Exome sequencing identified *RPS15A* as a novel causative gene for Diamond-Blackfan anemia

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Supplementary Appendix

Exome sequencing identified *RPS15A* as a novel causative gene for Diamond-Blackfan anemia

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A. Supplementary Methods

Whole-exome sequencing analysis

gDNA of individuals of the family (Table S1) was enriched for protein-coding sequences with a SureSelect Human all Exon V5 kit (Agilent Technologies, Santa Clara, CA, USA). This was followed by massive parallel sequencing with the HiSeq 2000 platform with 100 bp paired-end reads (Illumina, San Diego, CA, USA). Candidate germline variants were detected through our in-house pipeline for WES analysis with minor modifications for the detection of germline variants. The resultant sequences were aligned to the University of California Santa Cruz (UCSC) Genome Browser hg19 with the Burrows-Wheeler Aligner (Li & Durbin, 2009). After removal of duplicate artifacts caused by the polymerase chain reaction (PCR), the single nucleotide variants with an allele frequency >0.25 and insertion-deletions with an allele frequency >0.1 were called. With a mean depth of coverage of 116.3× (67× – 166×), more than 92% of the 50 Mb target sequences were analyzed by more than 10 independent reads. To validate the detected mutations, direct sequencing analysis was performed using genomic DNA derived from the leucocytes.

Cell lines

The human erythroleukemic cell line K562 was maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA) at 37℃ in a 5% CO2 atmosphere.

Genome editing using a CRISPR/Cas9 Vector

The CRISPR/Cas9 genome editing technique was performed using GeneArt CRISPR Nuclease Vector kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. An appropriate guide RNA (gRNA) sequence targeting the 3'-splice site of the third exon of *RPS15A* was identified using an online CRISPR Design Tool (http://tools.genome-engineering.org). Oligomers encoding gRNA were synthesized, annealed and cloned into the GeneArt CRISPR Nuclease Vector. The vector was transfected into K562 cells with two kinds of single-strand oligodeoxynucleotides (ssODNs) using Amaxa Nucleofector (Amaxa Biosystems). The ssODNs (90 base pairs) were designed to introduce the mutation observed in the

patients (c.213 G>A) or a silent mutation (c.207 A>C) as a negative control. The primer sequences are described below. To validate the effect of the RPS15A-deficiency on erythroid lineage cells, the allele frequencies of *RPS15A* mutations (c.213 G>A and c.207 A>C) were determined by targeted next generation sequencing with Miseq (Illumina) once every 3 days up to 12 days. The experiments were performed three times independently. From Day1 to Day3, the allele freqauency of the silent mutation and the DBA-associated *RPS15A* mutation were increased respectively. These results suggested that the genome editing progressed until Day3. We then performed paired t-test (Day3 vs Day12) to assess if the changes in the allele frequency are significant. Paired t-test showed that the allele frequency of the DBA-associated *RPS15A* mutation (c.213G＞A) was significantly decreased (p=0.008) (Table S2).

Transient transfection with small interfering RNA

To knock down the *RPS15A* gene, cells were transfected by using Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD, USA).

Oligonucleotide sequences

To validate the *RPS15A* mutations from the patients and their family as well as the K562 cell genome edited by the CRISP/Cas9 vector, we performed direct sequencing analysis using primers as follows:
RPS15A F2 (5'-AGCACAGAGGTTTAGCTCTCAAGT-3') and
RPS15A R2 (5'-ATTTCCAAAGGCAATTACAACATT-3').

For RT-PCR analysis of family members, we used the following primers located on the second and fourth exons:
RPS15 cDNA f1 (ATCCTGCAATCTAAGCCACAAT) and
RPS15 cDNA f2 (CATTTTTCCAGGTCTTTGAGTTG).

To construct the CRISPR/Cas9 vector, we used the following oligomers encoding the gRNA targeted at the 3'-splice site of the third exon of *RPS15A*:
CRISPR F (5'-CCTCACAGGCAGGCTAAACAAGTTTT -3') and
CRISPR R (3'-GTGGCGGAGTGTCCGTCCGATTTGTT-5').

To introduce a mutation observed in the patients (c.213 G>A) or a silent mutation (c.207 A>C) as a negative control in K562 cells, the following oligodeoxynucleotides (ssODN) were cotransfected with CRISPR/Cas9 Vector: ssODN (c.213 G>A),
5'-CAGAGCTGGGAAAATTGTTGTGAACCTCACAGGCAGGCTAAACAAAgtaagaacga gtgatctacacatttcaaagctttaagaatttttt-3');
ssODN (c.207 A>C),
(5'-CAGAGCTGGGAAAATTGTTGTGAACCTCACAGGCAGGCTCAACAAGgtaagaacg agtgatctacacatttcaaagctttaagaatttttt-3')

Full-length zebrafish *rps15a* (GenBank accession no. NM_212762) was amplified by PCR using forward and reverse primers appending *Eco*RI and *Xho*I sites (forward:
5'-CCGGAATTCCATCATGGTGCGCATGAAC-3'
and reverse: 5'-CGGCTCGAGTGTTGGCGACTTTACATGTTT-3').

