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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*.

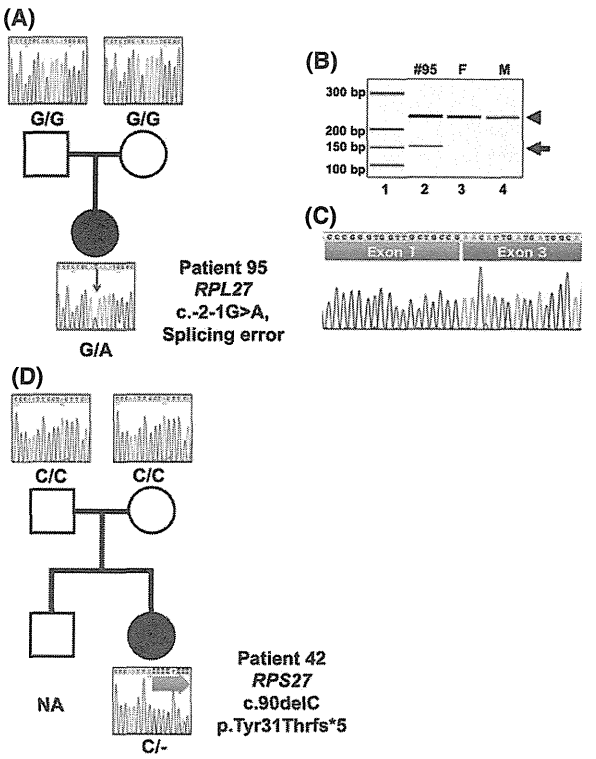


Fig 1. *De novo* mutations in *RPL27* and *RPS27*. (A) Family tree of Patient 95. Electropherograms indicate the gDNA sequence including the boundary between IVS-1 and the first exon of the *RPL27* gene. The red arrow indicates the position of the nucleotide substitution −2-1G>A observed in Patient 95. (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.

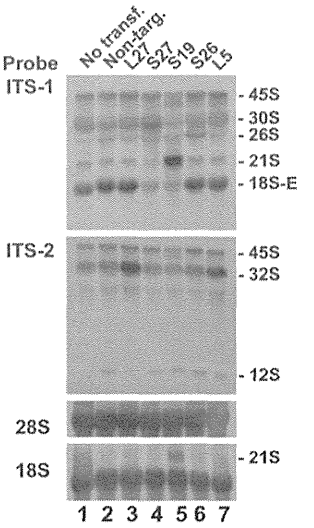


Fig 2. Perturbation of pre-rRNA processing by knockdown of the *RPL27* or *RPS27* gene. Northern blot analysis using K562 cells knocked down by siRNAs. The 5′ extremities of the internal transcribed spacer 1 (ITS-1) and internal transcribed spacer 2 (ITS-2) were used as probes to detect the precursors 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.

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 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

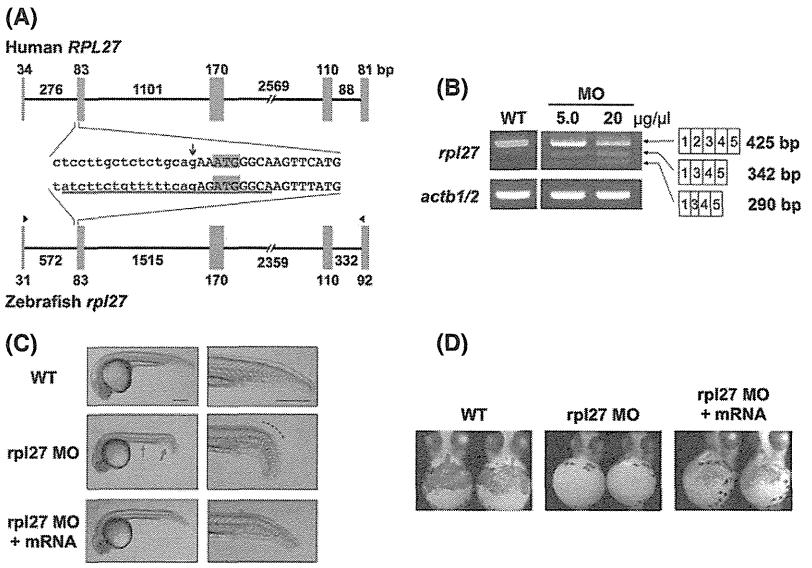


Fig 3. Morphological defects and decreased erythropoiesis in *rpl27* morphants. (A) The gene structures of human *RPL27* and zebrafish *rpl27*. The sequences of intron 1/exon 2 boundary regions are indicated. Uppercase and lowercase letters show the exon and intron sequences, respectively. The MO target 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

