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Ⅳ．研究成果の刊行物・別冊

bjh research paper

Loss of function mutations in *RPL27* and *RPS27* identified by whole-exome sequencing in Diamond-Blackfan anaemia

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Summary

Diamond-Blackfan anaemia is a congenital bone marrow failure syndrome that is characterized by red blood cell aplasia. The disease has been associated with mutations or large deletions in 11 ribosomal protein genes including *RPS7*, *RPS10*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPS29*, *RPL5*, *RPL11*, *RPL26* and *RPL35A* as well as *GATA1* in more than 50% of patients. However, the molecular aetiology of many Diamond-Blackfan anaemia cases remains to be uncovered. To identify new mutations responsible for Diamond-Blackfan anaemia, we performed whole-exome sequencing analysis of 48 patients with no documented mutations/deletions involving known Diamond-Blackfan anaemia genes except for *RPS7*, *RPL26*, *RPS29* and *GATA1*. Here, we identified a *de novo* splicing error mutation in *RPL27* and frameshift deletion in *RPS27* in sporadic patients with Diamond-Blackfan anaemia. *In vitro* knockdown of gene expression disturbed pre-ribosomal RNA processing. Zebrafish models of *rpl27 and rps27* mutations showed impairments of erythrocyte production and tail and/or brain development. Additional novel mutations were found in eight patients, including *RPL3L*, *RPL6*, *RPL7L1T*, *RPL8*, *RPL13*, *RPL14*, *RPL18A* and *RPL31*. In conclusion, we identified novel germline mutations of two ribosomal protein genes responsible for Diamond-Blackfan anaemia, further confirming the concept that mutations in ribosomal protein genes lead to Diamond-Blackfan anaemia.

Keywords: bone marrow failure, Diamond-Blackfan, genetic analysis, erythropoiesis, childhood.

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Diamond-Blackfan anaemia (DBA) is an inherited rare red blood cell aplasia that is characterized by normochromic macrocytic anaemia, reticulocytopenia and selective defects in erythroid progenitor cells in normocellular bone marrow. Patients usually present with anaemia in the first year of life, although there is a non-classical mild phenotype diagnosed later in life. Macrocytic anaemia is a prominent feature of DBA but the disease is also characterized by growth retardation and congenital anomalies, including craniofacial, upper limb/hand, cardiac and genitourinary malformations, that are present in approximately half of the patients. In addition, DBA patients have a predisposition to malignancies including acute myeloid leukaemia, myelodysplastic syndrome, colon carcinoma, osteogenic sarcoma and female genital cancer (Lipton *et al*, 2006; Vlachos *et al*, 2008, 2012; Ito *et al*, 2010).

DBA is associated with single, monoallelic, inactivating mutations in ribosomal protein (RP) genes. Except for rare germline *GATA1* mutations reported in two X-linked DBA families (Sankaran *et al*, 2012), all known causative mutations have involved RP genes. Approximately 20% of DBA patients are familial. However, most cases occur sporadically and have *de novo* mutations. In DBA, mutations in RP genes include *RPS7, RPS10, RPS17, RPS19, RPS24, RPS26* and *RPS29* (encoding RP for the small subunit) and *RPL5, RPL11, RPL26* and *RPL35A* (encoding RP for the large subunit). These mutations have been reported in up to 60% of DBA patients (Draptchinskaia *et al*, 1999; Gazda *et al*, 2006, 2008, 2012; Cmejla *et al*, 2007; Farrar *et al*, 2008; Doherty *et al*, 2010; Konno *et al*, 2010; Gerrard *et al*, 2013; Mirabello *et al*, 2014). To date, approximately 40% of patients have no known pathogenic mutation. In this study, we carried out whole-exome sequencing (WES) analysis of 48 patients without known causative mutations or deletions and found loss-of function mutations in the *RPS27* and *RPL27* genes.

Methods

Patient samples

Genomic DNA (gDNA) was extracted from peripheral blood leucocytes with the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The diagnosis of DBA was based on the criteria developed at an international clinical consensus conference (Vlachos *et al*, 2008). All clinical samples were obtained with informed consent from paediatric and/or haematology departments throughout Japan. The Ethics Committee of Hirosaki University Graduate School of Medicine and the University of Tokyo approved this study.

Whole-exome sequencing analysis

To identify the candidate disease variants including non-RP genes, we performed WES analysis. gDNA from patients was enriched for protein-coding sequences with a SureSelect Human All Exon V3, V4 or V5 kit (Agilent Technologies, Santa Clara, CA, USA). This was followed by massively

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 (Yoshida *et al*, 2011; Kunishima *et al*, 2013). 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 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 analysed by more than 10 independent reads.

