DBA患者末梢血：健常人およびDBA患者末梢血単核球(MNC)をPercoll密度勾配法で遠心分離した。回収したMNCをPBSで2回洗浄した。1%SDS/PBSでタンパク質を抽出した。BCA protein assay kit (Promega)でタンパク質の濃度を測定した。抽出タンパク質は、SDS-PAGE sample buffer中でボイルした。

・Protein assay

10ugのタンパク質をSDS-PAGEで分離し、イモビロン膜(Millipore)に転写した。3%スキムミルク/TBS-Tでブロッキング後にそれぞれ抗RPS19抗体(1:100, Santacruz)、抗RPL11抗体(1:100, Santacruz)、抗GAPDH-HRP抗体(1:100, Santacruz)で4℃、overnight反応させた。2次抗体としてanti-mouse-HRP(1:10000, Santacruz)、anti-goat-HRP(1:10000, Santacruz)を室温1時間反応させた。Supersignal Weat Duraで発光させ、LAS3000でシグナルを検出した。

（倫理面への配慮）

患者末梢血の取り扱いについては、国立感染症研究所倫理員会および東京医科歯科大学倫理委員会の承認の上に患者の同意を得た場合に限り検体を研究に使用した。

C．研究結果

・DBA遺伝子の培養細胞発現抑制系における発現解析

RPS19、*RPL5*、*RPL11*およびコントロールとして*Luciferase*に対するshRNAを発現するレンチウイルスベクターを作製しMCF7細胞とU2OS細胞に感染させ、Dox誘導型shRNA発現細胞株を作製した。DoxでshRNAの発現を誘導後3－4日間培養し、抽出したタンパク質でWestern Blotting法(WB)によりそれぞれのタンパク質の発現量を解析した。その結果、RPL11に関して、shRNAの発現により顕著な発現量の減少が認められた。興味深いことにRPL5の発現抑制によってもRPL11のタンパク質量の減少が観察された。また、RPS19に関してもshRNA発現によってRPS19タンパク質の減少が観察された。これらのことからDBAの培養細胞モデル系でDBA原因遺伝子の遺伝子発現が抑制されるとタンパク質レベルでも発現量が減少することが確認された。（図1）

・末梢血単核球（MNC）のRPL11およびRPS19の発現解析

患者検体は採血されてから検査室に届くまでにおよそ1日が経過すると考えられる。DBAの原因遺伝子の産物であるRPL11およびRPS19について、採血からの時間経過とタンパク質発現量の変化を調べた。健常人から採取した血液を使い採血直後または採血後室温で1日保存後にMNCを分離し、抽出したタンパク質でWBによりそれぞれのタンパク質の発現量変化を解析を行った。その結果、RPL11については採血後1日の室温保存によって発現量は変化しなかったが、RPS19は室温で1日保存した場合、発現量が減少した。（図2）

・DBA患者MNCでのタンパク質発現解析

遺伝子コピー数解析の結果からRPL11のアレル欠損が疑われたDBA患者血液から抽出したMNCのタンパク質を用いてRPL11の発現解析を行った。その結果、RPL11の発現量は、健常人と比べ有意な差はなく遺伝子変異による発現量の減少は認められなかった。（図3）

D．考察

DBAの診断マーカーとしてDBAの原因遺伝子の発現量の減少を指標にできるかどうか、培養細胞を用いた遺伝子発現抑制系とMNCで解析した。培養細胞においてDBAの原因遺伝子発現を抑制した場合、タンパク質量の顕著な減少が観察され、DBAの培養モデル系において発現量の減少の検出は可能であると考えられた。

しかしながら、Primary細胞においてDBAの原因遺伝子産物のうちRPS19タンパク質は、採血後の時間経過に伴って発現量の低下が認められた。このことから、実際に患者から採血された検体が検査機関に送られてくるまでの保存期間の差によってRPS19の発現量が大きく左右されることが考えられた。

