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Molecular pathogenesis in Diamond–Blackfan anemia

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Abstract Diamond–Blackfan anemia (DBA) is a congenital anemia and a broad spectrum of developmental abnormalities that presents soon after birth. The anemia is due to a failure of erythropoiesis with normal platelet and myeloid lineages. Approximately 10–20% of DBA cases are inherited. Genetic studies have identified heterozygous mutations in at least one of eight ribosomal protein genes in up to 50% of cases. Mutations in *RPL5* and *RPL11* are at a high risk for developing malformation. Especially, mutations in *RPL5* are associated with multiple physical abnormalities, including cleft lip/plate and thumb and heart anomalies. Recently, the 5q− syndrome, a subtype of myelodysplastic syndrome characterized by a defect in erythroid differentiation, is caused by a somatically acquired deletion of chromosome 5q, which results in haploinsufficiency of RPS14. These data indicate that abnormalities in ribosome function are broadly implicated in both congenital and acquired bone marrow failure syndrome in humans.

Keywords Ribosomal protein · Diamond–Blackfan anemia · 5q− syndrome · Congenital bone marrow failure syndrome

1 Introduction

Diamond–Blackfan anemia (DBA) is a rare congenital, inherited bone marrow failure syndrome (IBMFS) characterized by normochromic macrocytic anemia, reticulocytopenia and absence or insufficiency of erythroid precursors in normocellular bone marrow [1]. DBA was first reported by Josephs in 1936 and refined as a distinct clinical entity by Diamond and Blackfan in 1938 [2, 3]. Approximately, 90% of affected individuals typically present in infancy or early childhood, although a "non-classical" mild phenotype may not be diagnosed until later in life [4, 5].

Although macrocytic anemia is a prominent feature of DBA, 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 [4–6]. In addition, DBA patients have a predisposition to the development of malignancies [e.g., acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and osteogenic sarcoma] [4]. Diagnosis of DBA is often difficult due to incomplete phenotypes and a wide variability of clinical expression [4–7]. The central hematopoietic defect is characterized by an enhanced sensitivity of hematopoietic progenitors to apoptosis along with evidence of stress erythropoiesis, which includes elevations in fetal hemoglobin and mean red cell volume (MCV) [8]. The majority of patients also exhibit an increase in erythrocyte adenosine deaminase activity [9]. Corticosteroids remain the mainstay of treatment. Approximately 80% of patients respond to an initial course of steroids [4]. Bone marrow transplantation is the only curative treatment, but requires an HLA-matched sibling and is primarily reserved for patients with severe complications.

Recently, a number of mutations in ribosomal protein genes have been identified in DBA patients [4, 10, 11]. In addition, gene products mutated in the other IBMFS including dyskeratosis congenita (DKC) and Schwachman–Diamond syndrome are also predicted to be involved in ribosome biogenesis [12]. In this review article, we summarize the recent progress in understanding of the

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Table 1 Mutations in ribosomal protein genes in DBA patients (%)

	Gazda et al. [17, 19, 21] (American and European)	Cmejla et al. [22] (Czech)	Quarello et al. [23] (Italian)	Konno et al. [24] (Japan)
RPS19	25	21.4	28	11.1
RPL5	6.6	21.4	9.3	8.9
RPS10	6.4	ND	ND	ND
RPL11	4.8	7.1	9.3	4.4
RPS35A	3.5	ND	0	0
RPS26	2.6	ND	ND	ND
RPS24	2	ND	1.6	0
RPS17	<1	3.6	ND	2.2
Total	52.9	52.6	48.2	26.6

ND not done

molecular pathogenesis of DBA and new insight into the mechanism of a defect in erythropoiesis.

2 Inheritance and genetics

Diamond–Blackfan anemia is a rare disease with a frequency of 2–7 per million live births and has no ethnic or gender predilection [1]. DBA occurs in both familial and sporadic forms. Most cases of DBA are sporadic with equal sex ratio, but at least 10% of patients have positive family history for the disorder. In Japan, the annual incidence is 4.02 cases per million births, and 3 out of 56 cases have a family history [13]. Autosomal dominant inheritance is the most frequently observed pattern of inheritance. The basic molecular defects behind DBA were unknown until the discovery of the first DBA gene *RPS19* [11].

Proteins are universally synthesized in ribosomes, which consist of two subunits: one small (40S) and one large (60S). The mammalian ribosome comprises 4 ribosomal RNAs (rRNA) and 80 ribosomal proteins [14]. *RPS19* encodes a protein belonging to the small subunit of the ribosome. Following the observation that a DBA patient had an X;19 chromosomal translocation, a major DBA locus was mapped to chromosome 19q13, and the breakpoint was identified in the *RPS19* gene [10, 11, 15]. Subsequent large scale studies established that *RPS19* is mutated in approximately 25% of DBA patients [16].

Since the initial description in 1999, mutations in a number of genes that encode 40S ribosomal proteins have been identified in DBA patients. Mutations in *RPS24* on chromosome 10q22–q23 account for about 2% of DBA patients, while *RPS17* variants on chromosome 15q25.2 have been found in 2 patients [17, 18]. Doherty et al. [19] reported that variants of *RPS10* and *RPS26* were observed in 6.4 and 2.6% of cases, respectively. Mutations in genes encoding proteins of the large ribosomal subunit have also been found in DBA patients. *RPL35a* on chromosome 3q29 was detected in about 3.3% of patients with DBA; mutations in *RPL5* and *RPL11* on chromosome 1p22.1 and 1p36.12 were found in 6.6–21.4 and 4.8–9.3% of DBA patients, respectively [20–23]. Approximately 50% of DBA patients in Western countries have a single heterozygous mutation in a gene encoding a ribosomal protein (Table 1). In Japan, mutations in *RPS19, RPL5*, *RPL11* and *RPS17* were identified in 5 (11%), 4 (9%), 2 (4%) and 1 (2%) of 45 probands, respectively. In total, 12 (27%) of Japanese DBA patients had mutations in RP genes [24]. However, most of the studies did not include methods that are capable of detecting large deletions. Therefore, cases of DBA resulting from large chromosomal deletions or rearrangements are probably under diagnosed.

3 Genotype and phenotype correlation

Clinical data from European and American DBA patients show that the frequency of malformations is 31% in patients with *RPS19* mutations, which is not significantly different from that of the entire DBA population [25]. *RPS19* mutations are characterized by a wide variability of phenotypic expression. Even family members with the same mutation in *RPS19* can present with clinical differences [16]. *RPS19* mutations are found in some first degree relatives presenting only with isolated high erythrocyte adenosine deaminase activity and/or macrocytosis. However, large deletions at the 19q locus are always associated with mental retardation, which points to a contiguous gene syndrome [7].

Recent studies suggest that the patients with an *RPL5* and *RPL11* mutation are more likely to have craniofacial, thumb and heart anomalies [21–23]. Remarkably, patients with *PRL5* mutations tend to have cleft lip and/or plate or cleft soft palate, isolated or in combination with other physical abnormalities. Consistent with these reports, 3 of 4 Japanese patients with *RPL5* mutations also had physical malformations and 2 had cleft palate, whereas only 1 of 45 patients without an *RPL5* mutation presented with cleft palate [24].

4 Clinical features and diagnosis

Patients with DBA are usually seen in early childhood with profound macrocytic or normocytic anemia, reticulocytopenia and a reduction or absence of erythroid precursors in their bone marrow. More than 90% of DBA diagnoses are made before the age of 1 year [26]. .

Diagnosis of DBA is often difficult due to incomplete phenotypes and the wide variability of clinical expression [4–6]. The International Clinical Consensus Conference reported diagnostic and supporting criteria for the diagnosis of DBA [4]. Only the identification of pathogenic mutations in one of the DBA genes definitively establishes a diagnosis of DBA. Furthermore, molecular diagnosis enables the detection of carriers, and the avoidance of hematopoietic stem cell transplantation from sibling donors with the mutations. However, determining the effects of missense mutations may be difficult, whereas nonsense and frameshift mutations will probably be pathogenic in the majority of cases [26].

