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A new in-frame deletion in ribosomal protein S19 in a Chinese infant with Diamond-Blackfan anemia

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ABSTRACT

Diamond-Blackfan anemia (DBA) is a congenital erythroid aplasia that usually presents as macrocytic anemia during infancy. Ribosomal protein S19 (RPS19) is identified as the first gene associated with DBA. RPS19 is mutated in 25% of DBA patients, but its role in DBA pathogenesis remains to be elucidated. We have identified a novel heterozygous frameshift mutation in RPS19 gene in a DBA child presenting with profound anemia after birth. A single nucleotide heterozygous deletion (C.251delG) results in frameshift in RPS19 gene in exon 4 at codon 84 with possible premature stop codon (p.Arg84LysfsX21). The mutant allele was not detected in her parents, indicating de novo mutation. Both alleles were expressed at the same level. Using an immunofluorescence technique, the mutated-type RPS19 expressions were mostly localized to entire nuclei with little staining for nucleoli and its intracellular localization significantly differed from the wild-type RPS19, which was localized to both nuclei and nucleoli. This type of a mutation could be very helpful in further understanding the role of the RPS19 protein in DBA pathogenesis.

1. Introduction

Diamond Blackfan anemia (DBA) is a rare, congenital hypoplastic anemia with an estimated incidence of about six per million live births that usually presents early in infancy [1,2]. The disease is characterized by a moderate to severe macrocytic anemia, occasional neutropenia or thrombocytosis, and low numbers of erythroid precursors in the bone marrow. In addition to anemia, congenital anomalies of the head and upper limbs in particular are present in about 40% of reported patients [3]. Although sporadic cases remain the most frequent (~75%) ones, familial cases with both dominant and recessive patterns of inheritance have been described. Corticosteroids are the recommended first line of treatment, however, approximately 60% of patients are steroid dependent, whereas 30% to 40% of the cases do not respond to prednisone [1]. The mainstay of treatment in DBA patients who do not respond to steroid treatment is erythrocyte transfusion. The major complication of regular transfusion is iron overload. An iron chelator must be administered as soon as the evidence of increased iron accumulation is observed. Allogeneic bone marrow transplant from an HLA matched sibling donor provides a reasonable alternative option. Transplantation early in the course of the disease would be expected to present a better outcome and is generally more successful in younger patients. However, those patients with DBA often lack HLA-identical siblings.

DBA is also the first human disease associated with mutations in a ribosomal structural protein. Study of a patient with an X:19 chromosome translocation (46, XX, t(X;19)(p21;q13)) provided the first clue to the localization of the DBA gene [4]. In fact, following the finding that ribosomal protein S19 (RPS19) is involved in this disorder, >150 RPS19 mutations throughout the entire gene have been described in 25% of DBA patients, including missense and nonsense mutations, insertions, deletions, and also splice site defects. However, the genetic basis of DBA remains obscure, and whether haplo-insufficiency of RPS19 protein remains to be demonstrated in certain DBA cases. Therefore, screening DBA patients for RPS19 mutations and studying functional consequences of these mutations are warranted in further defining the role of RPS19 in cellular metabolism and DBA pathogenesis. Here, we report a novel frameshift mutation and its functional effects of its associate RPS19 protein. Our results demonstrate that the mutation can affect the nucleolar localization in peripheral blood mononuclear cells (PBMCs) although it does not affect the mRNA expression level of RPS19 protein in PBMCs.

2. Materials and methods

2.1. DNA isolation and sequencing

Peripheral blood mononuclear cells (PBMC) from a DBA patient, the patient's parents, and three control individuals were isolated using Lymphocyte Separation Medium (Shanghai Sangon Corp.). Genomic

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Table 1
Patient characteristics.