また、RPL11欠損のあるDBA患者全血から分離

したMNCからすぐにタンパク質抽出した場合では、健常人検体と比べRPL11のタンパク質量の減少は認められなかった。これは、MNCのほとんどが基本的にG0期(休止期)にあり細胞の遺伝子発現全体が低下していること、また遺伝子変異による発現量の低下の影響が正常アレルによって十分に補われている可能性があることが原因と考えられた。このため患者のMNCで原因遺伝子のタンパク質発現の減少を解析するには、培養細胞のように細胞周期を増殖期へと移行させる必要があると考えられた。

これらの結果から、患者の血液細胞で解析するには、MNCをIL-3やIL-2などのサイトカインを添加した培地で細胞増殖を促しながら数日間培養することで採血後の保存による細胞ストレスを除去し、また細胞周期を増殖期に移行させた状態で原因遺伝子タンパク質量の減少を検出する必要があると考えられた。現在MNCの増殖期の細胞での発現量解析を進めている。

E．結論

DBAの診断マーカーとして原因遺伝子のタンパク質量を測定することは、*in vitro*培養モデル系では可能であると予想されたが、患者のMNCからそのままタンパク質量の測定を行った場合、判定は困難であると考えられた。今後、血球分離後に一端培養するなど追加ステップを加える検討が必要であると考えられた。

F．健康危険情報

なし

G．研究発表

1．論文発表

なし

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H．知的財産権の出願・登録状況

なし

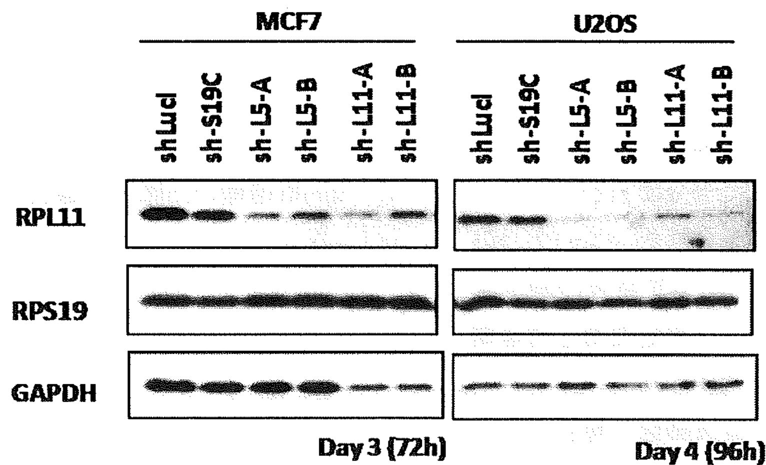


Figure 1. shRNA発現誘導細胞株におけるDBA原因遺伝子産物の発現解析
RPS19、RPL11、Luciferase (control)に対する発現誘導型shRNAを導入したMCF7およびU2OS細胞株にDoxycyclineでshRNA発現誘導後Day3およびDay4においてそれぞれのタンパク質発現をWestern Blotting法にて解析した。
RPL11およびRPS19に対するshRNAを誘導した場合、それぞれのターゲットとなるタンパク質の発現が減少を確認した。

Figure 2. 採血後の時間経過とRPL11およびRPS19の発現量変化
健常人の血液から採血直後または24時間室温保存後に末梢血単核球を分離し、RPL11およびRPS19の発現量を解析した。
RPL11の発現量に変化はなかったが、RPS19は24時間の保存でタンパク質発現量が減少した。

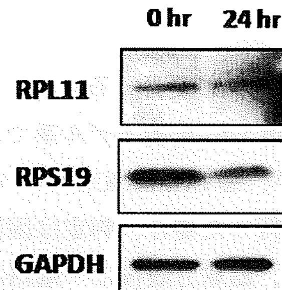
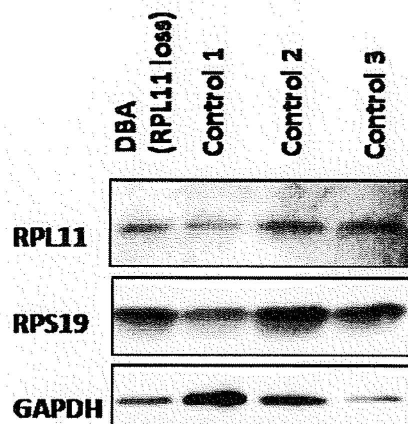