5 Impaired ribosomal biogenesis in DBA

The human ribosome is composed of 4 ribosomal RNAs and a minimum of 80 different ribosomal proteins. The catalytic component translating information encoded in RNA into polypeptides is RNA, not protein [27]. Ribosomal proteins have been added to the catalytic RNA backbone during evolution to improve ribosomal function. The 40S subunit contains 18S rRNA and the 60S subunit contains 28S, 5.8S and 5S rRNA. These mature rRNAs are transcribed as a single precursor (45S), which is subsequently processed into mature species by a complex series of cleavage and modification reactions (Fig. 1) [28, 29].

In eukaryotes, ribosomal biogenesis takes place in the nucleolus, a special compartment within the nucleus. RPS19 is one of the 33 ribosomal proteins that constitute the 40S ribosomal subunit in conjunction with 18S rRNA. Therefore, the highest concentration of RPS19 is within the nucleolus. Some patients with missense mutations in N- or C-terminal nucleolar localization signals (NOS) fail to localize RPS19 to the nucleolus. There is also a dramatic decrease in the expression of mutant DBA proteins in these patients [30].

The RPS19 protein plays an important role in 19S rRNA maturation and 40S synthesis in human cells [31, 32]. Knockdown of RPS19 expression by siRNA impairs 18S rRNA synthesis and formation of the 40S subunit. Cleavage site E is the major site affected in human cells depleted of RPS19 during pre-rRNA processing (Fig. 1). Thus, RPS19-deficient cells suffer from a relative deficiency of

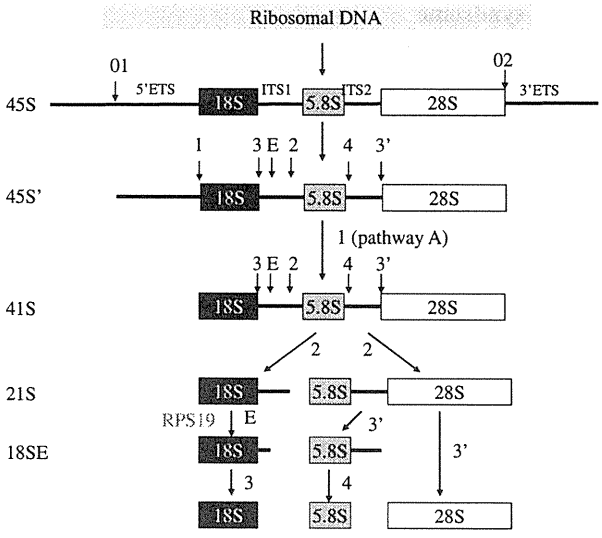

Fig. 1 Pre-rRNA processing in human cells. The major rRNA-processing pathways in human cells as initially derived from Hadjiolova et al. and modified by Rouquette et al. [28, 29]. The 18S, 5.8S and 28S rRNA is transcribed as a single precursor (45S), which is subsequently processed into mature species by a complex series of cleavage and modification reactions. The 45S precursor contains two external transcribed spacers at its 5′ and 3′ ends (5′-ETS and 3′-ETS) and two internal transcribed spacers (ITS1 and ITS2). From 45S′ pre-rRNA, there are two alternative pathways that differ in the order of cleavages 1 (pathway A) and 2 (pathway B). For simplicity, only the pathway A is shown below the 45S pre-rRNA. Cleavage site *E* is the major site affected in human cells depleted of RPS19 during pre-rRNA processing. Numbers *01*, *02*, *1*, *2*, *3*, *4* and *3* indicate cleavage sites. In the *right position* of the figure, pre-rRNA precursors containing 18S rRNA (45S, 45S′, 41S, 21S and 18SE) are shown

the 40S rRNA and have a reduced capacity for translation initiation. Deficiency of RPS19 leads to increased apoptosis in hematopoietic cell lines and bone marrow cells. Suppression of RPS19 inhibits cell proliferation and early erythroid differentiation, but not late erythroid maturation in RPS19-deficient DBA cell lines [33]. Haploinsufficiency of RPS19 has been demonstrated in a subset of patients and appears to be sufficient for the development of DBA [34].

The RPS24 and RPS7 proteins also play an important role in 18S rRNA maturation and small ribosomal subunit synthesis [17, 35]. However, in contrast to RPS19 involvement in the maturation of the internal transcribed spacer 1 (ITS1), RPS24 and RPS7 are required for processing of the 5′ external transcribed spacer. In addition, RPL53a, RPL5 and RPL11 play essential roles in 28S and 5.8S rRNA maturation and formation of the 60S subunit [20, 21]. Connections between mutations in these genes and the occurrence of DBA support the hypothesis that DBA is directly related to a defect in ribosome biogenesis.

6 5q− syndrome

5q− syndrome is a MDS with the 5q deletion, del (5q), as the sole karyotypic abnormality. The syndrome is characterized by refractory anemia, hypolobulated megakaryocytes and a low risk of transformation to AML [36]. Physical mapping methods have been used to narrow the region of recurrent somatic deletion on 5q to a 1.5-megabase common deleted region (CDR) containing 40 genes [37]. Recently, *RPS14* was identified as a 5q− syndrome gene in an RNA interference screen of each gene within the CDR [38]. Knockdown of RPS14 recapitulated the 5q− syndrome phenotype: a severe decrease in erythroid cells with relative preservation of megakaryocytic cells. Furthermore, decreased expression of *RPS14* results in a block in the processing of 18S rRNA and formation of the 40S ribosomal subunit. Notably, forced expression of RPS14 rescued the disease phenotype in patient-derived bone marrow cells. These studies suggest that acquired RPS14 haploinsufficiency in 5q− syndrome is analogous to the inactivating mutations in ribosomal genes in DBA. These findings have linked abnormalities in ribosome function to the occurrence of both congenital and acquired bone marrow failure syndromes.

7 Mechanisms of erythroid failure caused by RP deficiency

The fact that all of the causative genes for DBA are ribosomal proteins suggests that insufficiency in ribosomal function may be the underlying cause of red cell aplasia in patients with DBA. Although the mechanism whereby mutations in the ribosomal protein genes cause specific defects in red cell maturation is not fully understood, many lines of evidence indicate that p53 activation caused by ribosomal dysfunction may be central to DBA pathogenesis.

The murine double minute 2 protein (MDM2) acts as an ubiquitin ligase that targets p53 for degradation. Ribosomal proteins L5, L11 and L23 associate with MDM2 and activate p53 by inhibiting MDM2-mediated p53 suppression [39–41]. Disruption of 40S biogenesis leads to the release of RPL11 and other ribosomal proteins into the nucleoplasm, the binding of RPL11 to MDM2, the inhibition of MDM2 activity, and the consequent accumulation of p53 [42].

DBA murine models have been developed during a forward genetics screen in mice for pigmentation abnormalities [43]. The screen revealed missense mutations in *Rps19* and *Rps20* in two mutants with dominant inherited dark skin, *Dark skin 3* (*Dks3*) and *Dark skin 4* (*Dks4*), respectively. The *Rps19* mutant mice (*Dks3*) exhibited phenotypes consistent with those of DBA: growth retardation, macrocytic anemia with reticulocytopenia and increased apoptosis in bone marrow progenitors. In a cross with *p53* knockout mice, reduced dosage of *p53* rescued both the erythrocytic and body weight phenotypes caused by the *Rps19* mutation, suggesting a direct link between the DBA phenotypes and accumulation of p53.

8 Concluding remarks

Although DBA is currently the only known human congenital disease by defects in ribosomal proteins, the discovery of *RPS14* as a 5q− syndrome gene demonstrates that abnormalities in ribosome function are broadly implicated in both congenital and acquired bone marrow failure syndrome in humans.

The recent studies have provided fascinating insights into the pathogenesis of DBA. However, several important questions are yet to be answered. It remained to be determined, for example, how haploinsufficiency of ribosomal proteins expressing ubiquitously causes specific defects in erythropoiesis, and why mutations in some ribosomal proteins genes, such as *RPL5* have more profound impact on fetal development than mutations in other ribosomal protein genes. Although corticosteroids remain the mainstay of treatment of DBA more than half a century after the original report of their efficacy, their mechanism of action is still unknown. DBA have now begun to lead us towards a further understanding of bone marrow failure syndrome and potentially to novel ways of treatment.

