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Spontaneous remission in a Diamond-Blackfan anaemia patient due to a revertant uniparental disomy ablating a *de novo RPS19* mutation.

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Running title: DBA rescue by UPD

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SUMMARY

A combination of multilevel molecular analyses including whole exome sequencing (WES), SNP-arrays, microsatellite segregations, and primer extensions allowed us to explain an unsolved case of remitted Diamond-Blackfan Anaemia (DBA). In the patient's symptomatic DNA we identified a mosaic *de novo* pathogenic change, namely c.140C>T (p.Pro47Leu) in *RPS19*. We showed the mosaicism was due to the presence of a UniParental Disomy (UPD) involving the long arm of chromosome 19, where *RPS19* is mapped. We demonstrated the remission was associated with a reduction of mutant cells, likely due to the positive selection of UPD clones, ablating the mutation.

KEY WORDS: Diamond-Blackfan; uniparental disomy; complete remission; revertant mosaicism; RPS19.

MAIN TEXT

Diamond-Blackfan Anaemia (DBA; MIM#105650) is an inherited bone marrow failure syndrome that, in most cases, shows an onset in the first year of life and is characterized by hyporegenerative anaemia, congenital malformations in about one third of patients and elevated erythrocyte adenosine deaminase activity (eADA)(Da Costa, *et al* 2018).

Anaemia is corrected with steroids in about 50% of patients, with a minority of patients who achieve (often inexplicable) clinical spontaneous remission (Vlachos and Muir 2010).

DBA belongs to the large family of ribosomopathies and is usually caused by alterations in one of 20 ribosomal protein (RP) genes that lead to haploinsufficiency (Da Costa, *et al* 2018).

Here we used high density single nucleotide polymorphism (SNP)-arrays and whole exome sequencing (WES) to solve a clinically and molecularly intricate case of remitted sporadic DBA.

The proband was a 35-year male, the first of two siblings born full term after an uncomplicated pregnancy from healthy unrelated Caucasian parents (Fig.1A). His parents and brother had normal haematological findings. At birth, he was identified as having a severe macrocytic hyporegenerative anaemia needing red blood cell transfusion (Haemoglobin 76g/L, Mean Cell Volume 114.5fL, absolute reticulocyte count 8×10^9/L). No malformations were present except hypoplasia of the right thenar eminence; eADA was elevated (3U/gHb, normal values 1±0.2U/gHb) and bone marrow (BM) aspirate showed a selective decrease in erythroid precursors. He was clinically diagnosed with DBA. The patient did not respond to steroids and was regularly transfused. At 8 years of age, a few months after a steroid treatment, he achieved stable haematological remission, which was maintained at the last follow-up (35 yrs; Haemoglobin 130 g/L, Mean Cell Volume 98.5fL, absolute reticulocyte count 60×10^9/L, eADA 0.56U/gHb).

Molecular analysis of the patient and his family was performed after informed consent. High density SNP-arrays (CytoScan HD array, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were performed on II-1 at age 7 and 29 years old. They identified a mosaic segmental 29.7-Mb loss of heterozygosity on chromosome 19q12-q13.43 (chr19:29,348,081-59,097,752; hg19). This pattern was indicative of the presence of multiple clones with a uniparental disomy (UPD) limited to the long arm of chromosome 19 (Fig.1B). At 29 yrs old, the UPD involved almost the entire 19q and appeared practically complete (Fig.1C). Microsatellite analysis confirmed an allelic imbalance compatible with paternal chromosome 19q UPD (Fig.1A and

supplemental materials).

On blood-extracted genomic DNA of the proband at 7-yrs., WES identified the c.140C>T (p.Pro47Leu) heterozygous change in *RPS19* (Supplemental materials) predicted to be damaging according to several bioinformatics tools (https://varsome.com/), and reported as pathogenic in a Brazilian DBA patient (Angelini, *et al* 2007, Ramenghi, *et al* 2000). Sanger sequencing showed the mutation was *de novo* (Fig.1D).

B-allele frequency (BAF) analysis from WES data confirmed the UPD mosaicism detected by SNP-array with a Variant Allele Frequency of 31% (Fig.1E; Supplemental materials).

Somatic mosaicim of the c.140C>T variant was further measured by primer extension assay showing a reduction of mutant cells from ≅50% at 7 yrs in blood to ≅13% at 29 yrs, when the patient was in full remission (Fig.2A and Supplemental Fig.1).

Patients with DBA can enter a state of remission defined by an acceptable haemoglobin level without any treatment, lasting >6 months, independent of prior therapy without no obvious phenotypic or genotypic difference between remission and non-remission patients (Narla, *et al* 2011). Somatic mosaicism has been reported to explain remission (Biesecker and Spinner 2013). Farrar et al. described three DBA individuals with mosaic copy loss on chromosomes 3q and 15q, containing two well-established DBA genes, *RPL35A* (3q29) and *RPS17* (15q25.2) (Farrar, *et al* 2011). Interestingly, the two patients with low-level mosaicism experienced spontaneous remission of DBA in the second decade of life, whereas the subject with a higher fraction of mosaicism remained transfusion-dependent. These findings suggest that the fraction of blood mosaicism may correlate with the prognosis, and may also impact on the clinical outcome of patients with a mosaic point mutation in DBA genes.

