絶率に相関しないという結果も出ており，後述する抗ドナー抗体の力価と併せて血漿交換の回数を総合的に判断しているのが現状である[10]．このABO血液型不適合腎移植は，腎移植ドナー不足を解消する方策として普及してきている．現在ではわが国の生体腎移植の約20～25%がABO血液型不適合腎移植となっており[5]，血液型適合と不適合の移植成績はほとんど差がなく長期の移植成績が良好であることもABO血液型不適合腎移植の増加の要因となっている[11]．

一方，抗体関連拒絶反応（antibody-mediated rejection；AMR）は抗A，抗B抗体だけでなく，ドナーのHLAクラスI, II抗原に対する抗体で生じる[12]．このドナー抗体には，MHC-クラスI関連A鎖（MICA），B鎖（MICB）などに対して産生される場合もある．抗ドナー抗体は移植後の抗原感作により産生されることから，AMRはABO血液型適合腎移植でも生じうるため，移植後に血漿交換が必要となる場合がある．

当院では2011年のアルブミン製剤年間使用量は181 kg（181,438g）を超え，日本国内全体使用量約36,057 kgの0.5%を使用している．また，ALB/RBC比は2009年より増加傾向にある（図1）ことからアルブミン製剤の使用量削減は急務と考えられた．年間手術件数は1万件を超え，循環血漿量維持目的の使用が多い心臓血管外科手術が多いため，手術室での使用量が多いと予想したが，今回の集計では手術室8%と比べて血液浄化療法室の使用量が15%と多いことが判明した（図2）．病棟・ICU使用例の約1/4がアルブミン値3.0g/dlを超えている状況での使用であることが明らかになり，このうちの約40%が消化器科であった．慢性肝障害における難治性腹水等の適応があるものの，より一層の適正使用の推進が必要と考えられる．2.5g/dl以上3.0g/dl未満の使用例465例中の154例（33.1%）は急性のアルブミン値の変動が認められない病態でアルブミンが連続投与されており，今後慢性低アルブミン血症における適正使用に関して診療科に対する再度の周知徹底が必要と考えられた．

血液浄化療法室でのアルブミン製剤使用の内79%が単純血漿交換療法と比べアルブミンの損失を抑制することが可能とされているDFPP目的に使用されている[9]．実際にはABO血液型不適合腎移植におけるDFPPは前述のように抗A，抗B抗体価を目安として2から4回程度実施することが必要であり，ABO血液型適合腎移植の一部における血漿交換の頻度より明らかに多い．その結果，腎移植症例における移植前後を含めたアルブミン製剤の平均使用量はABO血液型適合腎移植に比べ不適合腎移植で有意に多くなっていた（図5）．2011年にDFPPを実施した67例の解析において，必要アルブミン量の120%以上のアルブミンを使用していた例が全体の30%以上になっていたことについては個々の症例における病態の詳細な分析が今後必要と考えられた．

我が国には約29万人（国民440人に1人）の慢性腎不全患者が人工腎臓透析を受けており，腎移植症例数は増加傾向にあるものの，未だドナー不足が解決されていないことから，今後もABO血液型不適合腎移植およびDFPP目的でのアルブミン製剤使用が増加することが示唆される．

当院におけるアルブミン製剤使用状況全般を詳細に検討すると，病棟・ICUでの高張製剤適正使用に問題があることは否めなかった．一方，ABO血液型不適合腎移植例が多い当院のような施設では移植前後の血漿交換におけるアルブミン製剤大量使用は不可避であり，この適応を適正使用とすべきかどうか，多施設共同研究で腎移植前後の血漿交換におけるアルブミン製剤の使用の実態調査を実施し，適正使用基準を新たに設定すべきと考えられた．

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THE NECESSITY FOR ALBUMIN PRODUCTS IN ABO-INCOMPATIBLE RENAL TRANSPLANTATION

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Abstract:

The Ministry of Health, Labour and Welfare evaluated the appropriateness of institutional standards for transfusion management systems and the adequate use of blood products in 2012. Our hospital fulfilled standards in a transfusion management system, but did not receive an additional credit for adequate use of blood products due to excess use of albumin as well as fresh frozen plasma in plasma exchange, cardiovascular surgery and organ transplantation. Regarding albumin consumption, total use of albumin products exceeded 180 kg per year, representing over 0.5% of the total use in Japan. In this study, we surveyed albumin consumption in each clinical department, and noted that approximately 12% of albumin was used in plasma exchange accompanying renal transplantation. When compared between ABO-matched and -mismatched renal transplantation, the former consumed 83.5±17.4 g and the latter used 325.6±30.4 g albumin per transplantation. In 2011, the total number of renal transplantations was 185, and about 1/3 were from ABO-mismatched donors. It is absolutely necessary for ABO-mismatched renal transplantation to remove anti-A or B antibody before operation through 2 to 4 rounds of plasma exchange. Over 200,000 patients suffer from chronic renal insufficiency in Japan, and the shortage of donors has become a serious problem. To overcome this, ABO-mismatched renal plantation must be encouraged. Against this background, we propose that a multi-institutional collaborative study is required to survey the amount of albumin used in plasma exchange in renal transplantation. We also propose that the guidelines for appropriate use of albumin for plasma exchange should be reconsidered.