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Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia

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ABSTRACT

Background
Diamond-Blackfan anemia is a rare, clinically heterogeneous, congenital red cell aplasia: 40% of patients have congenital abnormalities. Recent studies have shown that in western countries, the disease is associated with heterozygous mutations in the ribosomal protein (RP) genes in about 50% of patients. There have been no studies to determine the incidence of these mutations in Asian patients with Diamond-Blackfan anemia.

Design and Methods
We screened 49 Japanese patients with Diamond-Blackfan anemia (45 probands) for mutations in the six known genes associated with Diamond-Blackfan anemia: *RPS19*, *RPS24*, *RPS17*, *RPL5*, *RPL11*, and *RPL35A*. *RPS14* was also examined due to its implied involvement in 5q- syndrome.

Results
Mutations in *RPS19*, *RPL5*, *RPL11* and *RPS17* were identified in five, four, two and one of the probands, respectively. In total, 12 (27%) of the Japanese Diamond-Blackfan anemia patients had mutations in ribosomal protein genes. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. All patients with *RPS19* and *RPL5* mutations had physical abnormalities. Remarkably, cleft palate was seen in two patients with *RPL5* mutations, and thumb anomalies were seen in six patients with an *RPS19* or *RPL5* mutation. In contrast, a small-for-date phenotype was seen in five patients without an *RPL5* mutation.

Conclusions
We observed a slightly lower frequency of mutations in the ribosomal protein genes in patients with Diamond-Blackfan anemia compared to the frequency reported in western countries. Genotype-phenotype data suggest an association between anomalies and *RPS19* mutations, and a negative association between small-for-date phenotype and *RPL5* mutations.

Key words: protein genes, Diamond-Blackfan anemia, *RPL5* mutation.

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Introduction

Diamond-Blackfan anemia (DBA, MIM#105650) is a congenital, inherited bone marrow failure syndrome, characterized by normochromic macrocytic anemia, reticulocytopenia, and absence or insufficiency of erythroid precursors in normocellular bone marrow.[1] DBA was first reported by Josephs in 1936 and defined as a distinct clinical entity 2 years later by Diamond and Blackfan. Recent investigations have shown that the cellular defect in DBA fibroblasts is primarily caused by reduced proliferation and a prolonged cell cycle corresponding to the bone marrow characteristics of DBA.[2] DBA is a rare disease with a frequency of two to seven cases per million live births and has no ethnic or gender predilection.[1]

Approximately 90% of affected patients typically present in infancy or early childhood, although patients with a 'non-classical', mild phenotype are diagnosed later in life.[3,4] Macrocytic anemia 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.[3-5] In addition, DBA patients have a predisposition to malignancies including acute myeloid leukemia, myelodysplastic syndrome, and osteogenic sarcoma.[3] The diagnosis of DBA is often difficult because incomplete phenotypes and wide variability of clinical expression are present.[4-6] The central hematopoietic defect is enhanced sensitivity of hematopoietic progenitors to apoptosis along with evidence of stress erythropoiesis, including elevations in fetal hemoglobin and mean red cell volume.[2] The majority of patients have an increase in erythrocyte adenosine deaminase activity.[7]

Proteins are universally synthesized in ribosomes. This macromolecular ribonucleoprotein machinery consists of two subunits: one small and one large. The mammalian ribosome comprises four RNA and 80 ribosomal proteins.[8] The first genetic anomaly identified in DBA involves the *RPS19* gene, which is mutated in approximately 25% of DBA patients. This gene is located at chromosome 19q13.2 and encodes a protein belonging to the small subunit of the ribosome.[9,10] Haploinsufficiency of the *RPS19* gene product has been demonstrated in a subset of cases[11] and appears to be sufficient to cause DBA. The RPS19 protein plays an important role in 18S rRNA maturation and small ribosomal subunit synthesis in human cells.[12,13] Deficiency of RPS19 leads to increased apoptosis in hematopoietic cell lines and bone marrow cells. Suppression of *RPS19* inhibits cell proliferation and early erythroid differentiation but not late erythroid maturation in RPS19-deficient DBA cell lines.[14]

Mutations in two other genes, *RPS24* and *RPS17*, encoding proteins of the small ribosomal subunits have been found in approximately 2% of patients.[15,16] Furthermore, mutations in genes encoding large ribosomal subunit-associated proteins, *RPL5*, *RPL11* and *RPL35A*, have been reported in 9% to 21.4%, 6.5% to 7.1%, and 3.3% of patients, respectively.[17-19] To date, approximately 50% of DBA patients in western countries have been found to have a single heterozygous mutation in a gene encoding a ribosomal protein.[1,3] These findings imply that DBA is a disorder of ribosome biogenesis and/or function. However, there have been no studies of the incidences of these mutations in Asian DBA patients.

In this study, we screened 49 Japanese DBA patients (45 probands) for mutations of the six known DBA genes and *RPS14*, which has been implicated in the 5q- syndrome, a subtype of myelodysplastic syndrome characterized by a defect in erythroid differentiation.[20]

Design and Methods

Patients

Forty-nine patients were studied in order to define the frequency and type of mutations of ribosomal protein genes associated with DBA in Japan. Eight patients were from families with more than one affected member, whereas 41 were from families with only one affected patient. The diagnosis of DBA was based on the criteria of normochromic, often macrocytic anemia; reticulocytopenia; a low number or lack of erythroid precursors in bone marrow; and, in some patients, congenital malformations, without known causes of single cytopenia including acquired or congenital infection, transient erythroblastopenia of childhood, metabolic disorders, malignancies, or autoimmune diseases. All clinical samples were obtained with informed consent from 28 pediatric and/or hematology departments throughout Japan. Additional information was obtained by a standardized questionnaire including information on birth history, age of onset or diagnosis, family history, physical examination (especially regarding malformations), hematologic data, response to therapeutic procedures, and prognosis. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine.

Ribosomal protein gene analysis

DNA was extracted from peripheral blood using a standard proteinase K, phenol and chloroform protocol.[21] A polymerase chain reaction (PCR) was used to amplify fragments from genomic DNA using primer sets designed to amplify the coding exons and exon/intron boundaries of the *RPS19*, *RPS17*, *RPS24*, *RPS14*, *RPL5*, *RPL11* and *RPL35A*. PCR products were directly sequenced in the forward and/or reverse direction using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). *RPS19* was analyzed by determining the genomic DNA sequence of the non-coding first exon, with flanking regions, and the 450-base pair (bp) sequence upstream of the first exon (5'UTR) for each DNA sample, as previously described.[5]

To clarify the sequence of heterozygous insertion/deletion sequence variations, the respective PCR products were cloned into a TA pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and their sequences were confirmed.

Genotype-phenotype correlations and statistical analysis

Physical abnormalities in the Japanese DBA patients were evaluated from a viewpoint of correlations with genotype. Although growth retardation can be modified by several factors such as steroid therapy, chronic anemia, and iron overload, the retardation was considered pathognomonic for DBA if it was marked, being below -3 standard deviations (SD). Response to treatment is usually seen within 1 month of treatment in DBA, but a prediction of response has not been reported previously.[1,3] We, therefore, also examined the correlation between genotype and response to the first round of steroid therapy. Associations between two groups of variables were assessed with Fisher's exact test. All tests were two-sided and P levels less than 0.05 were considered statistically significant. Data were analyzed with SPSS 11.0J software (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics

Overall, 49 patients (45 probands) were available for analysis. The male to female ratio was 1:1.2. Forty-one index cases were classified as sporadic without unexplained anemia in first-degree relatives, while the remaining eight patients were from four families. All patients were Japanese except two cases: case 10 was Chinese and case 23 was a Brazilian of Japanese extraction. Case 15 had a Filipina mother and a Japanese father.