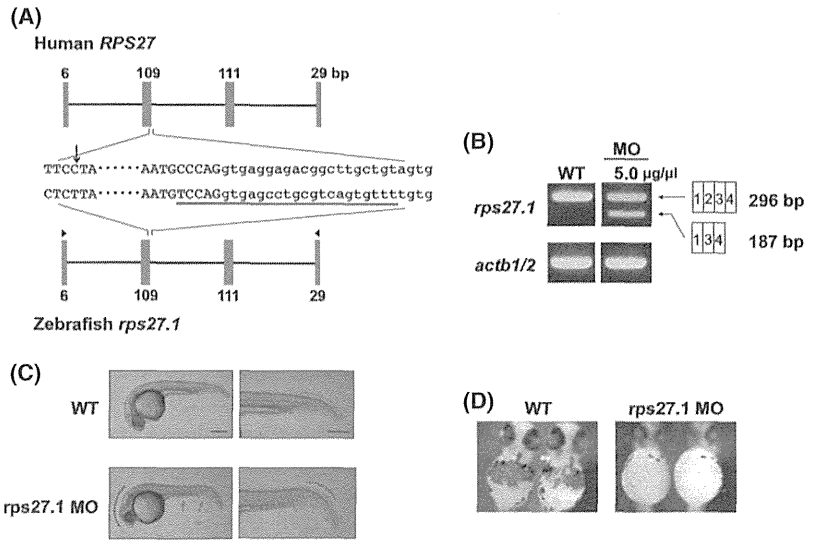


**Fig 4.** Morphological defects and decreased erythropoiesis in *rps27* morphants. (A) The gene structures of human *RPS27* and zebrafish *rps27.1*. The sequences of exon 2/intron 2 boundary regions are indicated. Uppercase and lowercase letters show the exon and intron sequences, respectively. The 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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# First-line treatment for severe aplastic anemia in children: bone marrow transplantation from a matched family donor *versus* immunosuppressive therapy

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## ABSTRACT

The current treatment approach for severe aplastic anemia in children is based on studies performed in the 1980s, and updated evidence is required. We retrospectively compared the outcomes of children with acquired severe aplastic anemia who received immunosuppressive therapy within prospective trials conducted by the Japanese Childhood Aplastic Anemia Study Group or who underwent bone marrow transplantation from an HLA-matched family donor registered in the Japanese Society for Hematopoietic Cell Transplantation Registry. Between 1992 and 2009, 599 children (younger than 17 years) with severe aplastic anemia received a bone marrow transplant from an HLA-matched family donor (n=213) or immunosuppressive therapy (n=386) as first-line treatment. While the overall survival did not differ between patients treated with immunosuppressive therapy or bone marrow transplantation [88% (95% confidence interval: 86-90) *versus* 92% (90-94)], failure-free survival was significantly inferior in patients receiving immunosuppressive therapy than in those undergoing bone marrow transplantation [56% (54-59) *versus* 87% (85-90); $P<0.0001$]. There was no significant improvement in outcomes over the two time periods (1992-1999 *versus* 2000-2009). In multivariate analysis, age <10 years was identified as a favorable factor for overall survival ($P=0.007$), and choice of first-line immunosuppressive therapy was the only unfavorable factor for failure-free survival ($P<0.0001$). These support the current algorithm for treatment decisions, which recommends bone marrow transplantation when an HLA-matched family donor is available in pediatric severe aplastic anemia.

## Introduction

Aplastic anemia is defined as peripheral blood pancytopenia caused by bone marrow failure; the pathogenesis of this disease is thought to involve autoimmune processes.[1-3] The principal interventions responsible for improved survival in aplastic anemia are bone marrow transplantation (BMT) and immunosuppressive therapy (IST). In children, BMT from an HLA-matched family donor (MFD) is the treatment of choice for severe aplastic anemia (SAA).[1,4-6] For children lacking an MFD, IST with a combination of antithymocyte globulin and cyclosporine has been used as a therapeutic option.[6-10] However, this treatment approach is based on the results of comparative studies between these therapies that were conducted mainly in the 1980s, and there have been few recent studies that compare the outcome of BMT recipients with comparable patients receiving IST.

The largest pediatric series in previous studies was reported by the European Group for Blood and Marrow Transplantation (EBMT) and included 304 children treated from 1970 to 1988; that study indicated survival was better following first-line BMT than after first-line IST (63% *versus* 48%; $P=0.002$) but did not compare failure-free survival after the two therapies.[6] Our previous analysis showed a significant advantage for patients receiving BMT from an MFD as first-line treatment in a study of 100 children with SAA who were treated between 1984 and 1998.[1] In patients who received first-line IST, 10-year overall and failure-free survival rates were 55% and 40%, respectively, both of which were markedly inferior to the rates in patients who initially underwent BMT, which was associated with 10-year overall survival and failure-free survival rates greater than 90%. Since the 1980s, the outcomes of both BMT and IST have improved, likely due to better supportive care and advanced treatment and transplantation protocols. A recently published Cochrane review regarding BMT from an MFD and IST as first-line treatment also pointed out that all studies included in the analysis

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had a high risk of bias due to their study design and were conducted more than 10 years ago and may not be applicable to the standard of care of today.[11] Updated evidence to aid treatment decisions in pediatric SAA is, therefore, required.