Keywords:
blood products, adequate use, plasma exchange, chronic renal insufficiency, organ transplantation

Journal Web Site: http://www.jstmct.or.jp/jstmct/

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.

First published online 25 November 2014
doi: 10.1111/bjh.13229

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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*.

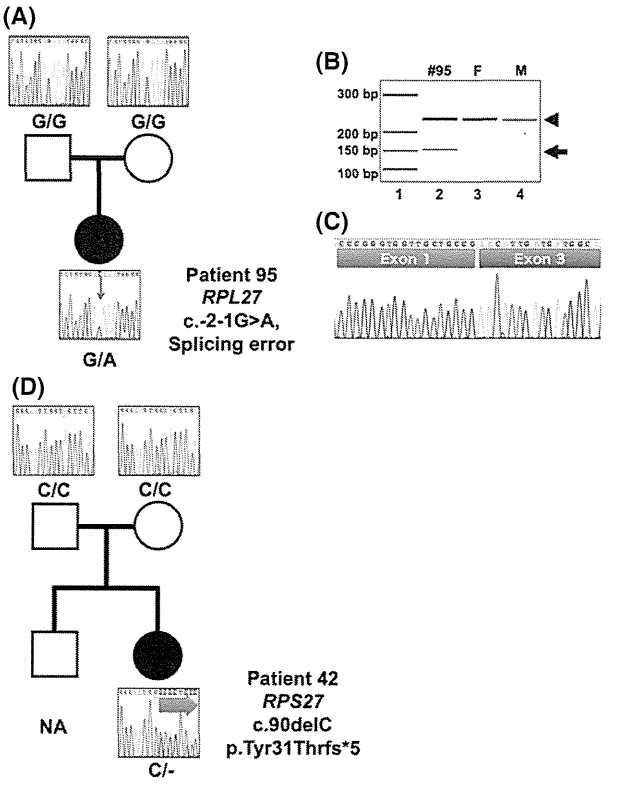

Fig 1. *De novo* mutations in *RPL27* and *RPS27*. (A) Family tree of Patient 95. Electropherograms indicate the gDNA sequence including the boundary between IVS-1 and the first exon of the *RPL27* gene. The red arrow indicates the position of the nucleotide substitution −2-1G>A observed in Patient 95.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

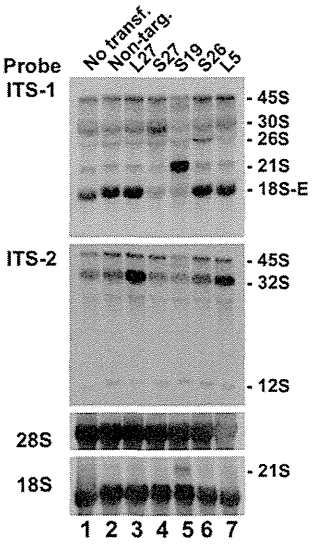

Fig 2. Perturbation of pre-rRNA processing by knockdown of the *RPL27* or *RPS27* gene. Northern blot analysis using K562 cells knocked down by siRNAs. The 5′ extremities of the internal transcribed spacer 1 (ITS-1) and internal transcribed spacer 2 (ITS-2) were used as probes to detect the precursors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

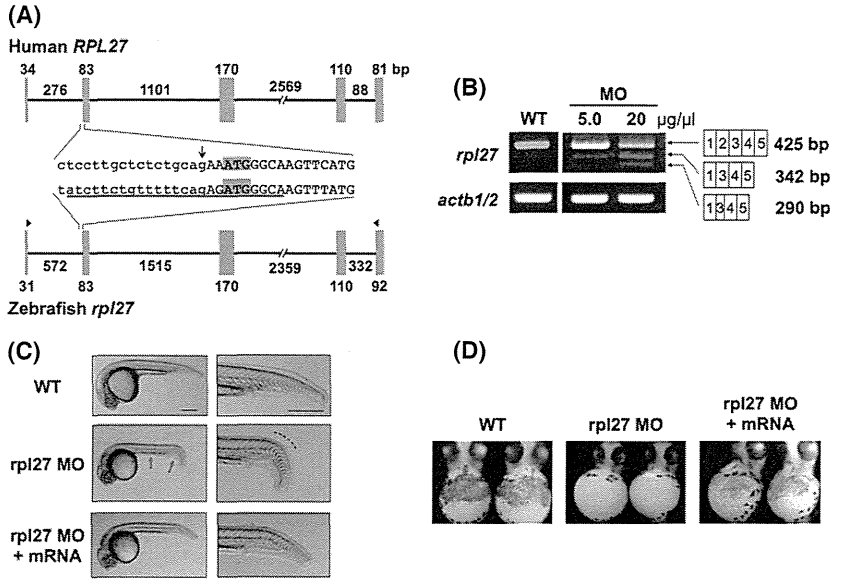

Fig 3. Morphological defects and decreased erythropoiesis in *rpl27* morphants. (A) The gene structures of human *RPL27* and zebrafish *rpl27*. The sequences of intron 1/exon 2 boundary regions are indicated. Uppercase and lowercase letters show the exon and intron sequences, respectively. The MO target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