Target deep sequencing analysis was performed for the RP genes with a low depth of coverage of <10×. Amplification of the genome was accomplished by long PCR reactions using KOD-FX-Neo DNA polymerase (TOYOBO, Osaka, Japan) using the primers described in Data S1. The PCR products were used for library preparation after determination of their quantity by the Qubit dsDNA HS Assay (Life Technologies, Invitrogen division, Darmstadt, Germany). Libraries were prepared using the Nextera XT DNA Sample Preparation Kit (Illumina) according to the manufacturer's recommendation. Sequencing reactions were carried out using the MiSeq v2 (2 × 150 bp) chemistries (Illumina). The MiSeq re-sequencing protocol for amplicon was performed. The sequences were mapped on the human GRCh37/hg19 assembly and quality-checked using the on-board software MiSeq Reporter, and analysed by AVADIS NGS software (Agilent Technologies).

To validate *RPL27* and *RPS27* mutations of patients and their families, we performed direct sequencing analysis using the primers described in Data S1.

Cell lines and transient transfection with small interfering RNA

The human erythroleukaemic 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°C in a 5% CO_2 atmosphere. To knock down the *RPL27* and *RPS27* genes, cells were transfected by using Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD, USA) (Nucleofector solution V, Nucleofector program T-16) with 5 μl of 40 nmol/l siRNA solutions per 2 × 10^6 cells. The siRNA purchased from Thermo-Fisher Scientific-Dharmacon (Waltham, MA, USA) were ON-TARGET plus SMART pool human *RPS19, RPL5, RPS27, RPL27* and a non-targeting pool.

Northern blot analysis

Total RNA was extracted from cells using the RNeasy plus kit (QIAGEN), and hybridized at high stringency. The probes used in the present study are described in Data S1.

Functional analysis using zebrafish

Morpholino antisense oligonucleotides (MOs) targeting zebrafish *rpl27* and *rps27*, orthologs of human *RPL27* and *RPS27* respectively, were obtained from Gene Tools, LLC (Philomath, OR, USA). They were injected at a concentration of 5·0 or 20 μg/μl into one-cell-stage embryos. The MO-injected embryos (morphants) were grown at 28·5°C. Haemoglobin staining was performed at 48 h post-fertilization (hpf) using *o*-dianisidine (Uechi *et al*, 2006; Torihara *et al*, 2011).

Full-length *rpl27* 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) and injected at 250 ng/μl into one-cell-stage embryos.

Total RNA was isolated from wild-types and the morphants. Reverse transcription (RT)-PCR was used to distinguish normal or cryptic sizes of the *rpl27* and *rps27.1* transcripts. This was performed by using primer pairs designed at exons 1 and 5 and exons 1 and 4, respectively. The MO and primer sequences are described in Data S1.

Results

Whole exome-sequencing analysis

A total of 98 Japanese DBA patients were registered and blood genomic DNA samples were collected. All samples were first screened for mutations in eight of 10 known DBA genes (*RPL5, RPL11, RPL35A, RPS10, RPS17, RPS19, RPS24* and *RPS26*) as well as *RPS14*, which had been implicated in the 5q- myelodysplastic syndrome, a subtype of myelodysplastic syndrome characterized by a defect in erythroid differentiation (Ebert *et al*, 2008). Screening was achieved by direct sequence analysis accompanied by high-resolution melt analysis (HRM) (Konno *et al*, 2010). Among these patients, 38% (38/100) had identifiable DBA mutations (Table S1). Some of the patients were described in our previous reports (Konno *et al*, 2010; Kuramitsu *et al*, 2012). Then, we screened for large gene deletions in the remaining 60 patients using synchronized-quantitative-PCR DBA gene copy number assay and/or genome wide single nucleotide polymorphism array analysis (Kuramitsu *et al*, 2012). We found that 20% (12 of 60) of samples had large deletions in previously identified DBA genes (Table S1).

WES was performed on the remaining 48 patients who lacked documented mutations or large deletions involving known DBA genes by screening. We found gene alterations in *RPS7, RPS27, RPL3L, RPL6, RPL7L1, RPL8, RPL13, RPL14, RPL18A, RPL27, RPL31* and *RPL35A* in 12 patients, whose WES data have been deposited in the European Genome-phenome Archive (EGA) under accession number EGAS00001000875. WES failed to identify a single *GATA1* mutation (Table I). The substitution mutations observed in

Table I. Characteristics of patients investigated by whole-exome sequencing.

