Table 3 Characteristics of Japanese DBA patients with mutations in *RPL5*

Patient	Malformation status	Response at first steroid therapy	Present therapy
10 proband	cleft palate, thumb anomaly, MR, rib defect, micrognathia, CHD	poor	Transfusion
41 proband	cleft palate, craniofacial abnormalities, CHD (GA, DORV, CoA, LSVC)	ND	Transfusion dependent
55 proband	thmub anomaly, polydactyly	response	Steroid dependet
65 proband	growth retardation	response	Steroid
73 proband	SFD	response	Steroid dependet
74 proband	None	None	Transfusion dependent

ちの2例は重症多発奇形を合併していた（Table 3）．注目すべきことに，口蓋裂を2例に認めた．欧米からの報告でも，*RPL5* 変異の症例は多発奇形の合併が多く，特に口蓋裂，口唇裂を合併することが多いことが示されていたが，今回の解析はそれを裏付ける結果であった[15,16]．

ステロイドは，DBA の治療の根幹であるが，評価可能な症例 61 例のうち，ステロイドに対する初回反応は 73% に見られた．しかし，遺伝子変異による差は認められなかった．

4. DBA の遺伝子コピー数解析法による RP 遺伝子の片アレルの欠失

欧米に比して本邦における RP 遺伝子の変異頻度は低く，約 70% の症例では原因遺伝子が不明であることが明らかになった．しかし，片アレルの大きな欠失がある場合，通常のシークエンス解析では，残った正常のアレルの塩基配列のみを読んでしまうため遺伝子異常を検出できない．そこで，Kuramitsu らは，独自に簡便かつ迅速な DBA の遺伝子コピー数解析法（DBA 同期的 q-PCR 法）を開発し，片アレルの欠失の同定を試みた[18]．この方法では，まず，遺伝子上の複数箇所に同一条件で増幅反応ができる PCR プライマーを設定する．もし，大きな欠失が存在し N の状態になっていると，正常の 2N に比べ DNA 量が半分であるため，2N と同じ増幅を得るのに PCR の反応回数が 1 回多く必要になることを利用して，片アレルの欠失を同定する（Figure 1）．例えば，症例 3 では，*RPL5* のみが正常に比べ，1 サイクルずれていることから，*PRL5* の片アレルの欠失が推定される．DBA で高頻度に変異報告のある遺伝子として，*RPL5*, *L11*, *L35A*, *S7*, *S10*, *S17*, *S19*, *S24*, *S26* の 9 遺伝子，また，DBA の原因としての重要度は未だ不明であるが論文や学会等において変異報告のあった遺伝子，*RPL9*, *L19*, *L26*, *L28*, *L36*, *S15*, *S27A* の 7 遺伝子につい