Genetics

RPS19

Five different mutations were detected in five probands out of 45 families (11%) (Table 1). The median age at presentation of the index cases with *RPS19* mutations was 1 month (range, 0 to 2 months). There appears to be a lower percentage of *RPS19* mutations in Japanese DBA patients than in patients from western countries. All mutations were in the coding region of the gene. Missense mutations resulting in amino acid substitutions were noted in four index cases. The three mutations, p.R62Q in case 30, p.R62W in case 44 and p.0 in case 43, have been reported in seven, ten and two families, respectively,[6,10,11,22-26] whereas one mutation, p.G95V in case 25, was novel, and could not be found in the Single Polymorphism Database (dbSNP at *www.ncbi.nlm.nih.gov/SNP*). Furthermore, the mutation was not observed in DNA from 50 normal individuals. An insertion of one nucleotide was found in one case (case 28), resulting in a novel frameshift mutation.

RPL5 and RPL11

The human *RPL5* gene consists of eight exons and is located on chromosome 1. Four novel mutations were found among the 45 probands (9%) (Table 1). The median age at presentation of the index cases with *RPL5* mutations was 10 months. A deletion of two nucleotides was found in case 10, and an insertion of one nucleotide was found in case 65, each affecting the reading frame. Two cases (cases 41 and 55) had point mutations that resulted in a loss of the translation initiation codon.

The human *RPL11* gene, which consists of six exons, is also located on chromosome 1. All exons and exon/intron boundaries were PCR-amplified and sequenced in DBA patients who were negative for mutations in *RPS19* and *RPL5*. Two mutations (4%) were found, and they were diagnosed at 18 and 20 months old, respectively (Table 1). A deletion of two nucleotides was found in case 9, and a deletion of one nucleotide was found in case 23, in each patient leading to a shift in the reading frame and the introduction of a premature stop codon.

RPS17

The *RPS17* gene is located on chromosome 15, and consists of five exons. *RPS17* mutations are rare and have been reported in only two patients with DBA. A novel one-nucleotide deletion in *RPS17* was identified in one patient (2%), resulting in the introduction of a premature stop codon (Table 1). The patient with the *RPS17* mutation (case 56) was born to healthy non-consanguineous parents and diagnosed as having DBA at the age of 1 month. He responded to the initial steroid treatment, and had a course of steroid-dependent therapy. No physical anomalies were seen in this patient.

RPL35A, RPS24 and RPS14

Mutations in *RPS24* and *RPL35A* are rare and have been reported in only eight and six patients with DBA, respectively. DBA patients were screened for *RPS24* and *RPL35A*, in addition to *RPS14*, which is implicated in the 5q- syndrome. No mutations were detected in *RPS24*, *RPL35A* or *RPS14* in Japanese DBA patients.

In total, sequence changes were found in four out of seven screened ribosomal protein genes (Table 2). Mutations in *RPS19*, *RPS17*, *RPL5*, and *RPL11* were detected in 11%, 2%, 9%, and 4% of the probands, respectively. The frequency of ribosomal protein gene mutations in Japanese DBA patients was 27%.

Genotype-phenotype correlations: congenital anomalies

The patients' characteristics are summarized in Table 3.

Table 1. Mutations identified in *RPS19*, *RPL5*, *RPL11*, and *RPS17* in Japanese DBA patients.

Patients (gender)	Inheritance	Age at diagnosis	Mutation	Predicted amino acid change
Mutations in the *RPS19* gene				
43 proband (F)	Sporadic	0 D	Exon2:g.3G>A	p.0
28 proband (M)	Sporadic	6 D	Exon3:g.130_131insA	E44fsX50
44 proband (F)	Sporadic	1 M	Exon4:g.184C>T	R62W
30 proband (F)	Familial	1 M	Exon4:g.185G>A	R62Q
30 father (M)	Familial	0 M	Exon4:g.185G>A	R62Q
25 proband (M)	Sporadic	2 M	Exon4:g.284G>T	G95V
Mutations in the *RPL5* gene				
10 proband (F)	Sporadic	0 M	Exon5:g.473_474delAA	K158fsX183
41 proband (F)	Sporadic	1 Y	Exon1:g.3G>T	p.0
55 proband (F)	Sporadic	3 Y	Exon1:g.3G>A	p.0
65 proband (F)	Sporadic	4 M	Exon2:g.37_38insT	F13fsX14
Mutations in the *RPL11* gene				
9 proband (F)	Sporadic	1 Y 10 M	Exon2:g.58_59delCT	L20fsX53
23 proband (M)	sporadic	1 Y 6 M	Exon5:g.460delA	R154fsX189
Mutations in the *RPS17* gene				
56 proband (F)	Sporadic	1 M	Exon2:g.26delT	V9fsX17

Anomalies associated with DBA were found in 27 patients (55%). Sixteen had two or more malformations (33%). All six patients with an *RPS19* mutation had physical anomalies, and three of them had multiple anomalies. In contrast, clinical data from European and American DBA patients showed that the frequency of malformations was 31% in patients with *RPS19* mutations, which is not significantly different from that of the entire DBA population.[26] *RPS19* mutations are characterized by a wide variability of phenotypic expression.[26] A mutation is frequently associated with various degrees of anemia, different responses to treatment, and dissimilar malformations. Even various family members having the same mutation in *RPS19* present with different clinical expressions. Cases 30, 44 and 43 harbored the same *RPS19* mutations reported in multicase families (p.R62Q, p.R62W, p.0).[6,10,11,22-27] Comparable to previous observations, no consistent clinical features were found in patients from different families displaying mutations in *RPS19*. For example, the father of case 30 harboring the same mutation had no finger anomalies, although case 30 had syndactyly and thumb polydactyly.

Consistent with reports that patients with *RPL5* and *RPL11* mutations are at high risk of developing malformations,[17,18] all four patients with *RPL5* mutations had physical anomalies. Furthermore, three of them had multiple physical anomalies, particularly case 41, who had very severe congenital heart disease (Table 3). One of two patients with *RPL11* mutations had physical anomalies. In contrast, of the 36 patients with no mutations, physical anomalies were seen in 16 (44%).

Nine patients had craniofacial anomalies. Of these, two had *RPL5* mutations, while the remaining patients had no mutations. Gazda *et al.* suggested an association between *RPL5/RPL11* mutation and cleft lip and/or palate.[17] Data in the Diamond-Blackfan Anemia Registry (DBAR) of North America also suggest that the DBA phenotype associated with cleft lip/palate is caused by non-*RPS19* mutations.[4] In our cohort, the frequency of cleft palate was significantly different between *RPL5*-mutated and *RPL5* non-mutated groups ($P<0.05$): cleft palate was seen in three patients, two of whom had *RPL5* mutations while the other patient belonged to the *RPL5* non-mutated group.

Thumb anomalies were seen in six patients, four of whom had *RPS19* mutations while two had *RPL5* mutations. There was a statistically significant difference in the frequency of thumb anomalies between *RPS19*-mutated and *RPS19* non-mutated groups ($P<0.05$). Flat thenar was seen in one patient with an *RPL5* mutation. In contrast to previous reports on patients with *RPL11* mutations, thumb anomalies were not found in our patients with these mutations.

A small-for-date phenotype was seen in seven patients (14%): one had an *RPS19* mutation, one had an *RPL11* mutation, and the four others had no mutations. None of the patients with *RPL5* mutations was born small-for-date.

Genotype-phenotype correlations: therapeutic response

Corticosteroids and transfusions are the mainstays of DBA treatment.[1,3] Of 45 patients evaluable for first treatment response, 73% responded to steroid therapy, 8% did not respond and 16% were never treated with steroids. The proportions of patients who responded to the first steroid treatment were 5/5 (*RPS19*), 2/3 (*RPL5*), 1/2 (*RPL11*), 1/1 (*RPS17*), and 22/27 (no mutation). There were no significant differences in the response rates among these patients.