Patient (UPN)	Age at diagnosis	Gender	Inheritance	Abnormalities	Mutation
5	1 year	F	Sporadic	None	*RPL18A* c.481C>T p.Arg161Cys
7	1 month	M	Sporadic	SGA, craniofacial abnormalities, skin pigmentation	ND
13	3 months	F	Sporadic	None	ND
21	1 year	F	Familial	None	*RPS7* c.75+1G>A Splicing error, *RPL13* c.547C>T p.R183C
26	Birth	F	Sporadic	Spastic quadriplegia, congenital hip dislocation, severe myopia, optic nerve hypoplasia, growth retardation	ND
35	18 months	M	Familial	None	*RPL6* c.253_255del p.Lys85del
36 (35 cousin)	Birth	M	Familial	Hypospadias, cryptorchidism	ND (No *RPL6* mutation was detected.)
37	4 years	M	Sporadic	Hypospadias, cryptorchidism, SGA	ND
42	2 months	F	Sporadic	None	*RPS27* c.89delC, p.Tyr31Thrfs*5
48	NA	NA	Sporadic	Fetal hydrops	*RPL3L* c.76C>G p.Arg26Gly
49	2 months	M	Sporadic	SGA, growth retardation	ND
50	2 months	F	Familial	Neutropenia	ND
52 (50 sister)	6 months	F	Familial	Neutropenia	ND
51	7 months	F	Sporadic	None	ND
53	8 months	F	Sporadic	SGA	ND
54	8 years	F	Sporadic	None	ND
61	9 months	M	Sporadic	None	ND
67	3 years	M	Sporadic	None	ND
68	16 months	M	Sporadic	None	*RPL14* c.446CTG(9), c.446CTG(15)
69	1 year	M	Sporadic	Flat thenar	ND
75	Birth	F	Familial	Acetabular dysplasia, total anomalous pulmonary venous connection	ND
76	Birth	M	Sporadic	IgG subclass 2 and 4 deficiency	*RPL35A* c.125A>G:p.Tyr42Cys *RPL7L1* c.G544A:p.V182I (His unaffected parents did not possess the mutation in *RPL35A*.)
77	Birth	M	Familial	None	ND
83	9 months	M	Sporadic	None	*RPL31* c.122G>A p.Arg41His
88	Birth	M	Familial	Cryptorchidism, hypospadias, learning disabilities	ND
89 (88 father)	NA	M	Familial	Skeletal malformation of fingers, growth retardation	ND
90	10 months	M	Sporadic	None	ND
91	Birth	F	Sporadic	None	*RPL8* c.413C>T p.Ser138Phe
93	11 months	M	Sporadic	Leucoderma, syndactyly	ND
95	Birth	F	Sporadic	Atrial septal defect, pulmonary stenosis	*RPL27* c.-2-1G>A Splicing error
96	28 months	F	Sporadic	None	ND
97	4 years	F	Sporadic	Growth retardation	ND
105	Birth	M	Sporadic	Growth retardation	ND
109	9 months	F	Sporadic	None	ND

Table I. (*Continued*)

Patient (UPN)	Age at diagnosis	Gender	Inheritance	Abnormalities	Mutation
112	4 months	F	Sporadic	Pulmonary atresia, tricuspid atresia, ventricular septal defect, hypoplasia of right ventricle, polydactyly of thumb, cerebellar hypoplasia, low-set ear, mandibular retraction, growth retardation	ND
116	4 months	M	Sporadic	Flat thenar	ND
117	NA	F	Sporadic	NA	ND
121	2 months	F	Sporadic	Growth retardation	ND
135	1 year	M	Sporadic	Xanthogranuloma	ND
136	Birth	M	Sporadic	None	ND
140	Birth	F	Sporadic	SGA	ND
144	2 months	F	Sporadic	Neutropenia	*RPL35A* c.125A>G p.Tyr42Cys (Her unaffected parents did not possess the mutation in *RPL35A*.)
151	9 months	M	Unknown	None	*RPL35A* c.113A>G p.Glu38Gly (His unaffected father was also heterozygous for the allele.)
152	NA	NA	Sporadic	None	ND
153	17 months	M	Sporadic	None	ND
154	NA	NA	NA	NA	ND
158	3 months	M	Sporadic	Patent ductus arteriosus	ND
159	8 months	M	Sporadic	None	ND

UPN, unique patient number; NA, not available; M, male; F, female; ND, not detected; SGA, small for gestational age.

RPL35A (Patients 76, 144 and 151) had escaped detection by the HRM analysis in the first step screening but were found by WES analysis. The mutations were confirmed by direct sequencing analysis. We speculated that the sensitivity of the HRM screening was insufficient for detection of these particular mutations because the size of the PCR amplicon containing the mutations was too large for the screening. A single missense mutation (c.125A>G: p.Tyr42Cys) observed in two of the sporadic DBA cases, Patients 76 and 144, was predicted to be causative because the unaffected parents of the two patients did not possess the mutation, suggesting that the mutations were *de novo* (Table I). Furthermore, tyrosine at position 42 is highly conserved among species. On the other hand, the pathological significance of the *RPL35A* mutation (c.113A>G p.Glu38Gly) observed in Patient 151 remains unknown because glutamic acid at position 38 is not well-conserved and the patient's unaffected father was also heterozygous for the allele (Table I).

The two known DBA genes, *RPS7* and *RPL26*, were not included in the first screening. Consequently, WES identified a *RPS7* mutation in Patient 21 and confirmed the mutation by direct sequencing. The mutation was predicted to be causative because it seemed to induce a splicing error in the gene. Mutations identified in the eight patients, including *RPL18A* in Patient 5, *RPL13* in Patient 21, *RPL6* in Patient 35, *RPL3L* in Patient 48, *RPL14* in Patient 68, *RPL7L1T* in Patient 76, *RPL31* in Patient 83 and *RPL8* in Patient 91, were missense mutations or in-frame deletions. Almost all of the causative variants of RP genes observed in DBA are loss-of function mutations (Gazda *et al*, 2012). Whereas analyses by SIFT, PolyPhen-2, Mutation Taster and CONDEL predicted that some of these mutations would probably damage the structure and function of ribosomal proteins, the pathological effects of the above-mentioned mutations were uncertain (Table S2). The substitution mutation of *RPL13* observed in Patient 21 seemed to be non-pathological because the *RPS7* splicing error mutation was also identified in this patient. The missense mutation in *RPL7L1T* found in Patient 76 also seemed to be non-pathological, because the *de novo* *RPL35A* mutation was identified in this patient. The in-frame deletion of *RPL6* observed in Patient 35 with familial DBA also might be non-causative, because the mutation was not identified in his cousin, Patient 36 (Table I).