Parameter	Patient 8 weeks of age	Normal values at age-related individuals
Hb (g/dl)	2.5 L	9–15
Hematocrit (%)	7.8 L	29–41
Leukocytes (10^9/l)	5.3	4.0–10
Platelets (10^9/l)	240	140–440
Reticulocyte (%)	0.2 L	0.7–2.5
Erythrocytes (10^{12}/l)	0.8 L	3.8–5.3
MCV (fl)	95 H	70–86
MCH (pg)	32	24–29
MCHC (g/dl)	36	32–36
CD34+ (%)	6.94 H	0.5–1.5
CD34+CD38+ (%)	1.810 H	0.450–1.350
CD34+CD38− (%)	5.130 H	0.050–0.150
CD20 (%)	14.79	21.0–33.0
CD3 (%)	59.77	60.0–71.0
CD4 (%)	34.20	37.0–48.0
CD8 (%)	16.12	16.0–21.0
CD3−CD16+CD56+ (%)	5.92	7.0–14.0
CD4/CD8	2.12	1.7–3.0
CD25 +/CD4+CD3+ (%)	12.8	0.00–10.00
HLA-DR +/CD4+CD3+ (%)	4.39	0.00–10.00
CD69+/CD4 + CD3 + (%)	2.76	0.00–2.00
CD25 +/CD8+CD3+ (%)	0.00	0.00–5.00
HLA-DR +/CD8+CD3+ (%)	16.34	0.00–10.00
CD69 +/CD8+CD3+ (%)	6.42	0.00–10.00

Control ranges according to Manual of Pediatric Hematology and Oncology. L: low; H: high.

DNA (gDNA) was isolated from PBMC by standard methods [5,6]. The DNA samples were amplified by polymerase chain reaction (PCR). Primers were used to amplify fragments that contained the 5′UTR and the six coding exons of the RPS19 gene. PCR fragments were amplified from 200 ng of gDNA in 50 μl reaction system using TaqMan polymerase [6]. Sequencing was performed using a BigDye Terminator Ready Reaction Kit (Perkin-Elmer, Foster City, CA), according to the manufacturer's instruction manual. All samples were sequenced using forward and reverse primers. Samples were analyzed on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA). Mutation was confirmed by the repeated sequencing of different PCR products.

2.2. Quantitative real-time RT-PCR

Total RNA was isolated from PBMCs (as described above) from the DBA patient and control individuals using an RNA isolation kit (Qiagen) according to the manufacturer's instructions, and XX total RNA was reverse transcribed into cDNA using M-MLV (Invitrogen Corp.). The expression level of RPS19 was analyzed by quantitative reverse transcriptase-polymerase chain reaction (Q-RT-PCR) using a LightCycler instrument (Roche Diagnostics GmbH) with LightCycler Software version 5.32. The cDNA was used for Q-PCR using SYBR Green I (Sigma-Aldrich) for detection of PCR products. cDNA (2 μl) was used in a 15 μl final volume reaction system. The LightCycler was programmed to run an initial denaturation step at 94 °C for 3 min followed by 40 cycles of denaturation (94 °C, 5 s), annealing (60 °C, 5 s), and extension (72 °C, 10 s), PCR products were measured at the end of the extension step of each cycle. The control reactions without the inclusion of reverse transcriptase were utilized to exclude any contamination. The control reactions examining the expression of the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene were run in the same tube as an endogenous reference. The outcome of each amplification was calculated with comparative methods. This procedure enabled calculations of the RPS19 expression level and fold changes between DBA and normal samples normalized to GAPDH in each RNA sample. The size of the RT-PCR products was confirmed on 1.2% agarose gels. The RT-PCR products were sequenced and analyzed as described above. The primers used for amplification of RPS19 gene were as follows: forward primer: 5′CAAGGAATTGTTTACCTGAGAC; reverse primer 5′CCATCTTGGTCCTTTTCCAC [7].