To detect *rps15a* transcripts, reverse transcriptase-PCR was performed using primers as follows:
(5'-TGCGCTGAAAAGCATCAATA-3') and
(5'-CCAGATCCTTCAACTGCACA-3').
The sequence of the MO targeting the splice junction of *rps15a* pre-mRNA was (5'-GCAAGTCACACTCACCTTGTTCAGC-3').

Conditions for Northern blot analysis.
Total RNA was extracted from cells using the RNeasy plus kit (QIAGEN). Total RNA was hybridized at 42ºC in RapidHyb buffer (GE Healthcare UK, Ltd. Buckinghamshire, England) and the membranes were washed in 0.1 x SSC, 0.1% SDS, 42°C. The probes used in the present study were as follows:
5′ITS1 (5′–CCTCGCCCTCCGGGCTCCGTTAATGATC-3′) and
5'ITS2 (5'-GGGGCGATTGATCGGCAAGCGACGCTC-3').

Functional analysis using zebrafish
A morpholino antisense oligonucleotide (MO) targeting the splice site of zebrafish *rps15a* was obtained from Gene Tools, LLC (Philomath, OR, USA). MO was injected at

concentrations of 0.5-2.5 μg/μL into one-cell stage embryos. The MO-injected embryos (morphants) were grown at 28.5°C. The morphological features of the morphants were compared with wild-type embryos at 24 hours post fertilization (hpf). Hemoglobin staining was performed at 54 hpf using *o*-dianisidine. Full-length *rps15a* was amplified by PCR and cloned into a pCS2+ vector for *in vitro* transcription. Capped mRNAs were synthesized from the linearized template using an mMessage mMachine SP6 kit (Life Technologies). The synthesized *rps15a* mRNA was injected at 400 ng/μL into one-cell-stage embryos with the splice site-targeting MO at 1.0 μg/μL. Total RNA was isolated from the wild-type embryos and the morphants. To distinguish normal or cryptic sizes of the *rps15a* transcript, RT-PCR was performed with primer pairs designed at exons 2 and 4.

B. Supplementary Table

Table S1. Clinical characteristics of the affected family members

Patient	Mother	Older sister	Proband
Age (years old)	46	18	17
Gender	Female	Female	Female
Age at diagnosis	0 month	1year 0 month	3 month
Malformation	No	No	Yes
Clinical data	at 42 years old	at diagnosis	at diagnosis
RBC (10^9/L)	580	1,680	2,870
Hb (g/L)	24	60	88
MCV (fL)	122	103	88.5
Reticulocyte (%)	1.8	3.9	1.0
WBC (10^9/L)	4.0	5.6	5.1
Plt (10^9/L)	557	691	306
Bone marrow	N/A	N/A	Normal cellularity, Erythroid 0%
Response to first steroid therapy	Yes	Yes	Yes
Present therapy	No	No	No

N/A: not available

Table S2. The allele frequency of the DBA-associated *RPS15A* mutation (c.213G>A) and a silent mutation (c.207A>C)

					Paired t-test	
Allele		Day 1	Day 3	Day 12	Day 1vs 12	Day 3 vs 12
c.207 A>C : Syn	Ave	18.90	27.38	37.00	0.033	0.030
	SD	4.46	6.00	8.93		
c.213 G>A : SE	Ave	9.76	16.67	7.70	0.199	0.008
	SD	5.08	2.84	4.16		

Syn : Synonimous substitution, SE : Splicing error, Ave : Average,

SD : Standard deviation

Data were derived from three independent experiments.