De novo *mutation in* RPL27 *and* RPS27

Next, we focused on novel loss-of function mutations in *RPL27* and *RPS27*, found in the screening. Almost all RP genes were sequenced with enough coverage for detecting germline mutations except for several RP genes (Table S3). Target deep sequencing analysis was performed for the RP genes with a low depth of coverage of <10× (Table S4 and

S5), and we confirmed that the mutations in *RPL27* and *RPS27* were the only ones found in these patients.

In Patient 95, we identified the substitution of c.-2-1G>A in the *RPL27* gene, a putative splicing error mutation (Fig 1A). To confirm the effect of the mutation, we performed RT-PCR analysis by using primers located on the first and third exons and total RNA derived from the patient's leucocytes. We found two transcripts in Patient 95: the full-length transcript and a shorter transcript lacking exon 2 by alternative splicing, a variant skipping exon 2, in which the translation initiation codon is located (Fig 1B,C). We performed a quantitative assessment of the levels of the full-length transcripts and the short transcripts, using the Experion automated electrophoresis system (Bio-Rad, Hercules, CA, USA). The calculated concentration of each product was 48·31 nmol/μl (7·49 ng/μl) and 31·69 nmol/μl (3·19 ng/μl), respectively. The results indicated that the extent of aberrant splicing accounted for about 40% of total *RPL27* transcripts in this patient. Patient 95 was a 2-year-old girl with no family history of anaemia, diagnosed with DBA at birth. She had an atrial septal defect and pulmonary stenosis. She responded to corticosteroid treatment and has been in remission for 2 years. Her clinical characteristics are presented in Table II. As she was thought to be sporadic type DBA, we examined the genotype of her parents. The direct sequencing analysis showed that the parents were homozygous for wild-type *RPL27* (Fig 1A). These results suggested the mutation observed in the patient was *de novo* and a probable pathogenic mutation of DBA.

In Patient 42, we found a single nucleotide deletion (c.90delC, p.Tyr31Thrfs*5) in the *RPS27* gene generating a premature stop codon by frameshift (Fig 1D). The patient was a 4-year-old girl with no family history of anaemia, diagnosed with DBA at 2 months of age. This patient had no abnormalities except for skin pigmentation, and responded to steroid treatment. Her clinical characteristics are presented in Table II. Her unaffected parents did not have the gene alteration observed in the patients (Fig 1D), indicating the mutation was *de novo*.

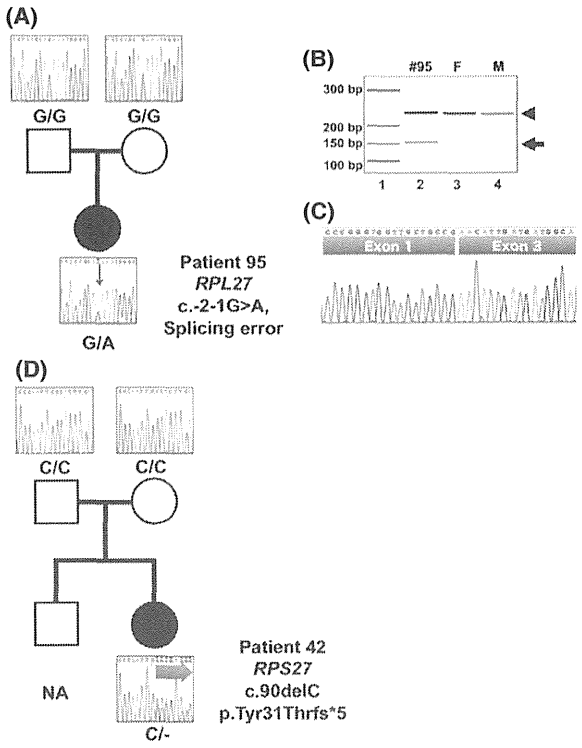

Fig 1. *De novo* mutations in *RPL27* and *RPS27*. (A) Family tree of Patient 95. Electropherograms indicate the gDNA sequence including the boundary between IVS-1 and the first exon of the *RPL27* gene. The red arrow indicates the position of the nucleotide substitution −2-1G>A observed in Patient 95. (B) RT-PCR analysis using the primer set located on the first and third exons of the *RPL27* gene. Arrowhead and arrow indicate PCR products for the full-length variant and the alternative splicing lacking the second exon, respectively. Molecular marker (lane 1), Patient 95 (lane 2), her father (F, lane 3) and mother (M, lane 4) are shown. (C) Sequence analysis of the short PCR product of Patient 95 showing the alternative splicing variants lacking the second exon. (D) Family tree of Patient 42. Electropherograms indicate gDNA sequence including a portion of the second exon of the *RPS27* gene. Blue arrow indicates the frameshift signals caused by single nucleotide deletion of c.90delC.