In children, the choice of an appropriate treatment is particularly influenced by the long-term sequelae of the disease and its therapy. Thus, failure-free survival is much more important than survival alone when analyzing the long-term outcomes of children with aplastic anemia. Lack of response, relapse, and clonal evolution are problematic in the IST setting, whereas graft failure, acute and chronic graft-*versus*-host disease (GVHD), and infectious complications limit the success of BMT. In the present study, we compared the outcomes of children with SAA who received IST or BMT from an MFD as first-line treatment using data from nationwide IST and BMT registries.

## Methods

### Patients

Between 1992 and 2009, a total of 599 consecutive children (younger than 17 years) with acquired SAA underwent BMT from an MFD or received IST as first-line treatment in Japan; 213 patients with an MFD underwent BMT and were registered in the Transplant Registry Unified Management Program (TRUMP) conducted by the Japanese Society for Hematopoietic Cell Transplantation, and 386 patients without an MFD were enrolled in two consecutive prospective multicenter trials (AA-92/97) conducted by the Japanese Childhood Aplastic Anemia Study Group and were initially treated with IST (Table 1). The disease severities were defined as previously reported.[12,13] Underlying inherited marrow failure disorders were excluded clinically and by chromosome fragility testing. Marrow cytogenetic studies were performed for all patients, and patients with clonal cytogenetic abnormalities were excluded from this study. Patients with paroxysmal nocturnal hemoglobinuria with clinical symptoms and positive findings on the Ham test/sucrose test were also excluded from this analysis. All treatments were performed after obtaining written informed consent from patients or their parents in accordance with the Declaration of Helsinki.

### Immunosuppressive therapy and bone marrow transplantation procedures

The characteristics of the treatment procedures are detailed in Table 2. Three hundred and eighty-six patients were enrolled in the AA-92 (n=84) and AA-97 (n=302) trials, and all the patients were initially treated with a combination of antithymocyte globulin and cyclosporine A. Response to IST and disease relapse were evaluated as previously reported.[12] Transplantation data were collected with the use of standardized forms provided by the TRUMP. A total of 213 patients underwent BMT from an MFD as first-line treatment following the local protocols for conditioning regimens and GVHD prophylaxis. Patients who did not reach neutrophil counts $>0.5\times10^9$/L for 3 consecutive days after transplantation were considered to have had primary graft failure. Patients with initial engraftment in whom absolute neutrophil counts subsequently declined to $<0.5\times10^9$/L were considered to have had secondary graft failure. Acute and chronic GVHD were evaluated according to standard criteria.[14-16] More details on methods are provided in the *Online Supplementary Methods section*.

### Statistical analyses

The date of analysis was July 30, 2012. Survival probabilities were estimated by the Kaplan-Meier method and compared between different groups of patients using the log-rank test. The influence of potential risk factors on overall survival and failure-free survival was assessed according to first-line treatment (BMT or IST), time period of treatment (1992-1999 or 2000-2009), age and other variables related to each treatment. Overall survival was defined as the time from diagnosis to death or last follow-up. Failure-free survival was defined as survival with treatment response. Death, primary or secondary graft failure, and secondary malignancy in the BMT group, and death, relapse, disease progression requiring stem cell transplantation (SCT) from an alternative donor or second IST, clonal evolution and evolution to paroxysmal nocturnal hemoglobinuria in the IST group were consid-

**Table 1. Patients' characteristics.**

| | First-line treatment | | |
|---|---|---|---|
| | BMT n=213 | IST n=386 | *P* |
| Age at diagnosis, year, median (range) | 10 (0-16) | 9 (0-16) | NS |
| Age at treatment, year, median (range) | 11 (0-16) | 9 (0-16) | NS |
| Gender | | | |
| Male / female | 119/94 | 217/169 | NS |
| Etiology, n. of patients (%) | | | |
| Idiopathic | 204 (96) | 312 (81) | <0.0001 |
| Hepatitis | 7 (3) | 67 (17) | |
| Others | 2 (1) | 7 (2) | |
| Severity, n. of patients (%) | | | |
| Very severe aplastic anemia | – | 227 (59) | – |
| Severe aplastic anemia | – | 159 (41) | |
| Interval diagnosis-treatment, days, median (range) | 84 (14-4605) | 15 (1-180) | <0.0001 |
| Time periods of treatment, n. of patients (%) | | | |
| 1992-1999 | 121 (57) | 155 (40) | 0.0001 |
| 2000-2009 | 92 (43) | 231 (60) | |

*BMT: bone marrow transplantation; IST: immunosuppressive therapy; NS: not significant.*

ered treatment failures. For multivariate analyses, the Cox proportional hazard regression model was used. *P* values less than 0.05 were considered statistically significant. This study was approved by the institutional ethics committee of the Japanese Red Cross Nagoya First Hospital.