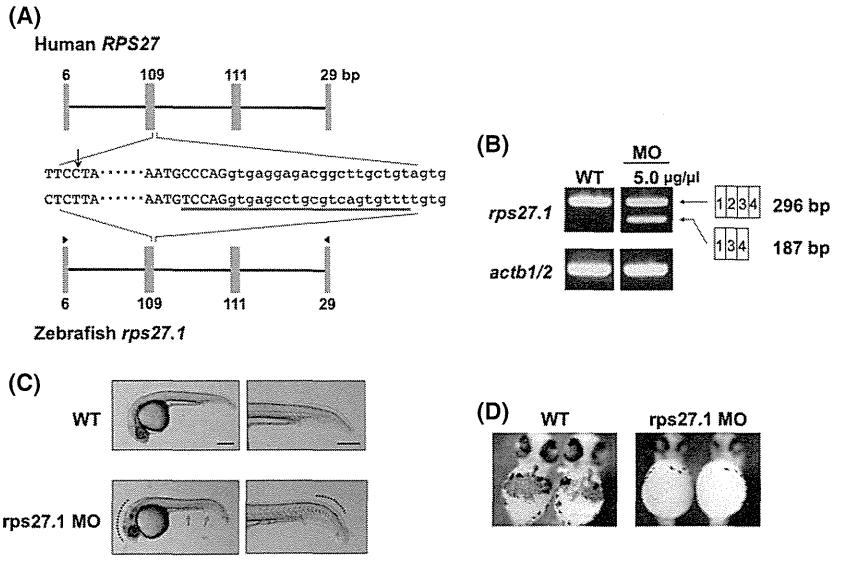

Fig 4. Morphological defects and decreased erythropoiesis in *rps27* morphants. (A) The gene structures of human *RPS27* and zebrafish *rps27.1*. The sequences of exon 2/intron 2 boundary regions are indicated. Uppercase and lowercase letters show the exon and intron sequences, respectively. The MO target 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.

Acknowledgements

We thank T. Kudo and A. Mikami for their technical assistance. This work was supported by the Research on Measures for Intractable Diseases Project and Health and Labor

Sciences Research grants (Research on Intractable Diseases) from the Ministry of Health, Labour and Welfare, by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan and by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (KAKENHI: 25291003).

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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Japanese Journal of Transfusion and Cell Therapy, Vol. 59, No. 3　59(3)：470—475, 2013

—【原　著】— Original —

腹水濾過濃縮再静注法（CART）の安全性確立に向けて

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青木　正弘　髙源　ゆみ　中林　恭子　今野マユミ　槍澤　大樹
入部　雄司　小倉　浩美　菅野　仁　藤井　寿一

腹水濾過濃縮再静注法（CART：Cell-free and Concentrated Ascites Reinfusion Therapy）とは，腹水症（又は胸水症）患者の腹水（又は胸水）を採取し，濾過濃縮後に再静注する治療法で，我が国で開発されて以来，保険診療の中で30年以上広く実施されている．

当院におけるCARTは各診療科が必要時に臨床工学技士に処理を委託していたが，院内統一の依頼・供給手順や製剤が存在せず，医療安全の面で問題があった．複数患者のCART実施時における患者取り違いのリスクを回避するために，輸血・細胞プロセシング部で申し込みから腹水・胸水処理，供給に至るまでを一括管理することとなった．具体的には，既存の輸血システムを流用し，電子カルテからの申込みと製剤固有バーコードの発行，バーコードによる製剤と患者の照合作業までの安全な供給体制システムを構築した．

次に輸血用血液製剤同様の製剤の安全性に関する基準を作成するために，濃縮前後，および一定条件下での保存後の腹水の性状やエンドトキシン検査に関して検討を行った．濃縮後のアルブミン量は26.5±2.7gであり，回収率は66.8%であった．処理前のアルブミン量に関わらず一定の回収率が得られた．また処理前腹水の4℃一晩保存，あるいは−30℃14日保存においてもエンドトキシンは検出されなかった．今後，冷蔵保存後の処理あるいは冷凍保存分割投与によってCARTを必要とする多くの患者へ適応可能になると考えられる．

キーワード：腹水，腹水濾過濃縮再静注法，エンドトキシン，凍結保存

はじめに

我が国において血液製剤，血漿分画製剤は，すべて厚生労働省が定める「輸血療法の実施に関する指針」及び「血液製剤の使用指針」に準拠して適正に使用することが求められている．アルブミン製剤も例外では無く，使用指針には肝硬変に伴う難治性腹水（肝性腹水）に対する治療が適応として認められている反面，末期患者への使用は延命効果のエビデンスが無く，生命尊厳の観点からも控えるべきとされており，緩和医療におけるアルブミン製剤の適応は否定的と考えられている[1]．