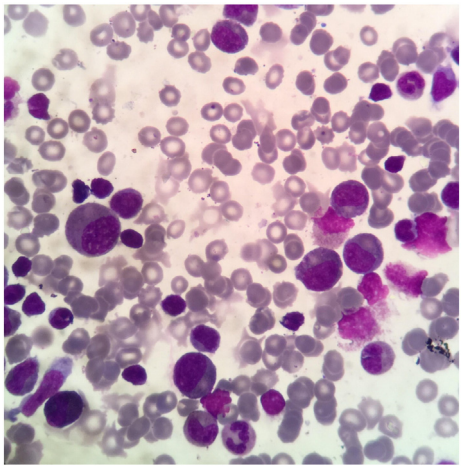

Fig. 1. Photomicrograph showing a selective serious reduction of red blood cell in direct marrow films. Giemsa ×400.

2.3. Immunofluorescence analysis

RPS19-WT and RPS19-MT cDNAs were cloned into *Bam*HI and *Eco*RI sites directly before eGFP tag into the expression vector pcDNA3.1-eGFP (constructed in our lab) to express the RPS19-WT-eGFP and RPS19-MT-eGFP fusion proteins from the T7 promoter. Resulting constructs were

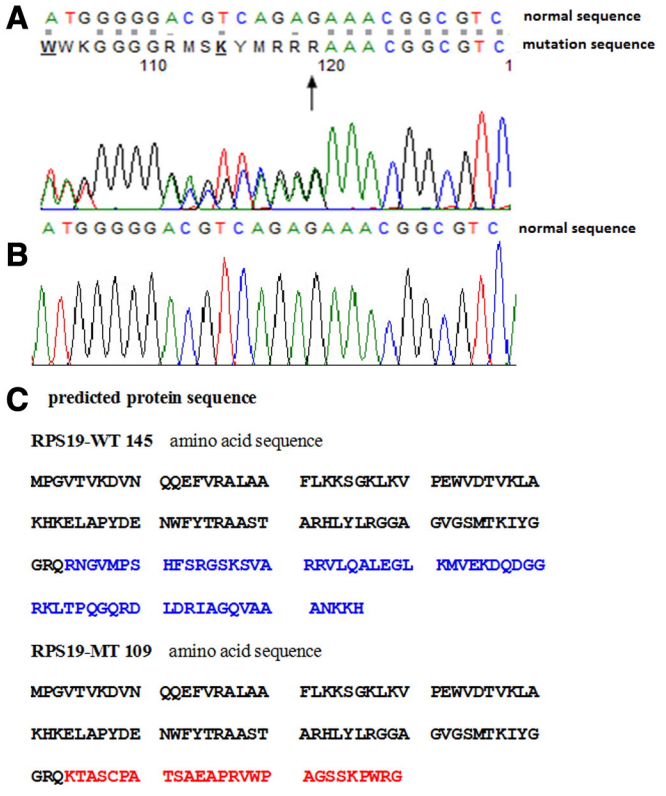

Fig. 2. Identification of a novel mutation in RPS19. (A) Sequencing analysis of all six RPS19 exons was performed at the genomic level in the DBA patient. In the sequence of RPS19 exon 4, a frameshift mutation was identified. (B) Sequencing analysis therefore was performed in both parents. No mutation was found. (C) The predicted amino acid sequence of the mutated RPS19 protein in the patient is in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

verified by sequencing. The CHO cells were transfected with the vector by Lipofectamine™ 2000 following the manufacturer's protocol.

After 2 days, the transfected cells were fixed by immersing in acetone for 3 min at −20 °C, washed with PBS, and further fixed with freshly prepared 4% paraformaldehyde in PBS for 15 min at room temperature. DNA was labeled with DAPI (1 μg/ml; Sigma-Aldrich). To verify the specificity, untransfected cells were used as a negative control. Nikon Eclipse E600 microscope and Olympus DP70 digital camera were used for visualization.

3. Results and discussion

The patient in whom we found this novel mutation is a girl born to parents in the first uneventful pregnancy. Both parents were healthy. The delivery was induced at the 38th week of gestational age because of fetal distress, and a Caesarean section was performed. The developmental milestones were small for gestational age. The weight at birth was 2300 g (≥2500 g in infants born appropriate for gestational age -AGA); body length was 45 cm (50 cm in AGA-born infants); head circumference was 24 cm (32–34 cm in AGA-born infants).