## Results

### Patients' characteristics

The characteristics of the 599 children are detailed in Table 1. The groups treated first-line with BMT (n=213) or IST (n=386) were similar with regards to age at diagnosis, age at treatment and male/female ratio. The majority of patients in both groups had a diagnosis of idiopathic disease, although the proportion of patients with non-idiopathic disease was higher in the IST group. Seven patients (3%) in the BMT group and 67 patients (17%) in the IST group suffered from hepatitis-associated disease. Nine patients had drug-induced or virus-associated disease. Information on the proportion of very severe disease was not available for 141 patients who underwent BMT because the severity of the SAA was not a required item for the registry. The clinical features of these patients were similar to those of the remaining patients. In the IST group, details regarding the severity of disease were provided for all patients: 227 (59%) had very severe disease and 159 (41%) suffered from severe disease. As expected, the time to treatment was significantly longer in the BMT group; the median interval between diagnosis and treatment was 15 days (range, 1-180 days) and 84 days (range, 14-4605 days) for those treated with IST and BMT, respectively. In accordance with decisions taken by the patients and the parents, ten patients underwent BMT more than 5 years after diagnosis. None of the patients who received IST before BMT from an MFD were included in the BMT group.

**Table 2. Treatment characteristics.**

| | |
|---|---|
| **Bone marrow transplantation** | **213** |
| Conditioning regimen, n. | |
| High-dose CY (200 mg/kg) -based | 158 |
| CY ± low-dose irradiation | 86 |
| CY + ATG ± low-dose irradiation | 72 |
| FLU + CY (100-120 mg/kg) -based | 44 |
| FLU + CY ± low-dose irradiation | 29 |
| FLU + CY + ATG ± low-dose irradiation | 15 |
| Myeloablative | 11 |
| CY + TBI (10-12 Gy) | 7 |
| BU + CY | 4 |
| GVHD prophylaxis, n. | |
| CyA + MTX | 174 |
| CyA alone | 23 |
| Tacrolimus + MTX | 6 |
| Others | 10 |
| **Immunosuppressive therapy** | **386** |
| IST trial, n. | |
| AA-92 | 84 |
| AA-97 | 302 |
| IST regimen, n. | |
| CyA + ATG | 140 |
| CyA + ATG + G-CSF | 246 |

*CY: cyclophosphamide; ATG: antithymocyte globulin; FLU: fludarabine; TBI: total body irradiation; BU: busulfan; CyA: cyclosporine; MTX: methotrexate; G-CSF: granulocyte colony-stimulating factor.*

### Immunosuppressive therapy

Response to IST at 6 months was not evaluable in 11 patients for the following reasons: early death (n=7) or BMT from an alternative donor within 6 months of IST (n=4). The causes of the early deaths were sepsis (n=3), interstitial pneumonia (n=2), hemolysis of unknown cause (n=1) and accidental ingestion (n=1). Of the patients who underwent BMT from an alternative donor within 6 months, two patients died of graft failure or cardiac toxicity related to the preconditioning regimen. Overall, 238 of the 375 evaluable patients (63%) improved with first-line IST and achieved a partial response (n=151) or complete response (n=87) at 6 months. All of these patients achieved transfusion independence.

For all 386 patients who received IST initially, the 10-year overall survival rate was 88% [95% confidence interval (CI): 86-90], as shown in Figure 1A, and the median follow-up time for living patients was 106 months (range, 22-224 months). In contrast to the high rate of overall survival, the result regarding survival with response was unsatisfactory, the 10-year failure-free survival rate being 56% (95%

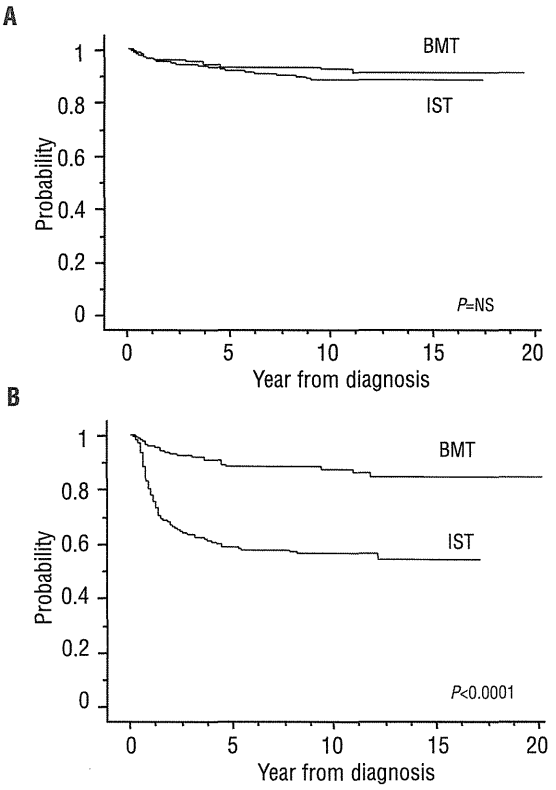


**Figure 1. Survival of 599 children with severe aplastic anemia according to first-line treatments with immunosuppressive therapy (IST) (n=386) or bone marrow transplantation (BMT) (n=213). (A) Overall survival. The 10-year overall survival was 88% (95% CI: 86-90) in the IST group and 92% (95% CI: 90-94) in MFD BMT recipients (*P*=NS). (B) Failure-free survival. The 10-year failure-free survival was 56% (95% CI: 54-59) in the IST group and 87% (95% CI: 85-90) in the BMT group (*P*<0.0001).**