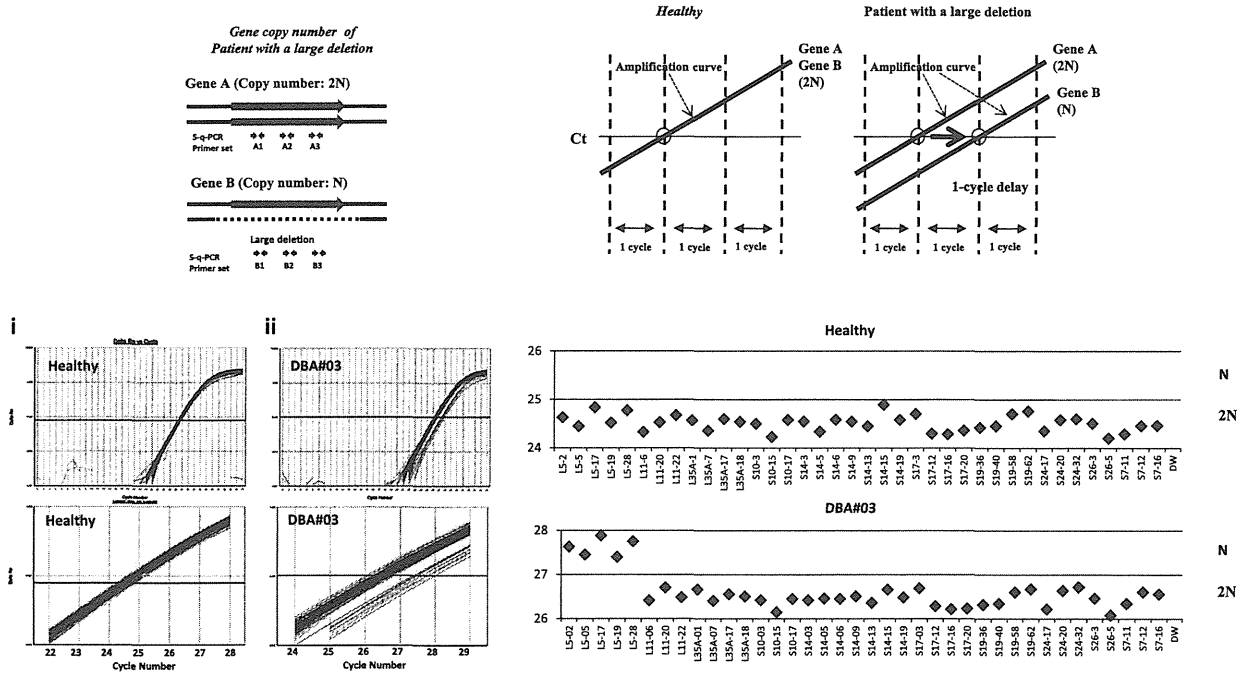

Figure 1 DBA Genomic Copy Number Assay

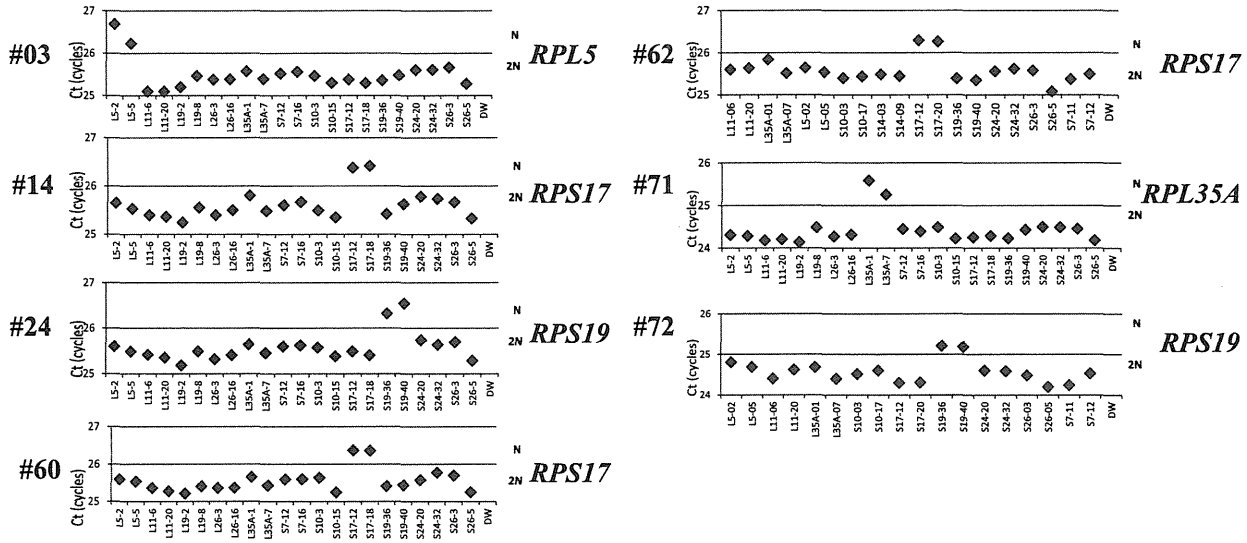

Figure 2　DBA Genomic Copy Number Assay

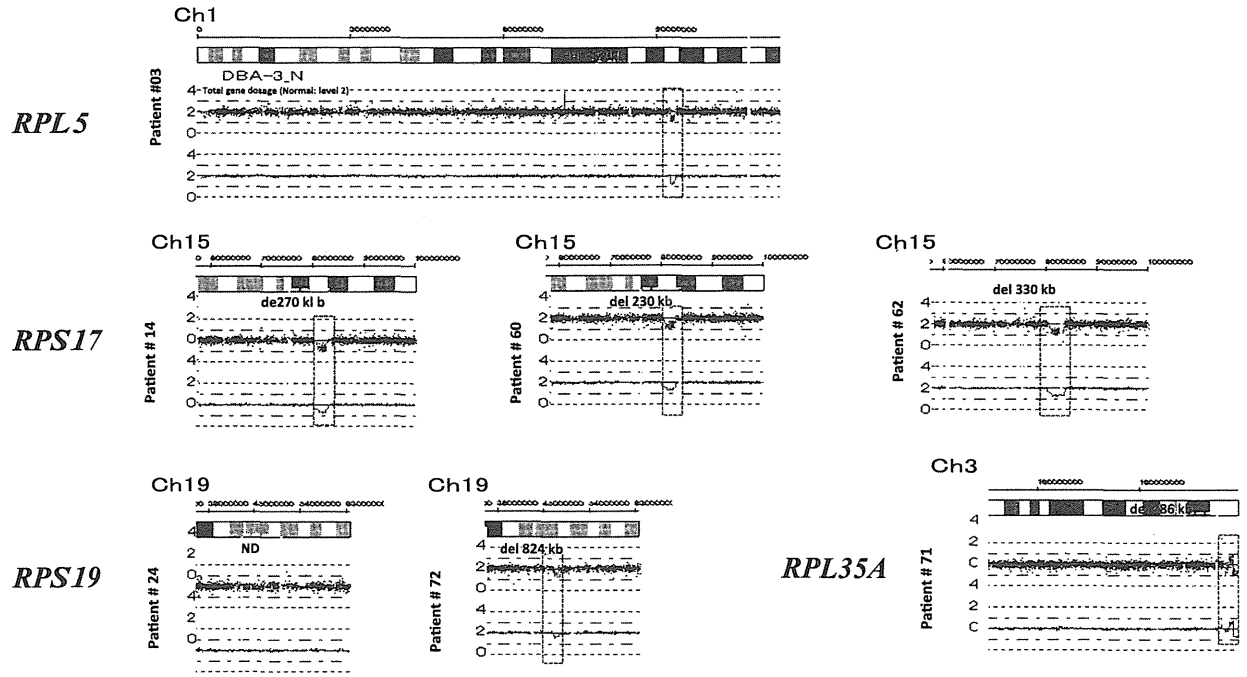

Figure 3　SNP Array

て，遺伝子コピー数測定用のq-PCRプライマーを設定した．弘前大学においてシーケンス解析の結果，変異が検出されなかった検体について，上記の遺伝子の遺伝子コピー数を測定した．その結果，27例中7例で特定の1遺伝子に対するプライマーセット全てが他の遺伝子の増殖曲線のCt値から1サイクルの遅れを示した（Figure 2）．つまり，これら1サイクル遅れた遺伝子は，その他の遺伝子（2N）のコピー数の半分（N）であることを示しており，片アレルの欠失が示された．7例の内訳は，*RPS17*欠失（3例），*RPS19*欠失（2例），*RPL5*欠失（1例），*RPL35A*欠失（1例）であった．

5. SNPアレイによる片アレル欠失の解析

上記の結果を確認するために，SNPアレイ（Affymetrx Gene Chip）とCNAGプログラムで遺伝子コピー数を測定した．その結果，27例中6例でRP遺伝子の片アレル欠損が確認された（Figure 3，Table 4）．これらは，q-PCRの結果と一致していたが，q-PCR法で検出された*RPS19*遺伝子