Sixty-nine percent of patients received red blood cell transfusions. Of 48 patients available for therapy in follow-up, 8 patients (17%) were transfusion-dependent, 18 patients (37%) were steroid-dependent, and 18 patients (37%) were transfusion-independent with no other treatment. Three patients received bone marrow transplants and were alive and well (Table 3). A malignancy was detected in one case (case 50, proband), who developed a myelodysplastic syndrome 1 year after the diagnosis of DBA.

Table 2. **Summary of sequence changes in seven ribosomal protein genes identified in Japanese DBA patients.**

Gene symbol	N. of tested DNA samples from unrelated probands	N. of probands with mutations	N. of subjects with mutations	Mutation types
RPS19	45	5 (11%)	6	missense, loss of 1st methionine, small insertion
RPL5	45	4 (9%)	4	loss of 1st methionine, small deletion, small insertion
RPL11	34	2 (4%)	2	small deletion
RPS17	45	1 (2%)	1	small deletion

Discussion

This is the first report of an investigation of DBA patients in Japan. Twelve types of mutations were detected in four ribosomal protein genes. These mutations occurred in 27% of Japanese DBA patients. Mutations in *RPS19*, which have been found in 25% of patients in western countries,[26] were detected in only five of 45 probands (11%) in Japan, and two of these mutations were unique. Novel mutations in *RPL5* (four probands; 9%), *RPL11* (two probands; 4%) and *RPS17* (one proband; 2%) were identified. The frequencies of mutations in *RPL5*, *RPL11* and *RPS17* were very similar to those in western countries.[16-19] These results may suggest that a lower incidence of mutations in ribosomal protein genes in Japanese patients with DBA is due to a lower incidence of *RPS19* mutations, although we might have missed large deletions or re-arrangements in this study.

Physical abnormalities and growth retardation were detected in 55% of the Japanese DBA patients, consistent with previous reports from western countries.[4-6] Recent studies suggest that patients with *RPL5* mutation are more likely to have physical malformations including craniofacial, thumb, and heart anomalies.[17,18] Remarkably, patients with *RPL5* mutations tend to have cleft lip and/or palate or cleft soft palate, isolated or in combination with other physical abnormalities.[17,18] We found that three of four patients with *RPL5* mutations had multiple physical malformations, and two had cleft palate, whereas only one patient without an *RPL5* mutation had cleft palate. In the general population, 0.1% to 0.2% of children are born with cleft lip and/or palate.[28] Our data, and those from previous findings, suggest that *PRL5* mutations are associ-

Table 3. **Characteristics of Japanese DBA patients.**

Patient	Malformation status	Response to first steroid therapy	Present therapy
Patients with mutation of *RPS19*			
25 proband	Thumb polydactyly, growth retardation (-2.0SD), etc.	ND	ND
28 proband	Thumb polydactyly, CHD, etc.	Response	Steroid-dependent
30 proband	Thumb polydactyly, syndactyly, growth retardation (-3.4SD)	Response	Steroid-dependent
30 father	Growth retardation (-3.6SD)	NA	CR
43 proband	Thumb polydactyly	Response	Steroid-dependent
44 proband	SFD	Response	CR
Patients with mutation of *RPL5*			
10 proband	Flat thenar, cleft palate, CHD, etc.	Poor	Transfusion-dependent
41 proband	Craniofacial abnormalities, cleft palate, CHD, etc.	ND	Transfusion-dependent
55 proband	Thumb polydactyly	Response	Steroid-dependent
65 proband	Growth retardation (-3.0SD)	Response	Steroid
Patients with mutation of *RPL11*			
9 proband	CHD, SFD, etc.	Response	CR
23 proband	None	Poor	Steroid-dependent
Patient with mutation of *RPS17*			
56 proband	None	Response	Steroid-dependent
Patients without mutation of seven RP genes			
1 proband	Growth retardation (-4.0SD)	Response	CR
1 daughter	None	Response	CR
3 proband	Growth retardation (-3.6SD)	Response	Steroid-dependent
4 proband	Craniofacial abnormalities, SFD, short stature, webbed neck	Response	Steroid-dependent
5 proband	None	Response	CR
6 proband	Cleft palate, SFD, etc.	Poor	BMT
7 proband	Craniofacial abnormalities, SFD, growth retardation, etc.	Response	CR
8 proband	Growth retardation, webbed neck	Response	Steroid-depndent
13 proband	None	NA	CyA, BMT
14 proband	None	Response	CR
15 proband	None	Response	Transfusion-dependent
20 proband	Craniofacial abnormalities, CHD, etc.	Rsponse	Transfusion-dependent
21 proband	None	Response	Steroid-dependent
22 proband	None	Response	CR
24 proband	Growth retardation (-4.0SD)	Response	Steroid-dependent
26 proband	Growth retardation (-4.1SD), craniofacial abnormalities, etc.	Response	Transfusion-dependent
33 proband	None	Response	BMT
36 proband	Hypospadias, cryptorchidism	Response	Steroid-dependent
36 cousin	None	Response	Steroid-dependent
37 proband	Hypospadias, cryptorchidism	ND	CR
42 proband	None	Response	CR
45 proband	Craniofacial abnormalities, growth retardation, etc.	Poor	Transfusion-dependent
48 proband	Fetal hydrops	ND	CR
49 proband	None	Response	Steroid-dependent
50 proband	None	Response	Steroid-dependent, CBT (due to MDS)
50 sister	None	Response	Steroid-dependent
51proband	None	Poor	CR
54 proband	None	ND	Transfusion-dependent
59 proband	None	ND	Transfusion
60 proband	SFD	ND	Transfusion
61 proband	None	Response	Cyclosporine
62 proband	CHD, SFD, growth retardation (-3.1SD)	Response	Steroid-dependent
63 proband	Craniofacial abnormalities, growth retardation (-7.5SD)	Response	Steroid-dependet
64 proband	None	Response	Steroid-depndent
66 proband	None	NA	Transfusion-dependent
67 proband	None	NA	NA

*ND: not done; NA: not available; SFD: small-for-date; CHD: congenital heart disease; MDS: myelodysplastic syndrome; BMT: bone marrow transplantation; CBT: cord blood stem cell transplantation; CR: complete remission. * RPS19, RPS24, RPS17, RPS14, RPL5, RPL11, RPL35A.*

ated with multiple physical abnormalities, especially cleft lip and/or palate.

Cmejla *et al.* reported that 87.5% of *RPL5*-mutated patients were born small-for-date, whereas only 42.9% of *RPS19*-mutated patients were born small-for-date.[18] However, in our series, the small-for-date phenotype was seen in seven patients, and all of them were *RPL5*-non-mutated patients. Our data suggest that *RPL5* mutations in Japanese DBA patients have no relevance to the small-for-date phenotype, which may be a unique characteristic of Japanese DBA.

According to recent studies, the frequency of malformation, particularly thumb anomalies, in *RPS19*-mutated patients, was relatively low compared to that in *RPL5*- or *RPL11*-mutated patients.[22-24,29] In Italian DBA patients, the risk of malformation was 7-fold higher in *RPL5*-mutated patients than in *RPS19*-mutated patients.[29] In contrast, all of the Japanese DBA patients with *RPS19* mutations had one or more malformations. The frequency of thumb anomalies was significantly higher in patients with *RPS19* mutations, as well as in patients with *RPL5* mutations, compared to in the other groups of patients.

Although steroid therapy is one of the established treatments for DBA, the mechanism of action is unknown and reliable prediction of response to initial steroid therapy is not available.[1,3] *RPS19* mutation status has not been predictive of response in any series.[3] In our cohort, responsiveness to first steroid therapy in Japanese DBA patients was as good as that reported in western populations.[1,3] In this study, no significant differences in response to initial steroid therapy were found between *RPS19*-mutated and *RPS19*-non-mutated groups, or between the groups with *RPS19* mutations and other ribosomal protein gene mutations.