CI: 54-59) (Figure 1B). The cause of treatment failure included death in 12 patients [due to intracranial hemorrhage (n=2), pneumonia (n=1), traffic accident (n=1) and sudden death (n=1) in addition to the seven early deaths], relapse in 23 patients, disease progression requiring second-line treatment in 109 patients, evolution to myelodysplastic syndrome in 15 patients, and appearance of paroxysmal nocturnal hemoglobinuria in two patients. After failed IST, a total of 113 patients underwent SCT from an alternative donor as second- or third-line treatment. The 10-year overall survival of these patients who received a transplant after failed IST was 79% (95% CI: 75-83) with a median of 435 days from diagnosis and SCT. We then analyzed the influence of potential risk factors for survival in the IST group. The prognostic significance of the clinical parameters is shown in Table 3. In the univariate analysis, age younger than 10 years at diagnosis was associated with a favorable overall survival rate [93% (95% CI: 91-95) *versus* 82% (95% CI: 78-86); *P*=0.012], and this was confirmed in a multivariate model. However, the rate of failure-free survival did not differ between patients in the two age groups. No other variables were significantly associated with survival after IST in either univariate or multivariate analyses.

### Bone marrow transplantation

In the BMT group, 209 patients (98%) achieved primary engraftment at a median of 16 days after transplantation. As shown in Figure 1A and 1B, the 10-year overall survival and failure-free survival rates for all 213 patients who were treated initially with BMT from an MFD were 92% (95% CI: 90-94) and 87% (95% CI: 85-90), respectively. When the analysis was applied to the patients who underwent BMT within 180 days from diagnosis, similar results were observed; the 10-year overall survival and failure-free survival rates were 94% (95% CI: 92-96) and 89% (95% CI: 86-92), respectively. The median follow-up time for living patients was 101 months (range, 18-213 months). The cause of treatment failure included primary graft failure in two patients, secondary graft failure in ten patients, second malignancy in one patient, and death due to other complications in 12 patients. Although both patients without primary engraftment died, nine of the ten patients with secondary graft failure remain alive; eight were saved by a second transplant, and one recovered spontaneously. Twenty-five of 209 patients (12%) who had achieved primary engraftment developed grade II to IV acute GVHD, and extensive chronic GVHD was observed in 13 of 209 patients (6%) alive 100 days after BMT.

The prognostic significance of the clinical parameters, including variables related to transplantation, was then assessed. We found no association between age, gender, etiology, interval between diagnosis and BMT, or time period of treatment and treatment outcome (Table 3). Of particular interest with regards to conditioning regimens is the fact that the addition of antithymocyte globulin produced an improvement of overall survival [96% (95% CI: 92-99) *versus* 87% (95% CI: 84-91); *P*=0.021], whereas the rate of failure-free survival was comparable. A fludarabine-based regimen did not affect outcome after BMT from an

**Table 3. Univariate analysis of 10-year overall survival (OS) and failure-free survival (FFS), according to first-line treatment.**

| | IST | | | | | BMT | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | OS | | FFS | | | OS | | FFS | |
| Variable | N. of patients | % (95% CI) | *P* | % (95% CI) | *P* | N. of patients | % (95% CI) | *P* | % (95% CI) | *P* |
| Age at diagnosis | | | | | | | | | | |
| Younger than 10 years | 219 | 93 (91-95) | 0.012 | 57 (54-61) | 0.754 | 89 | 95 (93-98) | 0.163 | 92 (89-95) | 0.200 |
| 10 years or older | 167 | 82 (78-86) | | 55 (51-59) | | 124 | 90 (87-93) | | 84 (81-88) | |
| Gender | | | | | | | | | | |
| Male | 217 | 87 (84-90) | 0.628 | 60 (56-64) | 0.089 | 119 | 91 (87-94) | 0.383 | 87 (83-90) | 0.679 |
| Female | 169 | 90 (87-92) | | 52 (48-56) | | 94 | 94 (91-97) | | 88 (84-91) | |
| Etiology | | | | | | | | | | |
| Idiopathic | 312 | 88 (86-91) | 0.661 | 54 (51-57) | 0.185 | 204 | 92 (90-95) | 0.568 | 87 (85-90) | 0.934 |
| Other | 74 | 87 (83-92) | | 66 (60-71) | | 9 | 88 (76-99) | | 88 (76-99) | |
| Severity | | | | | | | | | | |
| Very severe | 227 | 90 (88-92) | 0.600 | 57 (53-60) | 0.965 | | – | | – | |
| Severe | 159 | 85 (82-89) | | 56 (52-60) | | | | | | |
| Interval diagnosis-treatment | | | | | | | | | | |
| Less than median days | 187 | 91 (88-93) | 0.537 | 60 (57-64) | 0.170 | 105 | 95 (92-97) | 0.322 | 91 (88-94) | 0.362 |
| Median days or more | 199 | 86 (83-89) | | 53 (49-56) | | 108 | 90 (87-94) | | 85 (82-89) | |
| Time periods of treatment | | | | | | | | | | |
| 1992-1999 | 155 | 85 (82-88) | 0.119 | 54 (50-58) | 0.545 | 121 | 91 (89-94) | 0.510 | 87 (84-90) | 0.801 |
| 2000-2009 | 231 | 92 (90-94) | | 59 (56-63) | | 92 | 95 (93-98) | | 89 (85-93) | |
| Conditioning regimen | | | | | | | | | | |
| With ATG | | – | | – | | 87 | 96 (92-99) | 0.021 | 86 (83-90) | 0.648 |
| Without ATG | | | | | | 126 | 87 (84-91) | | 85 (82-89) | |
| GVHD prophylaxis | | | | | | | | | | |
| CyA + MTX | | – | | – | | 174 | 93 (90-95) | 0.924 | 88 (85-91) | 0.809 |
| Others | | | | | | 39 | 93 (88-98) | | 86 (80-93) | |