Our case parallels two recently described patients with a reversion of DBA, involving a deletion spanning the *RPS26* gene in one (Venugopal, *et al* 2017), and *RPL4*, a novel pathogenic gene in the other (Jongmans, *et al* 2018). These authors have suggested revertant mosaicism (RM) as a second rescue mechanism in DBA. RM refers to the co-existence of cells carrying disease-causing mutations with cells in which the inherited mutation is genetically corrected by a spontaneous event. Restoration of gene functions can be obtained by different genetic mechanisms, including gene conversion, intragenic crossover, back mutation, and second-site mutation (Biesecker and Spinner 2013, Venugopal, *et al* 2017).

The case described here confirms RM in patients with DBA remission. Our data suggest that

soon after zygote formation, a segmental paternal UPD of chromosome 19q reverted the maternal *de novo* mutation in a subset of embryonal cells (Fig.2B). SNP-array data show that the UPD had different extensions, suggesting the presence of different clones spanning *RPS19*, thus supporting the hypothesis of a selective advantage (Figs.1B-C). This biological process slowly led to a stable state of remission.

Remarkably, we noted that our patient reached stable normalization of eADA levels at remission showing complete rescue of the haematological phenotype. Indeed, eADA usually remains elevated in DBA cases, even in patients who have achieved remission or are haematologically stable on steroids (Vlachos and Muir 2010). On this basis, we strongly suggest the monitoring of eADA activity, especially in patients who experienced remission, in order to identify a possible RM. An alternative assay to monitor remission is the analysis of rRNA processing using Bioanalyzer, as described in (Quarello, *et al* 2016).

In conclusion, we have finally solved a 28-yr-long diagnostic puzzle, showing that the disease in our patient was due to a *de novo* maternal missense mutation in *RPS19*, and the rescue to a paternal UPD. However, unanswered questions remain on this case; for example, how and why did the revertant UPD occur and which factors determine the outgrowth of the reverting cells? Further work is required to understand the hidden mechanisms of this “natural gene therapy” phenomenon.

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AUTHORSHIP CONTRIBUTIONS: Brusco A., Garelli E., Giorgio E. conceived the experiments; Quarello P., Ramenghi U. performed the clinical evaluation and the follow up of the patient; N. Crescenzio performed BFU-E evaluation; Carando A., Carella M., Di Martino P.,

Garelli E., Di Gregorio E., Giorgio E., Mancini C., Menegatti E., Palumbo O., Pippucci T. performed and interpreted molecular biology experiments; Brusco A., Garelli E., Giorgio E., Quarello P. interpreted final data, wrote the manuscript and prepared the figures; all authors critically revised the manuscript.

FIGURE LEGENDS

FIGURE 1. Pedigree and molecular data. Panel A: family pedigree (proband in black). Segregation analysis of four representative microsatellite markers on chromosome 19q surrounding *RPS19* showed an imbalance in the patient, compatible with the Uniparental Disomy (UPD) found by Single Nucleotide Polymorphism (SNP)-array (dashed line). Haplotype analysis showed that the mutation is on the maternal chromosome 19. The patient and his brother inherited the same haplotype from their mother, confirming the mutation was *de novo*. The location of each marker on the human assembly (GRCh37/hg19) is shown in Mb. Panels B and C: SNP-arrays identified the presence of a segmental UPD mosaicism. The log_2 ratio indicates a normalized intensity value of each SNP across chromosome 19 compared to diploid individuals (ratio 1 is log_2 ratio of 0). The B allele can have values of 1 (BB), 0 (AB) and -1 (AA) in a diploid individual. Data show that the patient at 7 years (blood, panel B) had a mosaic segmental UPD spanning chromosome 19q. The B allele frequency shows a 29.7-Mb loss of heterozygosity at chromosome 19q12-q13.43 (29,348,081-59,097,752; hg19), interpreted as a somatic mosaicism for the UPD. In panel C, SNP-array data from DNA extracted from blood at 29 years old showed an increased mosaic segmental 19q UPD. Panel D: Sanger sequence electropherograms showing the c.140C>T mutation. As an example of normal sequence, we reported the mother I-2; the c.140C>T mutation is clearly visible at 7-yrs in blood, but is almost undetectable at 29-yrs in the same tissue. However, it can be detected at 34-yrs in buccal swab. A heterozygous c.140C>T DBA patient is also reported in the lower panel for comparison. Panel E: B-allele frequency (BAF) profile of biallelic SNVs along chromosome 19, calculated by H3M2 from WES alignments of patient's DNA from blood at 7 years old, confirmed UPD mosaicism detected by SNP-arrays. BAF profile is compatible with a state having averaged allelic heterozygosity of around 50% all along chromosome 19p, while it is clearly split into two states with an averaged allelic heterozygosity <50% and >50% along chromosome 19q.