Defective pre-ribosomal RNA processing due to repression of RPL27 *or* RPS27

A single pre-ribosomal RNA (pre-rRNA), called 45S is processed into mature 28S, 18S and 5.8S rRNAs (Hadjiolova *et al*, 1993; Rouquette *et al*, 2005). Among the mature rRNAs, the 28S and 5.8S rRNAs associate with the large ribosomal subunit (60S) and the 18S rRNA associates with the small subunits (40S) of the ribosome. It has been reported that the mutations in RP genes observed in DBA cause defects in pre-rRNA processing. For example, the loss-of-function of the small subunit of RP affects maturation of 18S rRNA (Gazda *et al*, 2006, 2012; Choesmel *et al*, 2007; Flygare *et al*, 2007; Idol *et al*, 2007; Doherty *et al*, 2010). To validate the effects of the knockdown of *RPS27* or *RPL27* on erythroid lineage cells, we introduced siRNA into the human erythroid cell line K562 cells and analysed pre-rRNA processing by Northern blotting analysis.

Consistent with previous reports, decreased expression of *RPS19* was associated with a defect in rRNA processing characterized by a decrease in 18S-E rRNA with accumulation of a 21S rRNA precursor, and decreased expression of *RPS26* resulted in accumulation of a 26S rRNA precursor. Reduction of *RPS27* led to the accumulation of 30S rRNA and a decrease in the 21S rRNA and 18S-E rRNA (Fig 2). These findings suggest that RPS27 is also essential for 18S rRNA processing, although RPS27 involves rRNA processing associated with the small subunit at different stages from RPS19 and RPS26. In contrast, knockdown of *RPL27* caused accumulation of 32S rRNA, which is very similar to the effects by *RPL5* siRNA, suggesting that RPL27 is important for the

Table II. Clinical characteristics of DBA patients with *RPS27* or *RPL27* mutation.

UPN	42	95
Mutated gene	*RPS27*	*RPL27*
Age (years)	4	2
Gender	Female	Female
Family history of anaemia	No	No
Onset	2 months of age	At birth
Malformation	Skin pigmentation	Atrial septal defect pulmonary stenosis
Clinical data at onset		
RBC ($\times 10^{12}$/l)	1·38	2·17
Hb (g/l)	49	71
MCV (fl)	105	92·3
Reticulocytes (%)	0·17	0·1
WBC ($\times 10^{9}$/l)	11·68	5·5
Platelets ($\times 10^{9}$/l)	373	446
Bone marrow	Hyper cellularity, erythroid 1%	Normo-cellularity, erythroid 7·4%
Response to first steroid therapy	Yes	Yes
Present therapy	NA	NA

UPN, unique patient number; RBC, red blood cell count; WBC, white blood cell count; NA, not available.

maturation of 28S and 5.8S rRNAs (Fig 2). These findings showed that decreased expression of *RPS27* and *RPL27* perturbed pre-rRNA processing associated with the small and large subunits, respectively.

To accurately model the degree of ribosomal haploinsufficiency, we titrated the dose of the siRNA to obtain approximately 50% of the expression compared with wild-type cells (Figure S1A). For this experiment, we used 50% *RPS19*, *RPS26* and *RPL5* knocked-down cells as positive controls. However, the rRNA processing defects were not clearly observed under these conditions even in the positive controls (Figure S1B). These results suggested that a more accurate functional assay was necessary to investigate the pathological significance of these mutations. For that reason, we turned to the zebrafish model.

Impairment of erythroid development in rpl27 *and* rps27*-deficient zebrafish*

To investigate the effects of *RPL27* mutations in DBA, we knocked down the zebrafish ortholog (*rpl27*) using MOs and analysed the morphology and erythropoietic status during embryonic development. The coding region of *rpl27* shares 84% nucleotide and 96% amino acid identities with its human ortholog. Although gene duplication is common in zebrafish, available information from public databases suggests that *rpl27* exists as a single copy in the genome. We inhibited expression of this gene using an MO designed to target the 3′-splice site of the first intron that corresponded

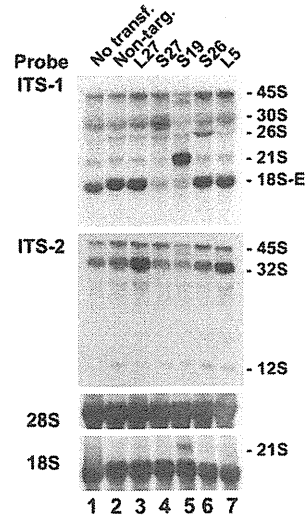

Fig 2. Perturbation of pre-rRNA processing by knockdown of the *RPL27* or *RPS27* gene. Northern blot analysis using K562 cells knocked down by siRNAs. The 5′ extremities of the internal transcribed spacer 1 (ITS-1) and internal transcribed spacer 2 (ITS-2) were used as probes to detect the precursors to the 18S rRNA associated with the small subunit and 28S rRNA and 5.8S rRNA associated with the large subunit of the ribosome, respectively. *RPS19*, *RPS26* and *RPL5* knocked-down cells were used as positive controls for the detection of defects in rRNA processing. ITS-1 and ITS-2 probes revealed the accumulation of 30S pre-rRNA in *RPS27* knocked-down cells and 32S pre-rRNA in *RPL27* knocked-down cells, respectively. Decrease of 18S-E pre-rRNA was also detected by the ITS-1 probe in *RPS27* knockdown cells. The mature 18S and 28S rRNAs were detected with specific probes.