*ATG: antithymocyte globulin; CyA, cyclosporine; MTX, methotrexate.*

MFD, although the number of patients treated with such regimens was too small to draw any conclusions. Multivariate analysis showed that none of the variables significantly influenced survival.

### Survival and prognostic factors

The overall outcomes of the 599 children with SAA, stratified according to their first-line treatment, are shown in Figure 1A and 1B. Our data clearly showed a significant advantage for children receiving BMT from an MFD as first-line treatment; the failure-free survival was significantly superior in patients treated with BMT than in those in whom IST was used ($P<0.0001$), whereas the overall survival of patients in these two treatment groups did not differ. Figure 2A and 2B show survival curves in all patients treated in the two sequential time periods, 1992-1999 and 2000-2009: results were comparable over time [10-year overall survival: 88% (95% CI: 86-90) *versus* 93% (95% CI: 91-95); 10-year failure-free survival: 67% (95% CI: 65-70) *versus* 68% (95% CI: 66-71)], indicating no significant improvement in the last two decades. When age groups were considered, overall survival at 10 years in the younger group (<10 years old) was significantly better than that in the other age groups [93% (95% CI: 92-95) *versus* 85% (95% CI: 83-88); $P=0.007$], although no difference in failure-free survival was observed (Figure 3A and 3B). The favorable overall survival in the younger group may be mostly due to that observed in the first-line IST group. In multivariate analysis, age younger than 10 years at diagnosis was identified as a favorable factor for overall survival ($P=0.007$), and choice of first-line BMT from an MFD was confirmed as an independent favorable factor for failure-free survival ($P<0.0001$), as shown in Table 4.

## Discussion

For children with SAA, BMT and IST have been accepted as standard treatments during the past three decades. The current guideline recommends BMT from an MFD as the treatment of choice for pediatric SAA[17-19] based on the results of comparative studies performed in the 1980s.[1,5,6,20,21] On the other hand, recent prospective studies with intensified IST for pediatric SAA have resulted in dramatic improvements in survival.[22,23] For example, a study from the EBMT showed a 100% overall survival rate at 6 years after first-line IST in 31 SAA patients younger than 20 years treated from 2002 to 2008.[22] These excellent overall survival results after IST have led to discussion about

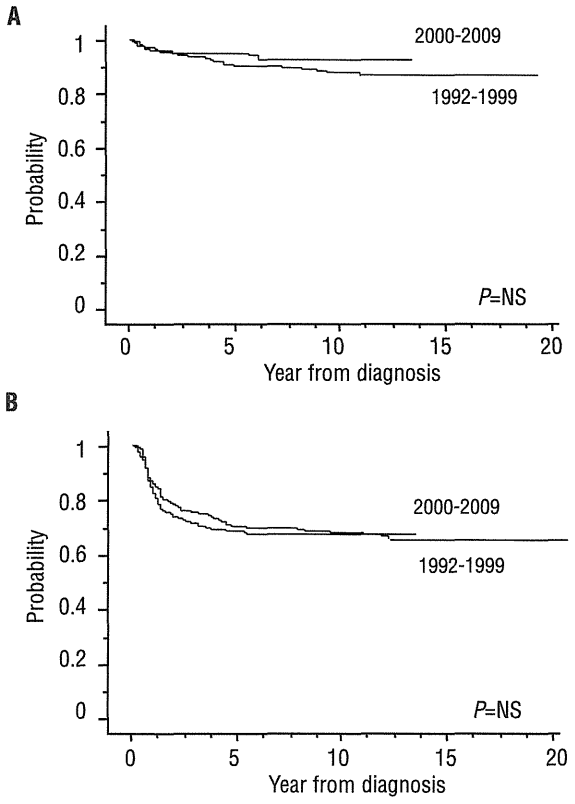


Figure 2. Survival of patients according to time periods of treatment: 1992-1999 (n=276) or 2000-2009 (n=323). (A) Overall survival. The 10-year overall survival was 88% (95% CI: 86-90) in 1992-1999 *vs.* 93% (95% CI: 91-95) in 2000-2009. (B) Failure-free survival. The 10-year failure-free survival was 67% (95% CI: 65-70) in 1992-1999 *vs.* 68% (95% CI: 66-71) in 2000-2009.