一般療法や薬物療法の効果のない難治性腹水（又は胸水）の治療法として，腹膜—静脈シャント（PVシャント）や経頸静脈的肝内門脈静脈短絡術（TIPS）などがあげられるが，外科的処置を要し，患者への侵襲が大きい．一方，従来，腹水濾過濃縮再静注法（CART：Cell-free and Concentrated Ascites Reinfusion Therapy）が知られている．CARTはがんや肝硬変などによって貯留した腹水（又は胸水）を最大孔径0.2μmの腹水濾過器で処理し，細菌やがん細胞，血球成分などを除去し，アルブミンやグロブリン等のタンパク成分を回収し患者自身に静注する治療法である（図1）．患者の全身・栄養状態の改善によるQOL（Quality of Life）の向上を図ると共に，血漿アルブミン製剤による感染性・免疫学的副作用が回避できるという特長がある．本法は濃縮腹水再静注（濾過なし）として1974年日本肝臓学会でその有効性が報告されて以来，1976年に濾過濃縮システムの臨床的検討が開始され，1981年難治性胸水・腹水症に保険収載されている．その後広く認知され，「肝硬変診療ガイドライン（日本消化器病学会編）」，「慢性肝炎の治療ガイド（日本肝臓学会編）」，厚生労働省の「重篤副作用疾患別対応マニュアル：卵巣過剰刺激症候群（OHSS）」などにCARTが推奨されるようになった．

CARTは血液浄化の一種として普及しているが，採取した腹水は一旦患者から切り離されてから処理され，

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〔受付日：2012年10月22日，受理日：2013年1月24日〕

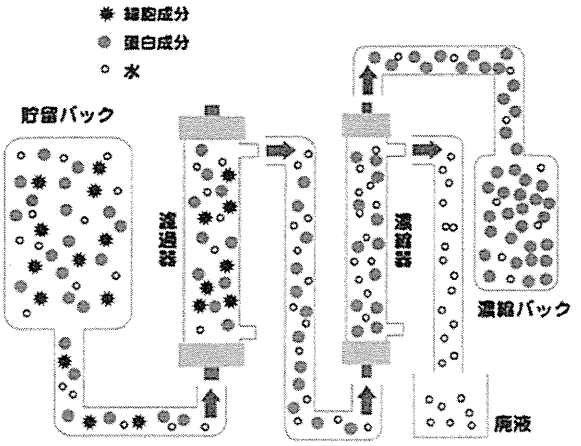

図1　Cell-free and concentrated ascites reinfusion therapy (CART)
CART — a therapy in which the ascitic fluid (or pleural fluid) of a patient with ascites (including hydrothorax) is collected, filtered, concentrated, and reinfused intravenously into the patient

腹水製剤として供給されるため，輸血部門による一括管理体制が必要と考えた．本論文では，(1) CART システム運用による管理体制の確立，(2) 濾過濃縮前後における腹水の生化学的性状の比較検討，(3) 各種保存条件における腹水中エンドトキシン濃度の検討について述べる．

対　象

対象は消化器科 17 名 23 件，一般外科 6 名 11 件，血液内科 1 名 5 件，婦人科 9 名 16 件で総数 33 症例(CART 実施数 55 件)で，疾患名別 CART 件数は卵巣がんが最も多く 14 件，次いで胃がん 12 件，肝硬変 9 件，急性骨髄性白血病 5 件，肝細胞がん 5 件，膵がん 4 件，悪性腹膜中皮腫 3 件，C 型肝炎 1 件，肺がん 1 件，腹膜がん 1 件であり，全件数の内がん性腹水が 80%，肝性腹水が 20% であった．

方　法

濾過濃縮には旭化成メディカル社製の腹水ろ過器 AHF-MOW と腹水濃縮器 AHF-UP，装置は ADP-01 を使用した．血清総ビリルビン値 5mg/dl 以上[2]，発熱・腹部症状・CRP 高値などより感染が強く疑われる場合，貯留処理前腹水量が 1,000ml 以下，血性腹水の著明な溶血所見などの場合は CART 適応不可とした．また当部に持ち込まれた時点で貯留バックの状態（クランプ部分からの漏れ等）を当部担当医が確認し適・不適の判定を行った．処理ポンプ速度は腹水再静注時の副作用（発熱）発生に影響することが報告されている[2]ため，最高処理速度を 50ml/min とした．濾過濃縮後の蛋白濃度が 5g/dl 以下，または濾過濃縮後の腹水容量が 1,000 ml 以上になる場合は再濃縮操作を行うことを部内基準とした．

検討項目は一般性状として処理前後の重量，比重，細胞数，pH を測定し，生化学データとして総タンパク，アルブミン，総ビリルビン，直接ビリルビン，LD，フィブリノゲンを，安全性の確認としてエンドトキシンを測定した．