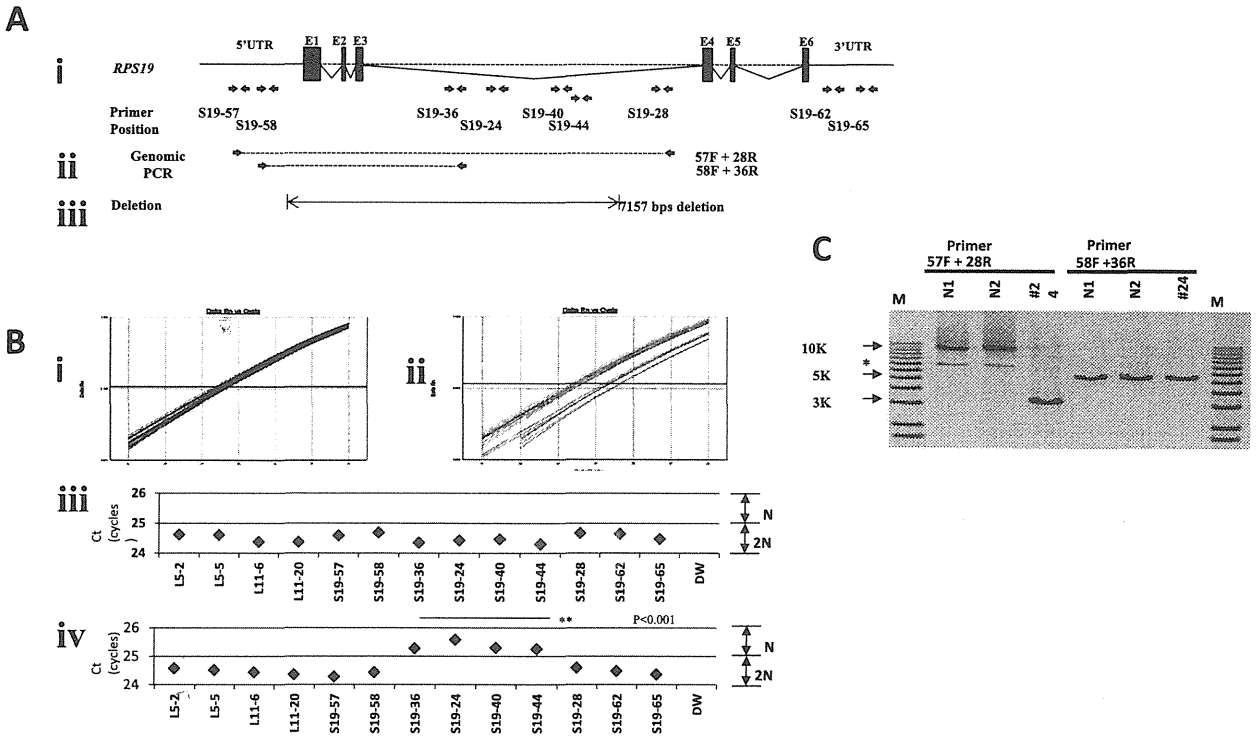

Figure 4　Large deletion in the *RPS19* gene

の大欠失（#24）は検出されなかった（Figure 3）．そこで，*RPS19* に対する q-PCR プライマーを増やし，*RPS19* の遺伝子コピー数を詳細に調べた（Figure 4A）．その結果，*RPS19* の 5'UTR 領域（S19-57, -58）および intron 3 内部から 3'UTR 領域に設定したプライマー（S19-28, -62, -65）ではコピー数が正常値を示したのに対して，intron 3 の内部より上流に設定されたプライマー（S19-36,-24, -40, -44）では片アレルの欠失を示した（Figure 4B）．このことから，*RPS19* 遺伝子の片方のアレルの intron 3 領域付近で小規模な欠失があることが考えられた．そこで，*RPS19* の 5'UTR と intron 3 にプライマーを設定し genomic PCR を行い，欠失領域の同定を試みた．その結果，#24 検体では約 7k 塩基対の欠失が想定されるバンドが電気泳動上で認められた（Figure 4C）．シーケンス解析の結果，5'UTR と intron 3 にある 23 塩基対の相同配列（CGGTGGCTCACACCTGTAATCCCAGCA, nt: −1400 から −1374 および nt: +5758 から +5784）の間で分子内相同組換えが生じ，結果として 7157 塩基の欠失が同定された（Figure 4A）．欠失した領域には，プロモーターおよび exon 1, 2, 3 が含まれることから，このアレルは正常な RPS19 タンパク質をコードしないと考えられた．また，SNP アレイの結果が，q-PCR の結果と一致しなかったのは，SNP プローブが欠失領域に存在しないためであった．