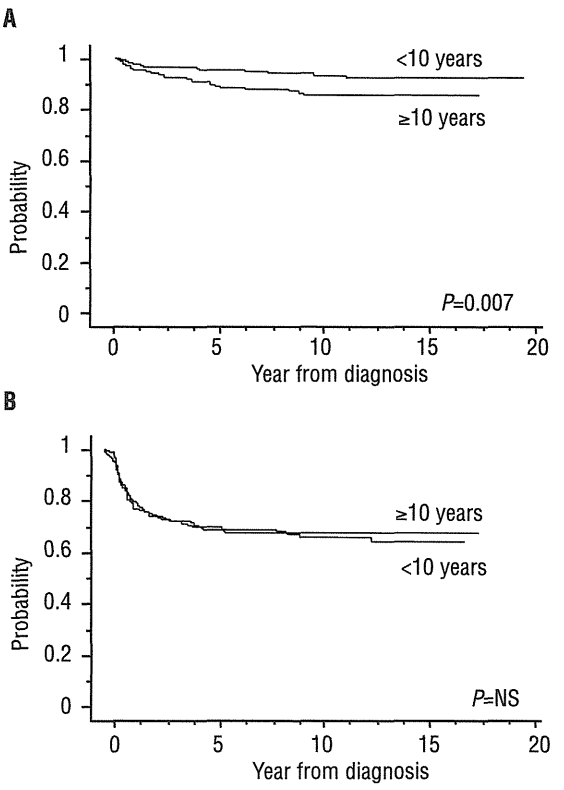


Figure 3. Survival of patients according to age at diagnosis: <10 years (n=308) or ≥10 years (n=291). (A) Overall survival. The 10-year overall survival in the younger group (<10 years) was significantly better than that in the other group [93% (95% CI: 92-95) *vs.* 85% (95% CI: 83-88); $P=0.007$]. (B) Failure-free survival. No difference in failure-free survival at 10 years was observed [67% (95% CI: 65-70) *vs.* 63% (95% CI: 59-67)].

the first-line treatment in children with SAA. To obtain solid evidence on which to base treatment decisions, ideally, a randomized controlled trial is required. However, because of the rarity of the disease, no randomized controlled trials comparing IST with BMT from an MFD as first-line treatment for SAA exist, and only retrospective studies using data from registries or relatively small cohorts of patients are available. Following the previous report of 304 children treated from 1970 to 1988,[6] the EBMT SAA Working Party (SAAWP) reported a consecutive study of 911 children younger than 16 years initially treated with IST (n=304) or BMT (n=607) between 1991 and 2002, which indicated that first-line IST gave an overall survival rate comparable to that of first-line BMT (81% *versus* 79%).[10] Unfortunately, the analyses had several limitations, because the drugs used for IST varied (e.g., antithymocyte globulin only, cyclosporine A only, or a combination of antithymocyte globulin and cyclosporine A) and the donor types used for BMT were not consistent (15% of the donors were mismatched family donors or matched/mismatched unrelated donors, although the majority of those were MFD). In addition, neither EBMT study provided results on failure-free survival,[6,10] which seems to be much more important than survival alone. Recent advances in supportive care and salvage therapies have effectively rescued non-responders to IST.[24] On the other hand, relapse, clonal evolution in the IST group and secondary graft failure and late malignancy in the BMT group are serious problems in long-term survivors. That is the reason why overall survival is no longer the only endpoint to determine optimal first-line treatment in children with SAA. In Japan, we have conducted consecutive prospective trials with a unified IST regimen consisting of antithymocyte globulin and cyclosporine A since 1992, enrolling 386 SAA patients younger than 17 years. During the same period, 213 SAA patients younger than 17 years underwent BMT from an MFD and were registered into the TRUMP, which provided a unique opportunity to investigate updated evidence for treatment decisions in pediatric SAA, although this study also had limitations due to its retrospective nature.

This study confirmed the excellent outcomes obtained in Japanese children with SAA treated with BMT from an MFD or IST. Consistent with the EBMT studies,[10,22] the survival of children with SAA initially treated with IST has improved markedly since the 1980s, when first-line IST gave greatly inferior survival (with overall survival rates of around 40-50%) when compared with first-line BMT[1,5,6,20,21]; in the current analyses, the probability of overall survival at 10 years in the patients treated first-line with IST reached 88%, which was comparable to that of the group treated first-line with BMT. Recent significant advances in second-line SCT, especially with a matched unrelated donor, may contribute to this marked improvement in survival after first-line IST.[25-27] In our series, a certain number of patients underwent SCT from an alternative donor after failed IST as a second- or third-line treatment. When patients were subdivided into three groups (first-line BMT from an MFD, IST only, and SCT after failed IST groups), the 10-year overall survival rates in these groups were 91%, 93% and 79%, respectively ($P$<0.0001), confirming that, in the case of failure of IST, SCT from an alternative donor is a very good salvage option, whereas MFD BMT and IST are excellent first-line treatments for children with SAA.