診療科側の要望にフレキシブルに対応するためには腹水貯留を午後や夜間に行い 4℃ に保存しておいた腹水・胸水を翌日処理する必要性などの可能性も考慮され，4℃ 翌日処理を想定した腹水の安全性確認のため，処理前腹水貯留バックに腹水の一部を残したまま 4℃ 保冷庫に保存し，24 時間後にエンドトキシン検査を実施した．また，処理後腹水の凍結保存による性状変化の確認のため，濾過濃縮後の腹水の一部を −30℃14 日間保存後 FFP 同様に 37℃ で解凍し，一般性状，生化学データ測定，エンドトキシン検査もそれぞれ実施した．

エンドトキシン検査は従来処理前腹水について外部検査機関へ外注していたが，供給前の安全性確認の必要性から院内で迅速に検査をする体制を導入した．腹水貯留直後，処理前腹水 4℃24 時間保存後，腹水処理直後，処理後腹水 −30℃14 日保存後の 4 ポイントについて院内検査でエンドトキシンを測定し，処理前腹水については従来の外注検査を平行して実施した．部内で使用したエンドトキシン検査試薬は生化学工業のエンドスペシー®ES24S セットを用い，測定装置として EG Reader SV-12 を使用しカイネティック比色法で判定した．検査は二重測定で行いその平均値を結果として採用した．エンドトキシン測定はタンパク濃度の影響を受ける為，処理前腹水については測定前に屈折計で簡易的にタンパク濃度を測定し，測定値が 3g/dl を超える場合は除タンパク処理を実施後測定サンプルとした．濃縮後腹水については全例除タンパク処理を実施し，除タンパク法は過塩素酸法[3]を用いた．

結　果

(1) CART システム運用の管理体制の確立（図 2）

当院における CART は各診療科が必要時に臨床工学技士に依頼しその処理を依頼していたが，院内統一の依頼・供給手順や製剤の安全性に関する基準がなかった．医療安全対策の面からも複数人数の患者の CART の実施時における患者取り違い等においては輸血用血液製剤同様の安全対策が必要と示唆されたことから，既存の輸血システムを流用し，輸血・細胞プロセシング部で申し込みから腹水・胸水処理，供給に至るまでを一括管理することにした．

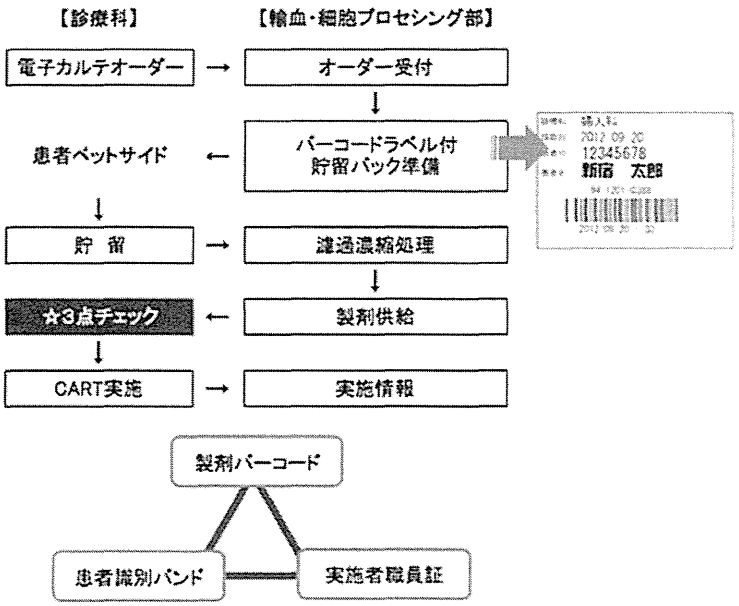

図2 Reinfusion system of ascitic preparation at our hospital
A safe reinfusion system was set up, making use of the existing blood transfusion system to place an order from the electronic medical chart, issue a bar code unique to the preparation, and cross-check the preparation and the patient using the bar code.

表1 Concentration efficiency and recovery rate

比較項目	処理前	処理後	濃縮倍数	回収率
容量	2,716 ± 135ml	410 ± 39ml	11.3 ± 1.4	
総タンパク	76.1 ± 6.7g	50.3 ± 5.1g		61.2 ± 2.2%
アルブミン	36.2 ± 3.3g	26.5 ± 2.7g		66.8 ± 2.4%

n = 53. mean ± SE
Mean volume after processing was 410 ± 39ml. Thus, in terms of volume, it was possible to achieve a concentration of about 11.3 ± 1.4 times. Recovery was 61.2% in the case of total protein and 66.8% in the case of albumin content.