to the position at which the mutation was identified in the patient (Fig 3A). Injection of this MO into the one-cell stage embryos perturbed the splicing and resulted in exclusion of exon 2 as observed in the patient (Fig 3B). When injected with 5 μg/μl MO targeted against *rpl27*, the expression level of a smaller transcript lacking exon 2 was comparable to that seen in Patient 1 (Figs 1B and 3B). Therefore, all of the following experiments were performed using 5 μg/μl MO.

We compared the morphological features of the morphants with wild-type embryos and found that the morphants showed abnormal phenotypes, such as a thin yolk sac extension and a bent tail at 25 hpf (Fig 3C). We also performed haemoglobin staining at 48 hpf and found a marked reduction of erythrocyte production in the cardial vein of the morphants (Fig 3D). All these abnormalities were rescued by the simultaneous injection of *rpl27* mRNA into the embryos, indicating that the morphological defects and decreased erythropoiesis observed in the morphants were caused by the aberrant splicing of *rpl27* in zebrafish (Fig 3B,D). These results suggested that the splice site mutation identified in human *RPL27* could be responsible for the pathogenesis of DBA.

We next investigated the effects of *RPS27* mutations in DBA. Public databases suggest that there are three copies of the zebrafish *rps27* gene, *rps27.1*, *rps27.2* and *rps27.3*, whereas

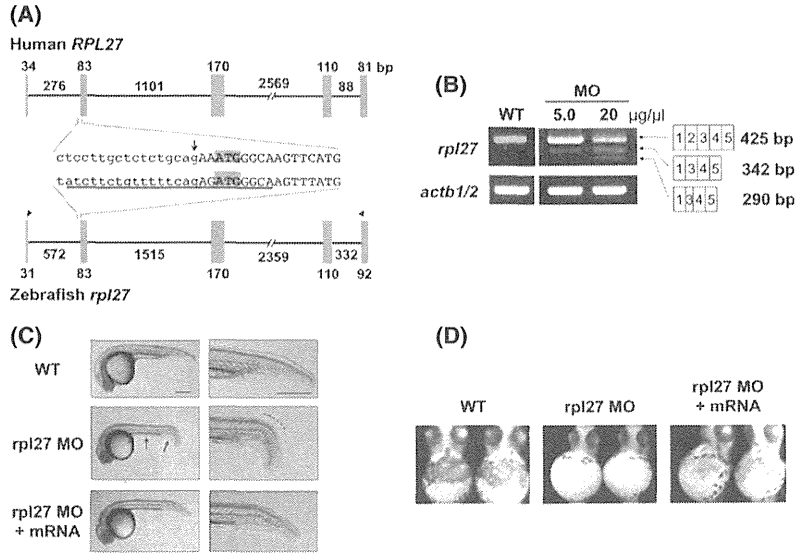

Fig 3. Morphological defects and decreased erythropoiesis in *rpl27* morphants. (A) The gene structures of human *RPL27* and zebrafish *rpl27*. The sequences of intron 1/exon 2 boundary regions are indicated. Uppercase and lowercase letters show the exon and intron sequences, respectively. The MO target site is underlined and the translation initiation codons (ATG) are shaded. The arrow indicates the position of the mutated nucleotide in the patient. Arrowheads show the primer positions for the RT-PCR. (B) The results of RT-PCR of *rpl27* and *actb* (control) in wild type and MO injected embryos. A smaller transcript without exon 2 was observed in the morphants as seen in the patient at a comparable level, when 5 μg/μl MO was injected into the one-cell-stage embryos. Injection with higher concentrations of MO (20 μg/μl) also produced a truncated exon 3. (C) Morphological features of wild-type and MO-injected embryos. A thin yolk sac extension and a bent tail are prominent in the morphants injected with 5 μg/μl MO (arrows), whereas these features are rescued in the embryos injected with *rpl27* mRNA. Scale bars: 250 μm. (D) The haemoglobin staining of cardial veins at 48 hpf. Compared to wild-type embryos, *rpl27* morphants injected with 5 μg/μl MO showed a drastic reduction in the number of haemoglobin-stained blood cells. Morphants co-injected with *rpl27* mRNA show recovery of the stained cells.

the human genome contains two copies, *RPS27* and *RPS27L*. We inhibited expression of the zebrafish *rps27.1*, which shares 96% amino acid identity with the human *RPS27*, using an MO designed to target the 5′-splice site of the second intron (Fig 4A). Injection of this MO into the embryos perturbed the splicing and resulted in exclusion of exon 2 (Fig 4B) that consequently introduced a stop codon in exon 3. The morphants showed abnormal phenotypes, such as a thin yolk sac extension, a bent tail and a malformed brain region at 26 hpf (Fig 4C). We also observed reduced erythrocyte production in about 60% of the morphants (Fig 4D). These results suggested that the frameshift mutation identified in human *RPS27* is a strong candidate for a causative mutation for DBA.