Figure 3. DBA患者末梢血単核球における原因遺伝子産物の発現解析
RPL11が欠損したDBA患者MNCより抽出したタンパク質でRPL11の発現を解析した。
MNCにおいては、患者に特異的なRPL11の発現量の減少は、認められなかった。



厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

先天性赤芽球癆（Diamond Blackfan貧血）の効果的診断法の確立に関する研究

Diamond Blackfan貧血診断法の開発

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研究要旨： Diamond-Blackfan貧血の簡便かつ高感度な診断法を確立するために、責任遺伝子産物のタンパクレベルでの解析、mRNAの定量を行えるシステム、FISHにより片アリル遺伝子欠失を同定できるシステムを構築した。今までの検討で責任遺伝子が明らかになっていない患者、新規患者から検体を収集し、1例においてRPS19遺伝を含む領域の片アリル欠損を示唆するデータを得た。

A．研究目的

DBAは、軽症例から最重症型まで広範囲な病像を示すことから、臨床所見のみで診断するのは容易ではない。本年は患者情報の詳細を把握すると共に、確定診断にいたるシステムの一環として、DBA責任遺伝子産物のmRNA及びタンパクレベルでの発現を検討できるシステムを開発する。

B．研究方法

1）遺伝子解析異常が判明していない患者検体の収集

DBA診療に当たる主要医療機関に連絡をとり、研究主任者の施設において遺伝子解析を行い、既知遺伝子に変異を認めなかった症例について、移植を行った症例については口腔粘膜スワブから核酸を、移植を行っていない症例についてはタンパク、核酸を抽出する。

2）新規変異解析法の確立

最も変異報告の多い*RPS19*について抗*RPS19*抗体でタンパク質発現量を測定し、バイオマーカーとしての有用性について検証する。また国立感染症研究所との共同作業により、*RPS19*を含む候補責任遺伝子の発現を定量化できるシステムを構築し、患者においての検査を試みる。

（倫理面への配慮）

本研究は、患者検体を用いて解析を行う。診療に役立つ情報が得られるが、採取量及び、採取時の苦痛には十分な配慮を行う。また遺伝子解析については各種指針に則り、患者個人情報の保護について十分な配慮を行う。

C．研究結果

1）遺伝子解析異常が判明していない患者検体の収集

8症例の情報を収集した。その中では全例において本班会議提唱するDBA診断基準を満たしていた。2症例において造血細胞移植が行われ、1症例においてはステロイドが投与されていた。また遺伝子解析がおこなわれていない新たな2症例も判明した。

2）新規変異解析法の確立

上記患者のうち造血細胞移植を行った1症例では口腔粘膜スワブより核酸を抽出した。まだ遺伝子解析がおこなわれていない1症例においては、RPS19 mRNAの定量を行い、1/2の発現量であることから片アリルのRPS19を含む領域の欠失であ

る可能性が示唆され、FISHプローブの構築を行った。

Western blottingについても国立感染症研究所に加えて本施設でも行える体制を構築したが、その定量化のために、Luminex法を利用可能かどうか検討を開始した。

D．考察

DBAの責任遺伝子解析では15%弱の患者においてのみその遺伝子異常が明らかになっている。ステロイドの使用、造血細胞移植などの治療方針決定の際に、その確定診断は極めて重要であるが、今回の解析により、タンパク発現欠損あるいは低下、mRNA定量、遺伝子領域欠損などが明らかになれば、さらに多くの患者において診断が確定し、診療上の福音となることが予測される。