In summary, we found that heterozygous mutations in *RPS19*, *RPL5*, *RPL11* or *RPS17* were present in 27% of Japanese DBA patients. No mutations were detected in *RPS14*, *RPS24* or *RPL35A*. We observed a slightly lower frequency of mutations in ribosomal protein genes in our cohort of Japanese DBA patients than the frequencies reported previously from western countries, although the data from both populations are based on relatively low numbers of patients and values showing significant differences between populations are lacking. Our data suggest an association between *RPL5* mutation and malformations, especially cleft palate, and between *RPS19* mutation and malformations, particularly thumb anomalies. This study also suggests that no association exists between *RPL5* mutations and the small-for-date phenotype or between *RPS19* mutations and non-responsiveness to initial steroid therapy in Japanese DBA patients.

Authorship and Disclosures

EI was the principal investigator and takes primary responsibility for the paper. YK, TT, ST, GX, RNW, KT, and SO performed the laboratory work for this study. SO, TH, AH, SK, DH, YK, RY, KK, RK, TI, TH, MHP, and KS enrolled the patients. EI and YK wrote the paper.

The authors reported no potential conflicts of interest.

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Relapse of aplastic anemia in children after immunosuppressive therapy: a report from the Japan Childhood Aplastic Anemia Study Group

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ABSTRACT

Background
Although the therapeutic outcome of acquired aplastic anemia has improved markedly with the introduction of immunosuppressive therapy using antithymocyte globulin and cyclosporine, a significant proportion of patients subsequently relapse and require second-line therapy. However, detailed analyses of relapses in aplastic anemia children are limited.

Design and Methods
We previously conducted two prospective multicenter trials of immunosuppressive therapy for children with aplastic anemia: AA-92 and AA-97, which began in 1992 and 1997, respectively. In this study, we assessed the relapse rate, risk factors for relapse, and the response to second-line treatment in children with aplastic anemia treated with antithymocyte globulin and cyclosporine.

Results
From 1992 to 2007, we treated 441 children with aplastic anemia with standard immunosuppressive therapy. Among the 264 patients who responded to immunosuppressive therapy, 42 (15.9%) relapsed. The cumulative incidence of relapse was 11.9% at 10 years. Multivariate analysis revealed that relapse risk was significantly associated with an immunosuppressive therapy regimen using danazol (relative risk, 3.15; P=0.001) and non-severe aplastic anemia (relative risk, 2.51; P=0.02). Seventeen relapsed patients received additional immunosuppressive therapy with antithymocyte globulin and cyclosporine. Eight patients responded within 6 months. Seven of nine non-responders to second immunosuppressive therapy received hematopoietic stem cell transplantation and five are alive. Eleven patients underwent hematopoietic stem cell transplantation directly and seven are alive.

Conclusions
In the present study, the cumulative incidence of relapse at 10 years was relatively low compared to that in other studies mainly involving adult patients. A multicenter prospective study is warranted to establish optimal therapy for children with aplastic anemia.

Key words: children, aplastic anemia, relapse, risk factors, immunosuppressive therapy.

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Introduction

Aplastic anemia (AA) is thought to be an immune-mediated bone marrow disease, characterized by bone marrow aplasia and peripheral blood pancytopenia. Currently, two effective treatments are available for this disorder: allogeneic bone marrow transplantation and immunosuppressive therapy. Bone marrow transplantation from a human leukocyte antigen (HLA)-matched sibling donor can cure the majority of transplanted patients with severe AA.[1] The outcome after bone marrow transplantation has been markedly better in children than in adults, with less frequent and severe graft-*versus*-host disease and better overall survival.[2,3] However, most children with severe AA have no matched sibling donor and rely on immunosuppressive therapy as first-line treatment.

The combination of antithymocyte globulin and cyclosporine is now considered the standard immunosuppressive regimen for children with severe AA who lack a matched sibling donor.[4] Recent large trials of combined immunosuppressive therapy for severe AA in children demonstrated that the response rate is greater than 60% and the 3- to 5-year survival rate is approximately 90%.[5-7] However, relapse and clonal evolution with transformation to myelodysplasia or acute myeloid leukemia remain significant problems after immunosuppressive therapy, and long-term, event-free survival is less impressive than after bone marrow transplantation.[4,8] We previously reported the results of a multicenter trial of immunosuppressive therapy for children with AA (AA-92 study).[5] In the AA-92 study, the response rate at 6 months was 71%, with the probability of survival at 4 years being greater than 90%. However, a significant proportion of patients subsequently relapsed and required second-line therapy. To select the optimal therapy for such patients, a detailed analysis concerning relapse after response to immunosuppressive therapy is very important; however, analyses of relapse of AA in children after the standard combined immunosuppressive regimen are very limited.[9-11] Although the European Group for Blood and Marrow Transplantation (EMBT) reported an analysis of relapse of AA after immunosuppressive therapy in a large number of patients, the study populations were primarily adults treated in the 1970s and 1980s with antithymocyte globulin monotherapy.[9] A report from the Italian Association of Pediatric Hematology and Oncology focused mainly on the response to cyclosporine and dependence after immunosuppressive therapy.[10] A single-center retrospective analysis from the National Institutes of Health showed excellent long-term survival with a 33% cumulative incidence of relapse at 10 years in children with severe AA who responded to the standard immunosuppressive therapy; however, a detailed analysis of relapse that included risk factors was not provided.[11]

We previously conducted two prospective multicenter studies: the AA-92 and AA-97, which began in November 1992 and October 1997, respectively.[5,12] From 1992 to 2007, 473 children with AA were treated with immunosuppressive therapy in these studies, and 441 of the children were treated with antithymocyte globulin plus cyclosporine. In the present study, we assessed the relapse rate, risk factors for relapse, response to second-line treatment, and prognosis after relapse in AA children treated with an antithymocyte globulin/ cyclosporine-based regimen.

Design and Methods

Patients

Two consecutive prospective studies were designed by the Japan Childhood Aplastic Anemia Study Group and involved 79 hospitals in Japan. The eligibility criteria have been described previously.[5] The severity of disease was determined according to currently used criteria.[13,14] Disease was considered severe if at least two of the following were present: (i) neutrophil count less than 0.5×10^9/L; (ii) platelet count less than 20×10^9/L; and (iii) reticulocyte count less than 20×10^9/L with a hypocellular bone marrow. AA was considered very severe if the above criteria for severe disease were fulfilled and the neutrophil count was less than 20×10^9/L. Non-severe disease was defined by at least two of the following: (i) neutrophil count less than 1.0×10^9/L, (ii) platelet count less than 50×10^9/L; and (iii) reticulocyte count less than 60×10^9/L with a hypocellular bone marrow. Allogeneic bone marrow transplantation was recommended for those patients with severe or very severe disease who had a matched sibling donor. This study was approved by the Ethic Committee of Hyogo Children Hospital.

Treatment

The details of the immunosuppressive therapy administered were described in previous reports.[5,12] Immunosuppressive therapy consisted of horse antithymocyte globulin (Lymphoglobulin; Genzyme Corp., Cambridge, MA, USA) (15 mg/kg per day, days 1 to 5), cyclosporine (6 mg/kg per day, days 1 to 180, with subsequent adjustments to maintain the whole blood cyclosporine concentration between 100 to 200 ng/mL), and methylprednisolone for prophylaxis against allergic reactions (2 mg/kg per day for 5 days, with subsequent halving of the dose every week until discontinuation on day 28). Patients with very severe AA were treated with immunosuppressive therapy plus granulocyte-colony stimulating factor (G-CSF) (Filgrastim; Kirin, Tokyo, Japan) [400 $\mu g/m^2$ on day 1, with responding patients (neutrophil count > 1.0×10^9/mL) receiving the same dose three times a week for 3 months in the AA-92 study and for 60 days in the AA-97 study]. In the AA-92 study, the addition of G-CSF to immunosuppressive therapy for patients with severe AA and non-severe AA was randomized, while in the AA-97 study, G-CSF was not given to patients with severe AA or non-severe AA except to those with documented severe infection. All patients in the AA-92 study received danazol at a dose of 5 mg/kg/day for 6 months, and danazol was discontinued without tapering.