Regarding survival with response after first-line treatment, we found that the failure-free survival rate in patients treated with IST plateaued over the past two decades after having slightly improved since the 1980s (from 40% in the 1980s to 56% currently).[1] Thus, unlike the overall survival results, failure-free survival in the IST group was significantly inferior to that in the MFD BMT group. Consistent with our observations, the EBMT group also demonstrated no significant improvement in outcomes in response to IST since the 1990s.[10] This may suggest that the IST regimen has not improved over time. Over the past decade, with the hypothesis that more intense IST might produce better outcomes, the addition of newer immunosuppressive agents, such as mycophenolate mofetil and sirolimus to antithymocyte globulin and cyclosporine A, has been tested, but has failed to improve responses.[28-31] The combination of antithymocyte globulin and cyclosporine A is, therefore, still regarded as the standard IST regimen. Another possibility is that we have reached a ceiling in the percentage of patients with the capacity to respond to IST.[18] In patients refractory to IST, the pathophysiology of the disease may be different from that in patients responsive to IST, which is thought to involve autoimmune processes, although there are no good markers to routinely or reliably distinguish non-responders from responders.[13,32-34] Further studies are needed to identify patients refractory to IST, because these patients might benefit from prompt alternative donor SCT.

Importantly, all patients in the current analyses were treated with horse antithymocyte globulin (Lymphoglobulin), which has recently been withdrawn from Asian and European markets and replaced by rabbit antithymocyte globulin. To date, there are only limited studies using rabbit antithymocyte globulin as first-line IST for pediatric aplastic anemia, and thus, the effectiveness of this form of antithymocyte globulin for pediatric patients remains controversial.[35-38] The change of product might result in different outcomes in response to IST for children with SAA.

Survival after BMT from an MFD in children with SAA has exceeded 90% for the past two decades, and this has remained unchanged when compared with our previous observation in the 1980s. In this study, the major causes of treatment failure were primary and secondary graft failure, but notably, most patients with secondary graft failure were rescued by second transplantation or careful observation. In addition to short-term complications, long-term sequelae, such as chronic GVHD and late malignancy, should be taken into consideration to make optimal treatment decisions, especially in children. Our results showed that acute and chronic GVHD were relatively uncommon

**Table 4. Multivariate analysis of favorable factors for survival in all 599 patients with SAA.**

| Overall survival | Hazard ratio | 95% CI | P |
|---|---|---|---|
| First-line treatment: BMT | 1.619 | 0.881-2.977 | NS |
| Treatment period: 2000-2009 | 1.536 | 0.556-2.753 | NS |
| Age: <10 years | 2.207 | 1.240-3.927 | 0.007 |
| **Failure-free survival** | **Hazard ratio** | **95% CI** | **P** |
| First-line treatment: BMT | 4.497 | 2.935-6.891 | <0.0001 |
| Treatment period: 2000-2009 | 1.090 | 0.812-1.464 | NS |
| Age: <10 years | 1.113 | 0.833-1.488 | NS |

*BMT: bone marrow transplantation; NS: not significant.*

in the setting of BMT from an MFD for pediatric SAA, which is consistent with recently reported results from the EBMT SAAWP, with 11% of grade II to IV acute GVHD and 4% of extensive chronic GVHD after BMT from an MFD for SAA in all age groups.[39] Regarding late malignancy, Kikuchi *et al.* recently published data from 329 Japanese children with SAA from the nationwide registry, confirming a low incidence of late malignancy after BMT from an MFD; the cumulative incidence of late malignancy was 0.8% at 10 years and 2.5% at 20 years, respectively, which was much lower than the cumulative incidences in reports from western countries.[40] In the present series, only one patient developed a late malignancy (myelodysplastic syndrome), and was saved by second BMT. These observations suggest that this approach has been already established as first-line treatment for children with SAA.

In conclusion, our updated data clearly demonstrate that children receiving BMT from an MFD as first-line treatment have a significant advantage over children managed with first-line IST, given the dramatically better failure-free survival and the lower incidence of associated long-term sequelae in the BMT group, which supports the current algorithm for treatment decisions that recommends BMT for pediatric SAA when an MFD is available. On the other hand, IST using the combination of antithymocyte globulin and cyclosporine A is the treatment of choice for children with SAA without an MFD considering the comparable overall survival with BMT from an MFD, which could possibly be ascribed to recent improvements in outcomes after SCT from an alternative donor. In other words, patients have an excellent chance of survival, even after failed first-line IST, when they undergo second-line SCT from an alternative donor.

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***Authorship and Disclosures***

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