FIGURE 2. Summary of the clinical and molecular findings and proposed mechanism of remission. Panel A: a time-line summary of the clinical data and molecular tests performed. Percentages of cellular mosaicism for the c.140C>T mutation obtained by primer extension assay and WES at different ages of the patient and in different tissues. PE: Primer Extension assay; WES: Whole Exome Sequencing. eADA values are reported as U/gHb. Panel B: rationale

for the DBA remission in our patient, as interpreted from clinical and genetic data. The c.140C>T mutation must have arisen in the maternal germline, or at the zygote stage. At a very early embryonal stage, multiple UPD events involved the long arm of chromosome 19. In specific cell types, such as hematopoietic precursors, UPD spanning RPS19, and reverting the c.140C>T pathogenic mutation, gave a proliferative advantage. After several years, the number of UPD clones were enough to sustain a normal red cell production, and the patient became clinically remitted. The overall mechanism can be named UPD-mediated revertant mosaicism.

SUPPLEMENTARY FIGURE 1. Primer extension assay set up. The percentage of mutation mosaicism in the proband has been determined with a specific primer extension assay investigating the c.140 C>T mutation in the *RPS19* gene. A standard curve was generated mixing DNA samples obtained from a heterozygous DBA patient for the c.140 C>T mutation (HET) and a control sample (wild-type for the variant, WT) at three different ratios (HET:WT): 75:25; 50:50 and 25:75. These mixes mimic a cellular mosaicism of 75%, 50% and 25%, respectively. By primer extension assay, we obtained a value of allelic ratio for each mix (WT:MUT or C:T). A standard curve was generated using GraphPad Prism 6 Software. The percentages of cellular mosaicism and the allelic ratio are reported on the Y-axis and X-axis, respectively. Results obtained from the standard mixes are shown in red. For each point (75, 50 and 25) a schematic representation of the primer extension result, the allelic ratio and the cellular mosaicism are reported. The primer extension assay was also performed on DNA from the heterozygous DBA patient, obtaining an allelic ratio of 1:1 as expected for a dominant mutation. All samples available from the proband (blood 7yrs and 29yrs, BFU-E 13yrs and buccal swab 34 yrs) were analysed by primer extension to obtain the allelic ratio and, for interpolation with the standard curve, the corresponding percentage of cellular mosaicism. Results are shown in green and are summarized in Figure 2A.

REFERENCES

Angelini, M., Cannata, S., Mercaldo, V., Gibello, L., Santoro, C., Dianzani, I. & Loreni, F. (2007) Missense mutations associated with Diamond-Blackfan anemia affect the assembly of ribosomal protein S19 into the ribosome. *Hum Mol Genet,* **16,** 1720-1727.

Biesecker, L.G. & Spinner, N.B. (2013) A genomic view of mosaicism and human disease. *Nat Rev Genet,* **14,** 307-320.

Da Costa, L., Narla, A. & Mohandas, N. (2018) An update on the pathogenesis and diagnosis of Diamond-Blackfan anemia. *F1000Res,* **7**.

Farrar, J.E., Vlachos, A., Atsidaftos, E., Carlson-Donohoe, H., Markello, T.C., Arceci, R.J., Ellis, S.R., Lipton, J.M. & Bodine, D.M. (2011) Ribosomal protein gene deletions in Diamond-Blackfan anemia. *Blood,* **118,** 6943-6951.

Jongmans, M.C.J., Diets, I.J., Quarello, P., Garelli, E., Kuiper, R.P. & Pfundt, R. (2018) Somatic reversion events point towards RPL4 as a novel disease gene in a condition resembling Diamond-Blackfan Anemia. *Haematologica*.

Narla, A., Vlachos, A. & Nathan, D.G. (2011) Diamond Blackfan anemia treatment: past, present, and future. *Semin Hematol,* **48,** 117-123.

Quarello, P., Garelli, E., Carando, A., Mancini, C., Foglia, L., Botto, C., Farruggia, P., De Keersmaecker, K., Aspesi, A., Ellis, S.R., Dianzani, I. & Ramenghi, U. (2016) Ribosomal RNA analysis in the diagnosis of Diamond-Blackfan Anaemia. *Br J Haematol,* **172,** 782-785.

Ramenghi, U., Campagnoli, M.F., Garelli, E., Carando, A., Brusco, A., Bagnara, G.P., Strippoli, P., Izzi, G.C., Brandalise, S., Riccardi, R. & Dianzani, I. (2000) Diamond-Blackfan anemia: report of seven further mutations in the RPS19 gene and evidence of mutation heterogeneity in the Italian population. *Blood Cells Mol Dis,* **26,** 417-422.

Venugopal, P., Moore, S., Lawrence, D.M., George, A.J., Hannan, R.D., Bray, S.C., To, L.B., D'Andrea, R.J., Feng, J., Tirimacco, A., Yeoman, A.L., Young, C.C., Fine, M., Schreiber, A.W., Hahn, C.N., Barnett, C., Saxon, B. & Scott, H.S. (2017) Self-reverting mutations partially correct the blood phenotype in a Diamond Blackfan anemia patient. *Haematologica,* **102,** e506-e509.

Vlachos, A. & Muir, E. (2010) How I treat Diamond-Blackfan anemia. *Blood,* **116,** 3715-3723.