The patient was brought to our hospital with a history of conspicuously pale needing oxygen therapy when she was 2-months old. She had received one blood transfusion at 6th day of life for moderate anemia (hemoglobin 9.3 g/dl, hemoglobin > 14.5 g/dl within 10 days after birth). There was no history of jaundice and the child showed no other associated anomalies.

Blood count analyses revealed severe macrocytic anemia with normal counts of leukocytes and platelets. The data at the age of 8 weeks is summarized in Table 1. HbF was within normal limits. We could not perform erythrocyte adenosine deaminase (eADA) determination because she had received blood transfusion.

Bone marrow smears in Fig. 1 exhibited grossly reduced red cell precursors with dyserythropoietic features (<1%; normal values: 23.2–27.4%). The other cell lineages were within normal ranges showing 59% granulocytic lineage cells and 33% of lymphocytes (normal ranges: 51.2–63.1% and 31.8–39.8%, respectively). Cytogenetic abnormalities were not detected. B19 parvovirus and EB virus infections were also excluded. Abdominal and cranial ultrasonography, Echocardiogram and Magnetic Resonance Imaging (MRI) of the brain did not reveal any abnormality. The proportion of $CD34^{+}$ cells in the bone marrow was 6.94%, which was close to five times the normal limit. As shown in Table 1, the proportion of T, B and NK cells in the peripheral blood is all decreased. However, the proportion of CD4 + CD25 + CD3 + Treg cells is increased.