Assessments

A complete response was defined for all patients as a neutrophil count greater than 1.5×10^9/L, a platelet count greater than 100×10^9/L, and a hemoglobin level greater than 11.0 g/dL. For patients with severe AA and very severe AA, a partial response was defined as a neutrophil count greater than 0.5×10^9/L, a platelet count greater than 20×10^9/L, a hemoglobin level greater than 8.0 g/dL, and no requirement for blood transfusions. For patients with non-severe AA, a partial response was defined as a neutrophil count greater than 1.0×10^9/L, a platelet count greater than 30×10^9/L, a hemoglobin level greater than 8.0 g/dL, and no requirement for blood transfusions.[5] In patients with a complete response on day 180, the cyclosporine dose was tapered down slowly (10% of adjusted dose per month). In those with a partial response, cyclosporine was continued for another 6 months to allow further improvement of blood counts. Tapering of cyclosporine was started on day 360 (10% every 2 weeks) regardless of response.

The hematologic response was evaluated 6 months after the

initiation of therapy. Relapse was defined by conversion to no response from a partial or complete response and/or the requirement for blood transfusions.[5]

Statistical analysis

Failure-free survival curves were calculated by the Kaplan-Meier method, and evaluated by the log-rank test. The Cox proportional hazards model was used to assess the risk factors for relapse after immunosuppressive therapy using both univariate and multivariate analyses. The estimated magnitude of the relative risk (RR) is shown along with the 97.5% confidence interval (CI). Cumulative incidence using the competing risk method, as described by Fine and Gray,[15] was used for the assessment of factors predicting relapse. The competing events of relapse were death and transplantation.

Results

Patients' characteristics

In the AA-92 and AA-97 studies, 441 AA children were treated with antithymocyte globulin plus cyclosporine between 1992 and 2007. The characteristics of all the patients studied are summarized in Table 1. There were 112 and 329 patients in the AA-92 and AA-97 studies, respectively. The median age of all these patients was 8.3 years (range, 0 to 17 years). Patients with very severe (n=210), severe (n=149) and non-severe disease (n=82) received initial immunosuppressive therapy consisting of antithymocyte globulin and cyclosporine. Six months after the initial immunosuppressive therapy, 264 patients (59.9%) had achieved a complete response (n = 91) or partial response (n=173). Among the 264 patients who responded to immunosuppressive therapy, 42 (15.9%) subsequently relapsed. The cumulative incidence of relapse was 11.9% at 10 years and the median time from diagnosis to relapse was 21 months (range, 6 to 138 months). The median time from response to antithymocyte globulin therapy to relapse was 22 months (range, 2 to 135 months).

Risk factors for relapse

Two hundred and sixty-four patients with a total of 42 events were eligible for analyses of risk factors for relapse. In univariate analysis, two parameters, non-severe disease (RR=2.98, 97.5% CI 1.40 - 6.34, P=0.0047) and use of danazol (RR=3.44, 97.5% CI 1.78 - 6.65, P=0.00023), were statistically significant risk factors (Table 2). In contrast, the relative risk of relapse for patients with post-hepatitis AA was significantly lower than the relative risk for patients with idiopathic AA (RR=0.234, P=0.043). Gender, age, duration of AA prior to initial treatment, early response (within 90 days after immunosuppressive therapy), use of G-CSF, and HLA-DR2 could not be identified as risk factors. In multivariate analysis, two factors, non-severe AA (RR=2.51, 97.5% CI 1.15 - 5.46, P=0.02) and use of danazol (RR=3.15, 97.5% CI 1.62 - 6.12, P=0.001) remained statistically significant. Figure 1A shows the cumulative incidence of relapse of patients with non-severe AA (35.3%), severe AA (12.9%), and very severe AA (12.0%) 10 years after the first immunosuppressive therapy. The cumulative relapse rate of patients with non-severe AA was significantly higher than that of patients with severe AA (P=0.025) or very severe AA (P=0.005). Figure 1B shows the actuarial risk of relapse at 10 years among patients treated with danazol (29.0%) and in the group not treated with danazol (9.8%) (P<0.001).

Repeated immunosuppressive therapy versus hematopoietic stem cell transplantation as second-line therapy

Among 42 relapsed patients, 17 received a second course of immunosuppressive therapy with antithymocyte globulin and cyclosporine. Eight of these 17 patients responded within 6 months and are alive. Seven of nine non-responders to second immunosuppressive therapy received hematopoietic stem cell transplantation (HSCT) as salvage therapy. The hematopoietic stem cell donors were HLA-matched unrelated bone marrow donors (n=4), unrelated cord blood donors (n=2) and one matched sibling donor. Five of seven patients are alive following HSCT. Eleven patients underwent HSCT directly from an alternative donor (unrelated bone marrow donor, n=7; unrelated cord blood donor, n=1, HLA-mismatched family donor, n=3) and seven are alive. The estimated failure-free survival from the beginning of second-line therapy was 63.6% in the HSCT group compared with 47.1% in the groups treatment with repeated immunosuppressive therapy (P=0.96).

Table 1. Patients' pretreatment characteristics.

	Very severe AA	Severe AA	Non-severe AA
Registered	210	149	82
Sex (male/female)	115/95	83/66	47/35
Median age, years (range)	8.1 (0-17)	8.3 (1-17)	8.5 (2-16)
Etiology of AA			
Ideopathic	168	125	74
Hepatitis	37	21	7
Viral infection	2	1	0
Drug	3	2	1
Median days from diagnosis to treatment (range)	20.4 (1-146)	30.6 (1-180)	44.8 (3-180)
Study (AA-92/AA-97)	46/164	38/111	28/54
Response (complete/partial) (%)	128 (40/88) (61.0%)	91 (38/53) (61.1%)	45 (13/32) (54.9%)
Relapse (AA-92/AA-97)	6/8	9/5	11/3

Table 2. Risk factors for relapse in patients with aplastic anemia by univariate analysis.

Variable	Relative risk (97.5% CI)	P
Sex, male	0.977 (0.514-1.86)	0.94
Age	1.01 (0.947-1.08)	0.78
Etiology of AA		
Ideopathic	4.97 (1.22-20.2)	0.025
Hepatitis	0.234 (0.0577-0.952)	0.043
Duration of AA prior to initial treatment	1.01 (0.998-1.02)	0.11
Response at 90 days	1.07 (0.517-2.21)	0.86
Severity of disease		
Non-severe	2.98(1.40-6.34)	0.0047
Severe	1.21(0.561-2.63)	0.62
Very severe	1	
Study, AA-92 (Danazol+)	3.44 (1.78-6.65)	0.00023
G-CSF (+)	0.915 (0.363-2.31)	0.85
HLA-DR2	0.905 (0.307-2.67)	0.86

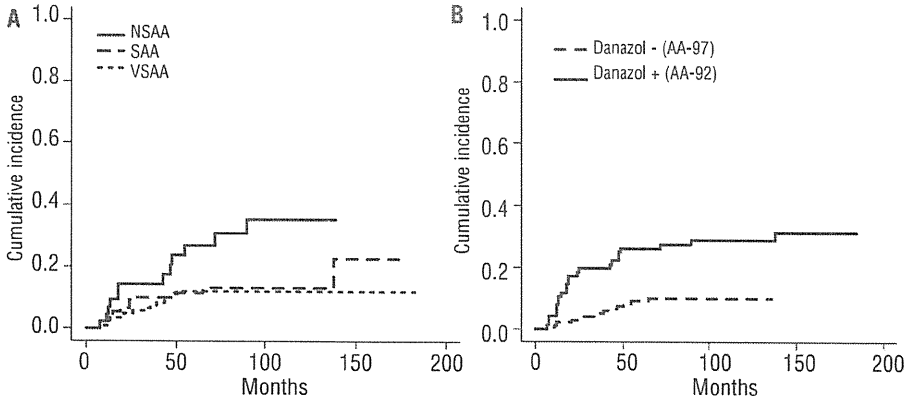

Figure 1. Cumulative incidence of relapse after immunosuppressive therapy in children with aplastic anemia. (A) The cumulative relapse rate of patients with non-severe aplastic anemia (NSAA) was significantly higher than that of patients with severe aplastic anemia (SAA) (P=0.025) and very severe aplastic anemia (VSAA) (P=0.005) 10 years after the first immunosuppressive therapy. (B) The actuarial risk of relapse at 10 years was significantly higher in the group treated with danazol (29.0%) than in the group not treated with danazol (9.8%) (P<0.001).