Discussion

WES analysis identified loss-of-function mutations in two RP genes. Each of the patients carrying one of these mutations was a sporadic case, and the mutations were *de novo*. Knockdown of *RPL27* and *RPS27* disturbed pre-rRNA processing for the large and small subunits, respectively. Although the zebrafish models cannot reproduce the exact features of DBA, such as macrocytic anaemia appearing after birth and skeletal abnormalities, the models of *RPL27* and *RPS27* mutations showed impairment of erythrocyte production. These results suggested that *RPL27* and *RPS27* play important roles in erythropoiesis, and that haploinsufficiency of either RP could lead to pure red cell aplasia. However, these findings only represent a single patient in relation to each gene. The identification of new DBA cases in the future with mutations in these genes will be important to confidently label *RPS27* and *RPL27* as DBA disease genes.

Interestingly, RPS27 binds to MDM2 through its N-terminal region, and overexpression of RPS27 stabilizes TP53 by inhibiting MDM2-induced TP53 ubiquitination (Xiong *et al*, 2011). Although the exact mechanism by which ribosome disruptions leads to DBA is unclear, a widely accepted hypothesis is that imbalances in expression of individual RPs trigger a TP53-mediated checkpoint, leading to cell cycle arrest and apoptosis of erythroid precursors (Narla & Ebert, 2010). Several animal models have demonstrated the role of TP53 in the pathophysiology of DBA (McGowan & Mason, 2011). In support of this conclusion, it was observed that certain RPs, such as RPL5, RPL11, RPL23, RPL26 and RPS7, bind to and inhibit the TP53 regulator MDM2, thereby inhibiting its ability to promote TP53 degradation (Zhang & Lu, 2009). Notably, like *RPL27*, many of the RP genes, including *RPL5*, *RPL11*, *RPL26* and *RPS7*, are mutated in DBA.

Here, we report the results of RP gene mutations observed in 98 Japanese DBA patients. The frequency of the patients harbouring probable causative mutations/large

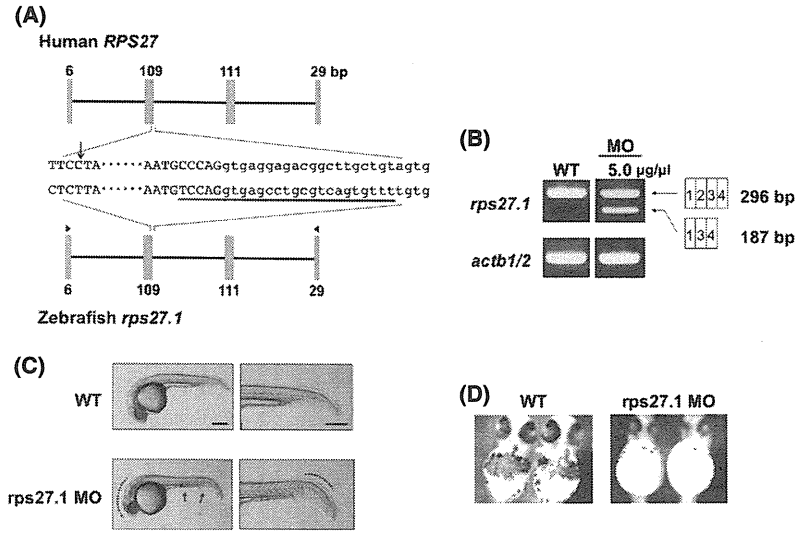

Fig 4. Morphological defects and decreased erythropoiesis in *rps27* morphants. (A) The gene structures of human *RPS27* and zebrafish *rps27.1*. The sequences of exon 2/intron 2 boundary regions are indicated. Uppercase and lowercase letters show the exon and intron sequences, respectively. The MO target site is underlined. The arrow indicates the position of the mutated nucleotide in the patient. Arrowheads show the primer positions for RT-PCR. (B) The results of RT-PCR of *rps27.1* and *actb* (control) in wild-type and MO-injected embryos. A smaller transcript without exon 2 was observed in the morphants. (C) Morphological features of wild-type and MO-injected embryos at 26 hpf. A thin yolk sac extension and a bent tail are prominent in the morphants (arrows). An abnormal development in the brain region was also observed. Scale bars: 250 μm. (D) Haemoglobin staining of cardial veins at 48 hpf.

