhibition of the nuclear export of p65 and IQCG in leukemogenesis by NUP98-IQCG. Front Med. 2016;10(4):410-419.
51. Pan Q, Zhu YJ, Gu BW, et al. A new fusion gene NUP98-IQCG identified in an acute T-lymphoid/myeloid leukemia with a t(3;11)(q29q13;p15)del(3)(q29) translocation. Oncogene. 2008;27(24):3414-3423.