今後さらに未検討患者においての解析を進める予定である。

E．結論

候補となる患者の検体を収集し、DBAをタンパク、mRNAレベルで診断できるシステムを開発した。

F．健康危険情報

なし

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H．知的財産権の出願・登録状況

１．特許取得

APPLICATION OF SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS (MSCs) FOR CARTILAGE OR MENISUCUS REGENERATION（米国国際特許出願中 YCT-1301）出願人：関矢一郎，発明者：宗田大，森尾友宏，清水則夫，黒岩保幸．

２．実用新案登録

該当なし

３．その他

該当なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

◎は本研究費によることが明記されている論文
○は本研究に関連する論文

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Ⅳ．研究成果の刊行物・別冊

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

by Yuki Konno, Tsutomu Toki, Satoru Tandai, Gang Xu, RuNan Wang, Kiminori Terui, Shouichi Ohga, Toshiro Hara, Asahito Hama, Seiji Kojima, Daiichiro Hasegawa, Yoshiyuki Kosaka, Ryu Yanagisawa, Kenichi Koike, Rie Kanai, Tsuyoshi Imai, Teruaki Hongo, Myoung-Ja Park, Kanji Sugita, and Etsuro Ito

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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 (DBA) is a rare congenital disorder, a clinically heterogeneous, red cellaplasia: 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 DBA patients.

Design and Methods

We screened 49 Japanese DBA patients (45 probands) for mutations in the 6 known DBA genes *RPS19, RPS24, RPS17, RPL5, RPL11,* and *RPL35A. RPS14* was also examined due to implication of its involvement in 5q- syndrome.

Results

Mutations in *RPS19, RPL5, RPL11* and *RPS17* were identified in 6, 4, 2 and 1 of the probands, respectively. In total, 13(29%) of Japanese DBA patients had mutations in RP 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 2 patients with *RPL5* mutations, and thumb anomalies were seen in 6 patients with an *RPS19* or *RPL5* mutation. In contrast, a small-for-date (SFD) phenotype was seen in 5 patients without an *RPL5* mutation.

Conclusions

We observed a slightly lower frequency of mutations in ribosomal protein genes in DBA patients when compared to previous reports from Western countries. Genotype-phenotype data suggest an association between anomalies and *RPS19* mutations, and a negative association between SFD and *RPL5* mutations.

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Introduction

Diamond-Blackfan Anemia (DBA, MIM#105650) 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 (1936) and refined as a distinct clinical entity by Diamond and Blackfan (1938). Recent study shows that the cellular defect in DBA fibroblasts is primarily caused by a 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 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] 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.[3-5] In addition, DBA patients have a predisposition to malignancy including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and osteogenic sarcoma.[3] Diagnosis of DBA is often difficult because incomplete phenotypes and wide variability of clinical expression are present.[4-6] The central hematopoietic defect is characterized by an enhanced sensitivity of hematopoietic progenitors to apoptosis along with evidence of stresserythropoiesis, including elevations in fetal hemoglobin and mean red cell volume (MCV).[2] The majority of patients exhibit 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 is comprised of 4 RNAs and 80 ribosomal proteins.[8] The first DBA gene, mutated in approximately 25% of DBA patients, was identified as *RPS19*, which is located at chromosome 19q13.2 and encodes the 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 encoding RPs of the small ribosomal subunits, *RPS24* and *RPS17*, have been found in approximately 2% of patients.[15,16] Furthermore, mutations in large ribosomal subunit-associated proteins genes, *RPL5*, *RPL11* and *RPL35A*, have been reported in 9% to 21.4%, 6.5% to 7.1%, and

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3.3% of patients, respectively.[17-19] To date, approximately 50% of DBA patients in Western countries have a single heterozygous mutation in a gene encoding a ribosomal protein.[1,3] These findings also implicate DBA as 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 RP genes associated with DBA in Japan. Eight patients were from multiplex families, 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 (TEC), 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 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.