deletions in RP genes was 55% (56/98), including *RPS19* 16% (16), *RPL5* 12% (12), *RPL11* 5% (5), *RPS17* 7% (7), *RPL35A* 7% (7), *RPS26* 4% (4), *RPS10* 1% (1), *RPS7* 1% (1), *RPL27* 1% (1) and *RPS27* 1% (1). No mutation of *RPS24*, *RPS29* or *RPL26* was identified in this study. In addition to above mutations, we found a missense mutation of *RPL35A* in a sporadic case (Patient 151). Mutations in RP genes are characterized by a wide variability of phenotypic expression. Even family members with the same mutation in the RP gene can present with clinical differences (Willig *et al*, 1999). For example, *RPS19* mutations are found in some first-degree relatives presenting only with isolated high erythrocyte adenosine deaminase activity and/or macrocytosis. Therefore, there is still the possibility that this *RPL35A* mutation is disease-causing, although the patients' father had the same heterozygous mutation without anaemia. To confirm the pathological effect of the substitution, a functional analysis is necessary. The zebrafish model might be very useful for this assay.

Recently, Gerrard *et al* (2013) found inactivating mutations in 15/17 patients by targeted sequencing of 80 RP genes. All mutations were in genes previously found to be DBA genes. The differences between these results and those in our study might be due to differences between human populations. In our cohort, all patients were Asian, whereas 80% were Caucasian in the cohort reported by Gerrard *et al* (2013). The frequency of RP gene mutaitons may vary between ethnic groups. However, the data from both cohorts are based on a relatively low number of patients and values showing significant differences between cohorts are missing. Interestingly, Gazda *et al* (2012) reported large-scale sequencing of 79 RP genes in a cohort of 96 DBA probands, none of whom had previously been found to have a pathogenic mutation. The study showed *c.* 53·9% of DBA patients had mutations in one of 10 known DBA-associated RP genes, including a novel causative *RPL26* gene. The results were very similar to ours, although their data did not contain large deletions of RP genes, which would escape regular sequencing analysis.

An additional five missense single nucleotide variants affecting single cases were identified in six patients, including *RPL3L*, *RPL7L1*, *RPL8*, *RPL13*, *RPL18A* and *RPL31* together with two in-frame deletions of *RPL6* and *RPL14* in two patients, which cause deletion of a single amino-acid (Table I). However, the pathological significance in these seven cases is uncertain. In the remaining 36 patients, no mutations were detected in *RP* genes. In conclusion, we identified novel germline mutations of two RP genes that could be responsible for DBA, further confirming the concept that RP genes are common targets of germline mutations in DBA patients and also suggesting the presence of non-RP gene targets for DBA. To identify the candidate disease variants in non-RP genes, we are now pursuing WES of their parents and planning to perform functional assays of these variants.

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Authorship and Disclosure

Y.O., Y. S., A.S.-O., K.C., H.T. and S.M. performed bioinformatics analyses of the resequencing data. R.W., K.Y., T.T. and R.K. processed and analysed genetic material, prepared the library and performed sequencing. R.W., K.Y., T.T. and R.K. performed the Northern blot analyses and RT-PCR analyses. M.K and I.H. performed DBA copy number analysis. T. S., T. U. and N.K. performed zebrafish experiments. K. K., I.K., S. Ohga, A.O., J.H., K.S., K.M., K. K., A.I., Y. K., S.K., K.T., T. S. and E.I. collected specimens and were involved in planning the project. Y.I. and H.K. analysed data and designed the study. E.I. and S.O led the entire project. T.T., R.W., N.K. and I.E. wrote the manuscript. All authors participated in discussions and interpretation of the data and results.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Perturbation of pre-rRNA processing by knockdown of the *RPL27* or *RPS27* gene when the extent of the knockdown was approximately 50%.

Data S1. Methods.

Table S1. Mutations identified in *RPS19*, *RPL5*, *RPL11*, *RPL35A*, *RPS17* and *RPS26* in Japanese DBA patients.

Table S2. Prediction of functional effects of mutations in ribosomal protein genes.

Table S3. Mean coverage of whole-exome sequencing of RP genes in Patients #42 and #95.

Table S4. Average coverage of target deep sequencing of RP genes in Patient #95.

Table S5. Average coverage of target deep sequencing of RP genes in Patient #42.

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Recurrent *CDC25C* mutations drive malignant transformation in FPD/AML

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Familial platelet disorder (FPD) with predisposition to acute myelogenous leukaemia (AML) is characterized by platelet defects with a propensity for the development of haematological malignancies. Its molecular pathogenesis is poorly understood, except for the role of germline *RUNX1* mutations. Here we show that *CDC25C* mutations are frequently found in FPD/AML patients (53%). Mutated CDC25C disrupts the G2/M checkpoint and promotes cell cycle progression even in the presence of DNA damage, suggesting a critical role for CDC25C in malignant transformation in FPD/AML. The predicted hierarchical architecture shows that *CDC25C* mutations define a founding pre-leukaemic clone, followed by stepwise acquisition of subclonal mutations that contribute to leukaemia progression. In three of seven individuals with *CDC25C* mutations, *GATA2* is the target of subsequent mutation. Thus, *CDC25C* is a novel gene target identified in haematological malignancies. *CDC25C* is also useful as a clinical biomarker that predicts progression of FPD/AML in the early stage.

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