まず，電子カルテより当部へ自己腹水オーダーを送ることにより，その患者個有の製造番号をもった製剤ラベルを出力し，貯留バッグと供給時の腹水濃縮バッグにそのラベルを貼付する．各診療科から持ち込まれる腹水について，貯留バッグからの漏れ，フィブリンの析出状態，血性腹水の場合は溶血の有無などを当部担当医が実施する．処理は当部の医師の監督の下，臨床検査技師が担当し，処理の終わった濃縮バッグは通常の輸血製剤同様のシステムにより払い出し，使用現場での3点チェック（製剤，患者，実施者バーコードの読み取りチェック）により確実に当該患者に静注する．以上のような安全な供給体制システムを構築しCARTを実施した．

(2) 濾過濃縮前後の腹水の生化学的性状（表1）

平均腹水処理容量（Mean ± SE）は2,716 ± 135ml，処理後容量は410 ± 39mlであり，容量としては約11.3 ± 1.4倍に濃縮し得た．処理前総タンパク量(g)は76.1 ± 6.7gで処理後が50.3 ± 5.1g，総アルブミン量としては処理前が36.2 ± 3.3g，処理後で26.5 ± 2.7gであり，それぞれの回収率は総タンパク量で61.2%，アルブミン量で66.8%であった．一般性状としての比重は処理前が1.016 ± 0.001，処理後が1.070 ± 0.002であった．pHは処理前が7.34 ± 0.02処理後が7.24 ± 0.01であり有意（$p < 0.05$，n = 51）に酸性側へ傾く傾向を認めた．処理後当日と−30℃，14日保存後の腹水の一般性状，生化学データについては全症例（n = 20）が全項目において有意差を認めなかった．

表2　Results of bacterial endotoxin testing

処理日	当日		4℃ 翌日	−30° 14日後
エンドトキシン EU/ml	処理前 n=55	処理後 n=52	処理前 n=55	処理後 n=20
<0.01	25	33	22	16
0.01	23	15	24	3
0.02	7	4	7	
0.03			2	
0.04				1

Reinfusion after guaranteeing safety was possible by satisfying our in-house standard (<0.1EU/ml) for cell preparations at all points.

(3) エンドトキシン検査（表2）

エンドトキシン検査は処理前55件について外注検査で全例0.8pg/ml以下，部内検査で0.1EU/ml未満であった．同腹水を未処理のまま貯留バックの状態で4℃，24時間保存後，再度貯留バックからサンプリングした腹水についても全例部内検査で0.1EU/ml未満であった．

考　察

貯留された腹水は一度患者から離れた部署で製剤として調製され，再度患者に輸注される．この流れは造血幹細胞移植や免疫細胞療法のための細胞製剤，自己血輸血の採取・調製・供給と同じであり，製剤を確実に当該患者に返すために取違い防止のための医療安全対策が重視されるべきと考えられる．現状ではCARTを実施している医療機関は少なく，CARTを組み入れた緩和医療を実施している医療機関に患者が集中する傾向にある．したがって，CART実施医療機関では同一日に複数の腹水を処理することも多々あり，腹水の採取，院内搬送，処理および輸注までの過程で複数の医療従事者が関わるために，当院では医療安全対策の観点から，取り違い防止策を講じるべきであるという意見があがった．さらに人による照合確認をどんなに周知徹底していても防ぎきれないのがヒューマンエラーであることから，ヒューマンエラー防止の目的でシステムによる照合を導入すべきという結論になった．今回当院で輸血部門が中心となってCARTに関わることにより，既に院内で確立している輸血システムを流用することが可能になり，バーコード管理による安全な濾過濃縮腹水供給を実施することが可能になった．但し，現状使用している製剤バーコードはCART処理から輸注までの管理では患者取り違い防止機能を果たしているものの，患者から貯留時の患者認証としての機能を有していない点が課題となっており，今後この点については採血時と同様の認証システムを適用するよう改善する予定である．

院内で各診療科がそれぞれの方法でCARTを実施していた体制を統一し，輸血部門が取り扱うことになったのを契機に，腹水濃縮の効率や処理前後の一般・生化学的性状，さらにエンドトキシン濃度の測定を行なった．ADP-01を使用した濾過濃縮効率を処理前後の容量からみると約3リットルの処理前腹水が平均で410mlに濃縮された．平均回収率は総タンパク量で61.2%(50.3g)，アルブミン量で66.8%（26.5g）であり，供給可能なアルブミン量としては血漿分画製剤の25%ヒト血清アルブミン50ml（12.5g），2本分に相当した．処理前後の総アルブミン量は高い相関（r=0.9725）を示し，処理前のアルブミン量に問わず一定の回収率が得られた(図3)．一方，処理前腹水の容量が1.5リットル以下の場合は回収率が30〜40%と低くなる傾向が認められ，総アルブミン量としても平均4.7g（n=5）であり，低アルブミン血症の是正の点で臨床的意義があるかどうか疑問が残り，現在の濾過濃縮フィルターのカラムサイズでは処理前腹水1.5リットル以上の処理が望ましいと考えられた．

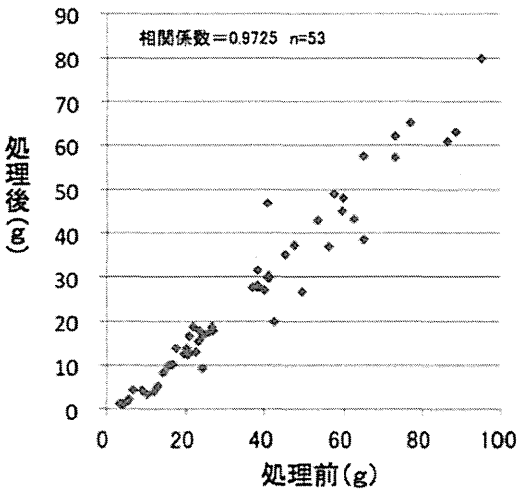

図3　Total albumin contene before and after processing
Total albumin content content before and after processing showed a hight correlation (r=0.9725).