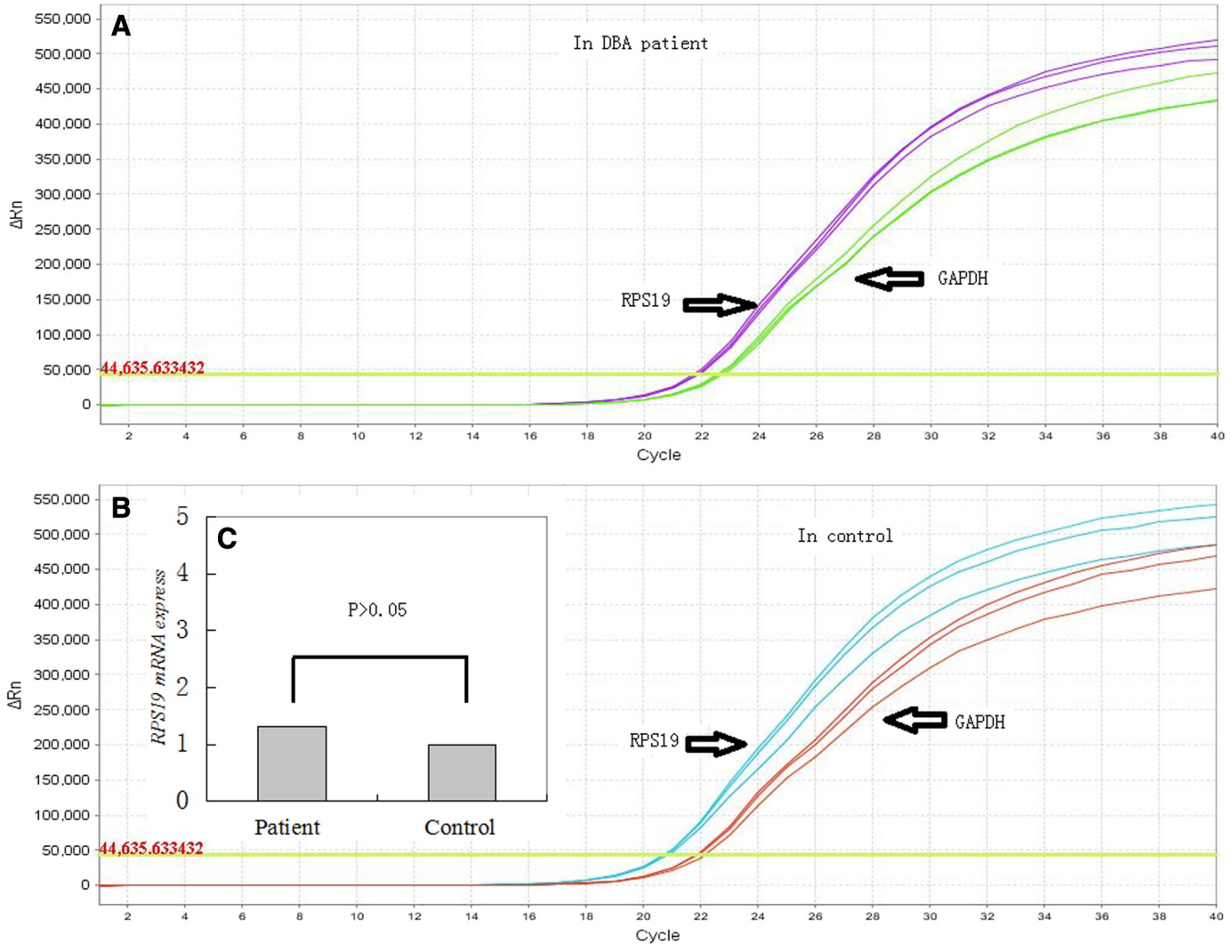

Fig. 3. Quantitative real-time RT-PCR shows no reduction of RPS19 mRNA normalized to GAPDH mRNA in PBMCs from the DBA patient with premature termination codon compared to control individuals. (A) In the DBA patient, multi-color lines on the left represent RPS19, lines on the right show GAPDH. (B) In the control sample, multi-color lines on the left represent RPS19, lines on the right show GAPDH. (C) The differential expression genes of RPS19 were not evident from the statistical analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Several recently published studies [6,8] suggest that 25% of DBA patients had RPS19 mutations. As laboratory and clinical findings from this patient suggested a possible diagnosis of DBA, we screened her for mutations in the RPS19 gene. Using direct sequencing of all six RPS19 exons at the genomic level, we found a single nucleotide heterozygous deletion (C.251delG) resulting in frameshift mutation in RPS19 exon 4 at codon 84 with possible premature or suppressed stop codon (Arg84LysfsX21) (Fig. 2A). The predicted RPS19 amino acid sequence is depicted in Fig. 2C. To determine the origin of mutation, DNA from both parents was also analyzed and the sequencing results confirmed that the mutation arose de novo as it was present only in the patient (in Fig. 2B). No coincident mutation has been described in any other cases so far. Similarly, a study from Europe revealed 2 base pairs deletion mutations in the exon 4 of RPS19 (C.250_251delAG; p.Arg84LysfsX69), [8]. And also, the patient reported in that study was a boy with high arch palate and facial asymmetry, presented a good response to steroid treatment. On the contrary, our case was a girl without any other congenital anomalies but with some signs of intrauterine growth delay. Increases in her hemoglobin were not seen within four weeks of administering corticosteroids at a dose of 2 mg/kg/day. So, she was steroid resistant and transfusion-dependent. Andrew Chatr-Aryamontri et al. [9] reported a frameshift mutation in exon 2 (C.13dupA). The patient with no malformation turned out no response to steroids and transfusion dependent.