The overall survival rate did not differ between the immunosuppressive therapy group (84.7%) and the HSCT group (63.6%) after second-line treatment (P=0.07). Other patients were treated with cyclosporine alone (n=6) or bone marrow transplantation from a matched sibling donor (n=6). Two patients did not receive second-line treatments. One patient developed clonal evolution to myelodysplastic syndrome after 65 months, and the second developed acute myeloid leukemia after 37 months. Two patients showed clonal evolution to paroxysmal nocturnal hemoglobinuria after 138 months and 55 months. There were seven deaths among the 42 patients who initially relapsed. The causes of death were HSCT-related complications (n=5), acute myeloid leukemia (n=1) and bacteremia (n=1). The overall 10-year survival rates for patients with very severe AA, severe AA, and non-severe AA were 82.2±3.3%, 82.1±4.7% and 98.2±1.8%, respectively.

Discussion

Analysis of relapse in children with AA responding to immunosuppressive therapy will provide valuable information for the management of childhood AA. Here, we present the results of a comprehensive analysis of the largest consecutive series of AA children treated with standard immunosuppressive therapy. Relapse of AA after immunosuppressive therapy is relatively common, with actuarial risks of 30 - 40% having been reported.[16-18] In the present study, the cumulative incidence of relapse at 10 years was 11.9%, which is relatively low compared with that found in other studies that primarily involved adult patients.[16-18] Differences in the study populations may explain the discrepancy between the results of our current study and those of the other studies. A recent Italian study of childhood AA showed a 16% cumulative incidence of relapse, which is comparable with that found in our study.[10]

Multivariate analysis of the data from this retrospective multicenter study shows that the use of danazol was the most statistically significant risk factor for relapse. From 1992 to 2007, 441 children with newly diagnosed AA were treated with immunosuppressive therapy consisting of antithymocyte globulin and cyclosporine with (the AA-92 study) or without danazol (the AA-97 study). There are several reports of the efficacy of anabolic steroids in the treatment of AA. A randomized trial from the EBMT SAA working party demonstrated that the addition of an anabolic steroid (oxymetholone) to antithymocyte globulin treatment improved the response rate of patients with treated AA.[24] In our study, consistent with that report, the response rate at 6 months was higher in the patients who received immunosuppressive therapy with danazol (67.9%) than in the group of patients who received immunosuppressive therapy without danazol (57.1%). Furthermore, our results also showed that the cumulative relapse rate was significantly higher in the patients treated with immunosuppressive therapy plus danazol (Figure 1B). The reason danazol has an impact on relapse is unknown. However, it is possible that a number of cases with an androgen-responsive congenital bone marrow failure syndrome such as dyskeratosis congenita were hidden in our series of AA patients, and discontinuation of danazol was responsible for relapse. Recent reports have shown that a bone marrow failure syndrome of variable severity due to dyskeratosis congenita may be present in otherwise phenotypically normal individuals, and can masquerade as acquired AA.[19-22] We found mutations in the telomerase reverse transcriptase (*TERT*) gene, which is one of the genes causing dyskeratosis congenita, in two of 96 Japanese children with acquired AA.[23] Recently, more dyskeratosis congenita genes have been discovered. It is possible that more cases with an androgen-responsive dyskeratosis congenita were hidden in our series of AA patients. Alternatively, danazol may inhibit complete eradication of pathological T-cell clones by antithymocyte globulin through an unknown mechanism. Understanding the effects of androgens and developing androgen-mimetic drugs could be of significant benefit.

In our cohort of patients with non-severe AA, most patients were transfusion-dependent. In the AA-92 and AA-97 studies, 82 patients with non-severe AA were treated with the standard immunosuppressive regimen consisting of antithymocyte globulin and cyclosporine. Six months after the initial immunosuppressive therapy, 13 patients had achieved a complete response and 32 patients achieved a partial response. Among the 32 patients who achieved a partial response, 14 patients later relapsed. However, 18 patients with non-severe AA patients who achieved a partial response maintained their hematologic response, and 12 of them subsequently achieved a complete response. When childhood non-severe AA is treated with supportive care, 67% of patients progress to develop severe AA, suggesting that it is important to consider early immunosuppressive therapy.[24] Our data indicate that

immunosuppressive therapy is beneficial for some patients with non-severe AA.

A previous Japanese study showed that the addition of G-CSF to immunosuppressive therapy increased the hematologic response rate after 6 months and reduced the relapse rate in adult patients with severe AA.[25] Recently, Gurion *et al.* conducted a systematic review and meta-analysis of randomized controlled trials comparing treatments with immunosuppressive therapy with or without hematopoietic growth factors in patients with AA. The addition of hematopoietic growth factors did not affect mortality, response rate, or occurrence of infections, but did significantly decrease the risk of relapse.[26] The data from our AA-92 trial were included in this meta-analysis. In contrast to the other five studies in the meta-analysis, only our study included patients with non-severe AA, who had a significantly higher relapse rate than that of patients with either severe AA or very severe AA. Differences in the study populations may explain the discrepancy between the results of our current study and those of the other studies in the meta-analysis. To compare our results with the other studies, we excluded patients with non-severe AA from the statistical analysis, and compared the risk of relapse between patients who did or did not receive G-CSF. The results again showed no significant differences in the relative risk between them (RR=2.71, 97.5% CI 0.614 - 12.0, *P*=0.19).

The majority of patients who experienced relapse responded to reintroduction of immunosuppressive agents.[27] Our present study also demonstrates that a second course of immunosuppressive therapy was a safe and effective treatment for the patients who relapsed after the first immunosuppressive therapy. However, an optimal second immunosuppressive therapy regimen has not yet been established. Furthermore, about half of the relapsing patients eventually received HSCT in our study. The treatment choice was based on center-related preferences or on anecdotal evidence. A multicenter prospective study is warranted to establish optimal therapy for these patients.

Appendix

The following centers and persons participated in the Japan Childhood Aplastic Anemia Study Group: Japanese Red Cross Nagoya First Hospital (K. Kato); Kyoto Prefectural University of Medicine (S. Morimoto); Kobe University School of Medicine (Y. Takeshima); Hyogo College of Medicine (Y. Ohtsuka); Tokai University (H. Yabe); Shizuoka Children's Hospital (J. Mimaya); Fukushima Medical University (A. Kikuta); Tokyo Metropolitan Children's Medical Center, Tokyo (T. Kaneko); Osaka City General Hospital (J. Hara); Nagoya University (S. Kojima) ; Jichi Medical School (T. Yamauchi); Kagoshima University (Y. Kawano); Okayama University (M. Oda); Hokkaido University (R. Kobayashi); Hiroshima University (S. Nishimura); Kanazawa University (S. Koizumi); Keio University (T. Mori); Hiroshima Red Cross Atomic Bomb Hospital (K. Hamamoto); Chiba University (T. Sato); Hirosaki University (E. Ito); Teikyo University School of Medicine (F. Ohta); Tottori University (T. Kawakami); Dokkyo University School of Medicine (K. Sugita); Kumamoto National Hospital (K. Takagi); Seirei Hamamatsu Hospital (T. Matsubayashi); Hyogo Children's Hospital (Y. Kosaka); Yokohama City University (K. Ikuta); Yamaguchi University (H. Ayukawa); Kanagawa Children's Medical Center (T. Kigasawa); Hirakata City Hospital (C. Kawakami); Nakadohri General Hospital (A. Watanabe); Gumma Children's Hospital (T. Shitara); National Defence Medical College (I. Sekine); Gifu University School of Medicine (K. Isogai); Kumamoto University School of Medicine (S. Morinaga); University of Ryukyu (N. Hyakuna); Narita Red Cross Hospital (K. Sunami); Asahikawa Medical College (M. Yoshida); Nagoya City University (Y. Ito).

Authorship and Disclosures

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