RP gene analysis

DNA was extracted from peripheral blood using a standard proteinase K, phenol and chloroform protocol.[21] The 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). Analysis of *RPS19* was performed 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]

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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 correlation and statistical analysis

Physical abnormalities were evaluated from a viewpoint of correlations with genotype in Japanese DBA patients. Although growth retardation can be modified by several factors such as steroid therapy, chronic anemia, and iron overload, the patients were consideredpathognomonic for DBA if there was marked growth retardation below -3 standard deviation (SD). Response to treatment is usually seen within 1 month of treatment in DBA, but the prediction for response has not been reported previously.[1,3] We also examined the correlation between genotype and responsiveness 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 significant for $p < 0.05$. Data were analyzed with SPSS 11.0J software (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

A total of 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 8 patients were from 4 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 Japanese father.

Genetics

RPS19

Six different mutations were detected in 6 probands out of 45 families (13%) (Table 1). The median age at presentation of the index cases with *RPS19* mutations was 1 month (range, 0 to 4 months). There appears to be a lower percentage of *RPS19* mutation in Japanese DBA patients compared to patients in Western countries. All mutations were in the coding region of the gene. Missense mutations resulting in amino acid substitutions were noted in 5 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 7, 10 and 2 families,[6,10,11,22-26] whereas two mutations, p.D118G in case 20 and p.G95V in case 25, were novel, and could not be found in the Single Polymorphism Database (dbSNP at www.ncbi.nlm.nih.gov/SNP). Furthermore, these mutations were not observed in DNA from 50 normal individuals. An insertion of one nucleotide was found in 1 case (case 28), resulting in a novel frameshift mutation.

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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 initiationcodon.

The human *RPL11* gene, which consists of six exons, is also located on chromosome 1. Allexons 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 one case 23, each 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 fiveexons. *RPS17* mutations are rare and have been reported in only 2 DBA patients. A novel 1-nucleotide deletion in*RPS17* was identified in 1 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 parentsand diagnosed as DBA at a month old. 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 8 and 6 patients with DBA, respectively. DBA patients were screened for*RPS24* and *RPL35A*, in addition to *RPS14*, which is implicated in 5q- syndrome. No mutations were detected in*RPS24*, *RPL35A* or *RPS14* in Japanese DBA patients

In total, sequence changes were found in 4 out of 7 screened RP genes (Table 2). Mutations in*RPS19*, *RPS17*, *RPL5*, and *RPL11* were detected in 13%, 2%, 9%, and 4% of the probands, respectively. The frequency of RP gene mutations in Japanese DBA patients was 29%.

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Genotype-phenotype correlations

Congenital anomalies

Patient characteristics are summarized in Table 3. Anomalies associated with DBA were found in 27 patients (55%). Sixteen were affected with two or more malformations (33%). All 7 patients with an *RPS19* mutation had physical anomalies, and 4 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 multi-families (p.R62Q, p.R62W, p.0).[6,10,11,22-27] Comparable to previous observations, no consistent clinical features were found within 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 for developing malformations,[17,18] all 4 patients with *RPL5* mutations had physical anomalies. Furthermore, three of them had multiple physical anomalies, especially case 41, who had very severe congenital heart disease (**Table 3**). One of 2 patients with *RPL11* mutations had physical anomalies. In contrast, of the 35 patients with no mutations, physical anomalies were seen in 15 (43%).

Nine patients had craniofacial anomalies. Of these, one had *RPS19* mutations and 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] The Diamond-Blackfan Anemia Registry (DBAR) of North America also suggested that the DBA phenotype associated with cleft lip/palate is caused by non-*RPS19* mutations.[4] In our data, the frequency of cleft palate was significantly different between *RPL5*-mutated and *RPL5* non-mutated groups ($p < 0.05$): cleft palate was seen in 3 patients, 2 patients with *RPL5* mutations and only one patient with *RPL5* non-mutated patients.

Thumb anomaly was seen in 6 patients, 4 of whom had *RPS19* mutations while 2 had *RPL5* mutations. There was a statistically significant difference between *RPS19*-mutated and *RPS19* non-mutated groups in the frequency of thumb anomalies ($p < 0.05$). 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. SFD was seen in 7 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 were born SFD.