Further studies have showed that RPS19 mutations had different effects on its functions. We were therefore interested in whether the frameshift deletion could influence mRNA level of mutated RPS19. We quantitated the RPS19 mRNA levels in PBMC from the DBA patient by qRT-PCR and compared with those from three normal control individuals after being normalized to GAPDH level followed by the sequencing analysis of the RPS19 RT-PCR products from the DBA patient as well as his parents. Interestingly, our results displayed that the patient did not reveal a significant reduction of the RPS19 mRNA as compared with the control individuals, $P > 0.05$ (Fig. 3); but the patient was detected both the wild-type and mutated type transcripts by sequencing the RT-PCR products, suggesting that the frameshift deletion of a single nucleotide found in gDNA was confirmed at the cDNA level (Fig. 4). This is in agreement with the observation by Hamaguchi et al. [10], showing that the presence of premature translation-termination codons (PTC) in a single DBA patient with a frameshift mutation (TT157-158AA, 160 in. CT) in the RPS19 mutated allele does not cause a decrease in mRNA levels. However, Andrew Chatr-Aryamontri et al. [9] found that the predicted PTC-RPS19 mRNAs (C.13dupA in exon 2) showed the amount of RPS19 mRNA was reduced, to about 50 to 60% of controls. The difference between our result and the other studies may depend on the possibility that part of our reported mutated mRNA is NMD (nonsense mediated decay)-insensitive. However, since we have not experimentally addressed NMD-insensitive, this remains a speculation. It could be that mutated mRNAs are retained in an untranslatable pool, although PTC-containing mRNAs have been observed associated to polysomes [11]. In fact, in autosomal dominant disease, most of the mutations presumed to result in synthesis of truncated proteins or frameshifted products probably cause haploinsufficiency. We do not know if, or how much of, the mutated transcripts present in the cells are translated into proteins. It is still debated whether NMD affects nucleus-associated or cytoplasmic mRNA [12]. Our result indicates that, at least in the single examined patient, the altered messenger is not subject to NMD.

Ribosomal protein involvement is well known in ribosome assembly and protein synthesis. In the nucleus, ribosomal protein assembles in the nucleolus, along with the 4 rRNA molecules, to build the complex and coordinated structure of the ribosome. None of the mutated RPS19 is able to be assembled into mature ribosome. The ribosome biogenesis and assembling were disrupted when RPS19 nucleolar localization was impaired [13–14]. Therefore, we were also interested in whether the deletion could influence the intracellular localization of the mutated RPS19. Mutated and wild-type RPS19 cDNAs were fused with the eGFP tag in the expression vector, and the vectors were transiently transfected into CHO cells. Intracellular localization of RPS19 was studied by immunofluorescence. As shown in Fig. 5, the RPS19-