処理前後の一般性状において唯一有意差を認めたのはpHで，処理後有意（$p<0.05$，n=51）にpHが酸性側にシフトしたが，市販の赤十字アルブミン製剤の添付文書によればpH範囲は6.4〜7.4であることから，平均pH7.2の供給製剤としてのpHの変化は特に問題ないと思われる．細胞成分が多い場合，濾過フィルターの目詰まりの原因となるが，一般的な目詰まり対処(動脈圧300mmHg→アラーム表示でポンプ停止→約100mlの廃液：以上を2回繰り返したら処理終了)を加味しても3リットル前後の腹水の濾過濃縮処理が平均約80分であり特に問題はなかった．また，処理後の腹水中には細胞成分は認められず，混濁状態の腹水であっ

ても供給製剤の色調は混濁のない黄色，ビリルビン値の高い場合や多少の溶血の影響のある場合で褐色様となった．

凍結保存前後の総蛋白・アルブミン濃度はほぼ変化がなく，エンドトキシン濃度も上昇を認めず凍結保存後の供給も可能と考えられた．また，保存自己血同様に患者本人のみに使用可能な製剤で，使用前の溶解においては新鮮凍結血漿，適正使用においてはアルブミン製剤と同等の扱いという製剤としての特殊性があることの院内周知が必要と思われる．

安全性検査としてエンドトキシン検査は，処理前腹水について外部検査機関で実施されていたため，その結果は以降になり当日供給する腹水製剤自体の安全性の保証を得ることは出来ていなかった．濾過濃縮後の供給製剤からのサンプルでエンドトキシン検査を実施し，処理後の腹水の安全性を担保した上で供給する体制を確立する必要があった．従来より院内でのがん免疫療法等の細胞製剤供給時のエンドトキシン基準は 0.1 EU/m*l* 未満とされていることから，同基準を適応することにした．濃縮後のサンプルの測定には除タンパク処理が必須とされるため，検査は除タンパクに約 40 分，測定に約 40 分を要するが，CART は供給においては緊急性が低いため検査時間としては問題とはならず，安全性確認後の供給に十分な検査法と言える．今回の検討では腹水貯留直後，処理前腹水 4℃24 時間保存後，腹水処理直後，処理後腹水－30℃14 日保存後の全ポイントで 0.1EU/m*l* 未満で当院での細胞製剤供給時の基準を満たしたことで，供給製剤の安全性保証後供給の実施が可能となったと共に，処理前腹水の 4℃，24 時間保存を許容期間とすることで患者から腹水を貯留する時間帯を広げることが可能と考えられた．また，分割凍結保存を実施した場合もエンドトキシン値の変化は認められず安全性は保持されると考えられた．

現在，腹水の保存に関して，処理前および処理後腹水を凍結保存して再静注した報告は数件あるが[4)~6)]，処理前および処理後腹水の冷蔵・凍結保存に関して有効性や安全性は明確になっていない．遠心機や成分採血装置で細胞成分を除去した後に腹水を一度凍結し自然解凍した際の初期の融解液中の蛋白濃度が高いことに着目し，フィルターを用いない濃縮方法の検討もされているが，現状のフィルターによる CART では濃縮されない尿酸や尿素窒素も濃縮されることで適応については検討が必要とされている[6)]．原則として当日に濾過濃縮，再静注を行っているが，自己腹水の冷蔵保存後の濾過濃縮・再静注，および CART 後の腹水製剤の凍結保存，分割投与を導入することで，CART を必要とする多くの患者へ適応可能になると考えられる．今後，それらの臨床的有用性について確認していきたいと考えている．

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TOWARDS THE ESTABLISHMENT OF A SAFE CELL-FREE AND CONCENTRATED ASCITES REINFUSION THERAPY

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Abstract:

Cell-free and concentrated ascites reinfusion therapy (CART) has been applied for over 30 years in the treatment of patients with intractable ascites (or pleural effusion): collected ascites (pleural effusion) is filtered, concentrated, and reinfused intravenously into the patient. Since the purified ascites might be accidentally administered to other patients in large-scale hospitals, we decided to establish a computer-aided system, similar to that used in blood transfusion, to enable safer management by using bar-code labels.

We next examined biochemical properties, including total protein and albumin concentration, of ascites before and after filtration/concentration, and found that the mean recovery of albumin was 66.8%, and mean albumin content was 26.5 ± 2.7 g after processing.

We also introduced a rapid testing system for endotoxin, which enabled us to show that purified ascites does not contain harmful levels of endotoxin (over 0.1 EU/m*l*). Finally, biochemical properties as well as endotoxin concentration were not significantly altered after storage either overnight at 4℃ or for 14 days at −30℃, suggesting that it may be possible to perform CART after overnight storage in refrigerators or provide purified ascites after cryopreservation.