C. Supplementary Figures

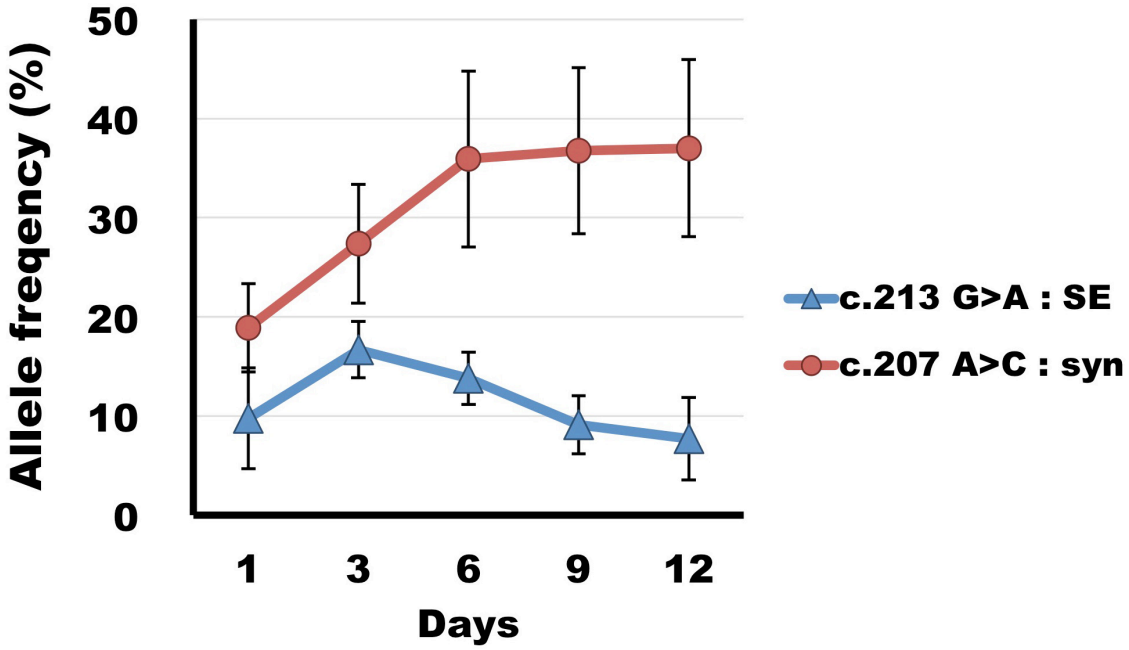

Figure S1. Erythroid cell line K562 after CRISPR/Cas9 gene editing.

The CRISPR/Cas9 vector was co-transfected into K562 cells with two kinds of single-stranded oligodeoxynucleotides using Amaxa Nucleofector (Amaxa Biosystems). We analyzed allele frequency in bulk DNA of each culture once every 3 days. From Day1 to Day3, the allele frequency of the silent mutation and the DBA-associated RPS15A mutation were increased respectively. After Day 3, the allele frequency of the DBA-associated RPS15A mutation (c.213G＞A) was decreased. The data were derived from three independent experiments.

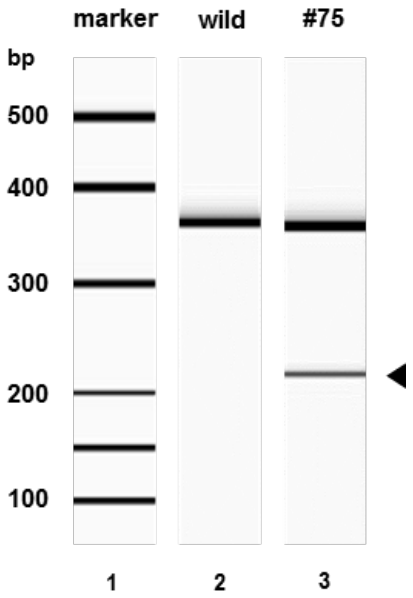

Figure S2. K562 subclone #75 with heterozygous mutation in *RPS15A*

This cell line expresses normal and alternative splicing form lacking second exon of *RPS15A* (black arrow head).

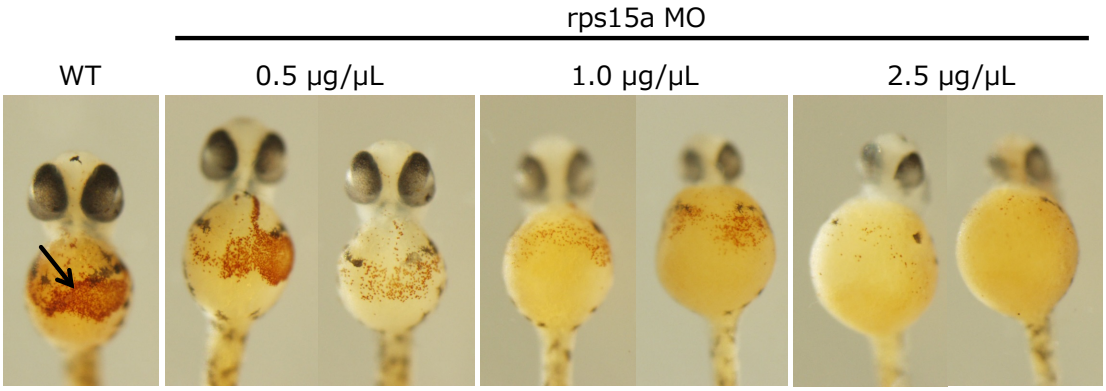

Figure S3. Hemoglobin staining of Rps15a-deficeint zebrafish

A reduction of erythrocyte production was more prominent in the morphants that were injected with MO at higher concentrations.