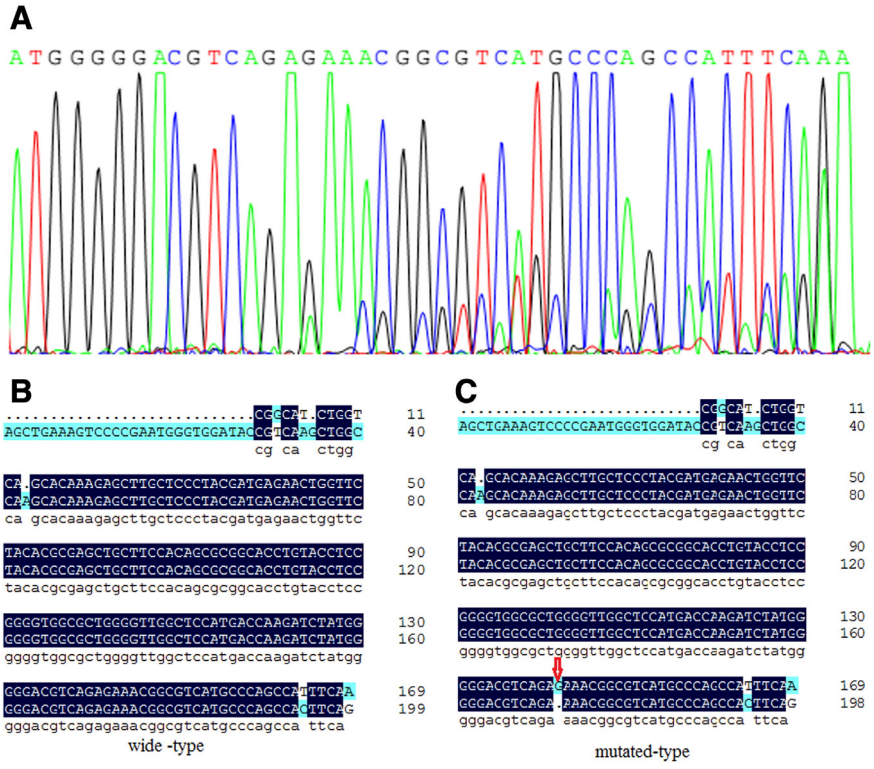

Fig. 4. Detection of a mutation in RPS19 RT-PCR products. (A) Sequencing analysis of all six RPS19 exons was performed at the cDNA level. In the sequence of RPS19 exon 4, a frameshift mutation was identified. (B) Sequence alignment of RPS19-WT at the cDNA level. (C) Sequence alignment of RPS19-MT at the cDNA level.

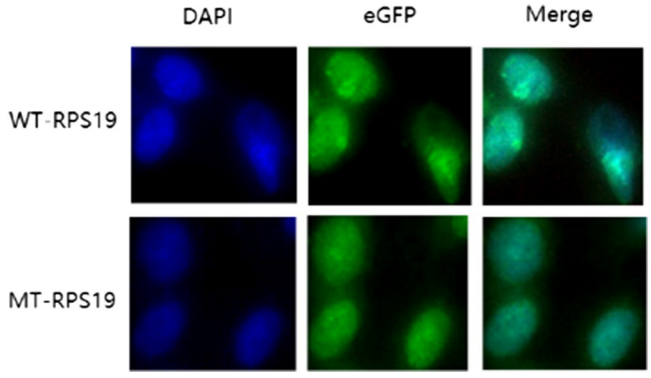

Fig. 5. Immunofluorescence analysis of RPS19 protein localization for wild type (WT-RPS19) and mutated type (MT-RPS19) proteins. Vectors expressing either WT-RPS19 or MT-RPS19 fused to the eGFP tag were transiently transfected into CHO cells. Subcellular localization of RPS19 was visualized by using eGFP (middle panel) and nuclei were stained by DAPI (left panel). The right panel represents the merged picture of DAPI and eGFP staining. The mutated-type RPS19 was mostly localized to entire nuclei with little nucleoli staining while the wild-type RPS19 was predominantly localized to nucleoli. Original magnification: ×200.

WT-eGFP exhibits an evident nucleolar staining as indicated by the co-localization with nucleolin, but RPS19-MT-eGFP does not localize into the nucleolus in the transfected cells and shows the dispersion in the nucleus. This is in good agreement with the finding that two regions in RPS19, the first 15 NH2-terminal amino acids (Met1 to Val15), and the 22 COOH-terminal amino acids (Gly120 to Asn142) are necessary for nucleolar localization of the proteins [9,15]. Strikingly, the mutation (Arg84LysfsX21) identified in our DBA patient removed the 22 COOH-terminal amino acids from one of the 2 NoS (nucleolar localization signals). Thus, it perturbed RPS19 nucleolar localization. Our reported mutant RPS19 could be translocated into the nucleus but not into the nucleolus, confirming that for RPS19, the frameshift mutation (C.251delG) plays a key role in the protein-protein and/or protein-rRNA interactions involved in RPS19 nucleolar localization. Mutations affecting such interactions would cause a failure of the mutated proteins to be retained in the nucleolus.

In summary, we described a novel frameshift deletion in a Chinese infant which has never been reported in the literature and the deletion changes the intracellular localization of the mutated protein. This finding could be very helpful in deciphering the mechanistic understanding of the role of RPS19 in DBA pathogenesis.

Disclaimer statements

Contributors: J.Y.Z. is the first author of this article who was responsible for study design, data analysis, manuscript writing and revision. M.J., H.Z.Z. and Z.B.L. reviewed and revised the manuscript. W.Q.X. and H.P.S. assisted in completing the statistical analysis. Y.M.T. is the guarantor.

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Conflicts of interest

The authors declare no conflict of interest.

Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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