Since the present guideline suggests that albumin products should be avoided for end-stage patients, CART might be useful in cancer patients with intractable ascites in palliative care units.

Keywords:

ascitic fluid, CART, endotoxin, cryopreservation

Journal Web Site: http://www.jstmct.or.jp/jstmct/

The Japanese Journal of Pediatric Hematology/Oncology vol. 51(5): 446–451, 2014

【第55回日本小児血液・がん学会学術集会】教育セッション3：赤血球系疾患

先天性溶血性貧血の病型および鑑別診断法の進歩と今後の課題

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要　旨

先天性溶血性貧血は赤血球膜，赤血球酵素，ヘモグロビンの構造・機能障害により赤血球寿命の短縮を来す単一遺伝子病である．我々は非免疫性溶血性貧血について特殊検査を実施しており，2004～2013年の10年間に469件について病因検索を行った．先天性溶血性貧血の約60%が学齢期までに診断されているが，40歳以上の症例が10%程度あり，小児科・内科の境界を越えた幅広い年齢構成を示すのが特徴と云える．病型で最も頻度の高いのは遺伝性球状赤血球症（HS）であり，家族歴・赤血球形態や浸透圧抵抗試験によって容易に診断できる例がある一方，非典型例の診断は困難であった．近年開発された赤血球膜表面積の定量検査である赤血球eosin 5′-maleimide（EMA）結合能検査の導入により，診断精度が格段に向上した．EMA結合能の測定により，「非球状性溶血性貧血」として依頼を受けたものの赤血球酵素やヘモグロビンに異常を認められなかった症例のなかに，少なからず赤血球膜異常症が含まれていることを経験した．一方で奇形・破砕赤血球が目立つ非典型的HSや遺伝性熱変形性赤血球症疑い症例では7種の赤血球膜骨格蛋白遺伝子の遺伝子検査が有用であることが明らかとなった．またピルビン酸キナーゼ（PK）活性低下があるものの*PKLR*遺伝子に変異を認めない症例に赤血球特異的転写因子である*KLF1*遺伝子に変異を認める例があり，今後これらの先天性溶血性貧血診断に網羅的遺伝子検査の導入が必要と考えられた．

キーワード：赤血球酵素異常症，不安定ヘモグロビン症，遺伝性球状赤血球症，遺伝性熱変形性赤血球症，赤血球膜骨格
Key words: erythroenzymopathies, unstable hemoglobinopathies, hereditary spherocytosis, hereditary pyropoikilocytosis, red cell membrane cytoskelton

先天性溶血性貧血の疫学・主要徴候・患者背景（表1，表2，表3，図1）

溶血性貧血は赤血球寿命の短縮によって発症する貧血の総称であり，先天性溶血性貧血はその病因が両親（のいずれか）から遺伝する場合と新生変異によって児に発症する単一遺伝子病である．1998年の調査結果では，推計受療患者数は，溶血性貧血全体で2,600人（95%信頼区間2,300～2,900人）であり，先天性溶血性貧血16.6%と報告されている．自己免疫性溶血性貧血（AIHA）や発作性夜間ヘモグロビン尿症（PNH）後天性溶血性貧血の病因が主として免疫学的機序による赤血球の破壊であるのに対し，先天性溶血性貧血は赤血球が骨髄を出て約120日間末梢血中で生存するための生理機能に破綻を来した場合に発症し，赤血球膜異常症，赤血球酵素異常症，ヘモグロビン異常症に大別される（表1）．AIHAやPNHが直接抗グロブリン試験（DAT）や赤血球CD55/CD59表面マーカー検査など，院内検査や臨床検査センターで検索が可能であるのに対し，これらの病因による溶血性貧血に関しては専門施設への検索依頼が必要であり，我々の検査室でも年間50件程度の依頼を受けている．

2004～2013年の10年間に検査依頼があった469件について，その年齢分布を示す（図1）．約半数の症例は新生児溶血性疾患として発症し，1歳未満に全体の1/3（33.9%）が検索を依頼されていた．約60%は慢性溶血性貧血を指摘され，学齢期までに検査を実施されていた．中高年になってから指摘される例も少数ながらあり，小児科・内科の境界を越えた幅広い年齢構成を示すのが特徴と云える．

感染・薬剤・食物によって誘発された急性溶血発作で発症する例が全体の46.7%であった．溶血性貧血の三主徴として貧血・黄疸・脾腫が挙げられるが，脾腫を指摘されていたのは9.8%，胆石を認めた例が4.9%に過ぎないことから，現在は貧血の鑑別プロセスのなかで溶血性貧血が早期に診断されていると考えられた．

家族歴では，両親・兄弟その他の親族に溶血性貧血が指摘されている例は全体の1/3程度であった．また後述するグルコース-6-リン酸脱水素酵素（G6PD）異常症やサラセミアなど，両親のどちらかが外国籍であるケースが全体の約1/6（14.1%）に達しているのも特筆すべき点と云える．子宮内胎児発達遅延（IUGR）や早産を認めた例は6.0%，

2014年7月18日受付，2014年7月18日受理
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