以上の結果より，s-qPCR を用いた片アレル欠失の解析法は，優れた方法であることが示された．

6. 大欠失と表現型

我々が同定した 7 例の DBA の原因遺伝子の片アレル欠失は，その多くがこれまでに報告のないものであった（*RPL5*（1 例），*RPS17*（3 例））．また，今回の解析では，*RPS17* の遺伝子異常が 3 例発見され，これまで欧米では極めて稀とされていたが，興味深いことに日本ではむしろ高頻度で *RPS17* に変異があることが明らかになった．片アレル欠失を有する症例は，共通の表現型を有し，7 例全例が，子宮内発育遅延や低身長などの発育遅延を有していた（Table 5）．欠失を示した 6 検体（#3, #24, #60, #62, #71, #72）について，両親の欠失を同様に DBA 同期 q-PCR 法で調べた．その結果，全ての両親で DBA 原因遺伝子の欠失は認められなかった．つまり，片アレルの欠失の場合は家族性ではなく，孤発性の変異タイプである可能性が示唆された．

7. 未知の遺伝子の検索

上記の解析を行っても原因遺伝子が不明な臨床検体について，次世代シークエンサーを用いた全エクソン解析を行い，新規原因遺伝子の同定を開始した．既知の SNP を除去した後，家族内罹患者や陰性コントロール（非罹患同胞や両親）の全エクソンデータと比較検討することにより，責任変異の候補となる遺伝子を絞り込み，得られた候補遺伝子の変異は，サンガーシークエンス法を用いて確認を行

Table 4 Genes that were found to be deleted using SNP arrays

Patient No.	Large deletion (Q-PCR)	Large deletion (SNP array)	Deletion length	Mutation	Genes deleted
#3	*RPL5*	*RPL5*	858 kb	whole allele loss	*EVI5, RPL5, FAM69A, MTF2, TMED5, CCDC18, DR1*
#14	*RPS17*	*RPS17*	270 kb	whole allele loss	*RPS17, CPEB1, AP3B2, FSD2, WHMM, HOMER2, FAM103A1, C15orf40, BTBD1, TM65F1, HDGFR3, BNC1, SH3GL3, ADAMTSL3, ZSCAN2, SAND2, WDR73, NMB*
#24	*RPS19*	*ND*	7157 bp	Ex 1-3 deletion	*RPS19*
#60	*RPS17*	*RPS17*	230 kb	whole allele loss	*RPS17, CPEB1, AP3B2, FSD2, WHMM, HOMER2, FAM103A1, C15orf40, BTBD1, TM65F1, HDGFR3, BNC1, SH3GL3, ADAMTSL3, ZSCAN2, SAND2, WDR73, NMB*
#62	*RPS17*	*RPS17*	330 kb	whole allele loss	*RPS17, CPEB1, AP3B2, FSD2, WHMM, HOMER2, FAM103A1, C15orf40, BTBD1, TM65F1, HDGFR3, BNC1, SH3GL3,ADAMTSL3, ZSCAN2, SAND2, WDR73, NMB, SEC11A, ZNF592, ALPK3, SLC28A1, PDE8A*
#71	*RPL35A*	*RPL35A*	786 kb	whole allele loss	*BDH, FYTTD1, LRCH3, IQCG, RPL35A, LMLN, FAM157A*
#72	*RPS19*	*RPS19*	824 kb	whole allele loss	*CEACAM5, CEACAM6, CEACAM3, LYPD4, DMRTC2, RPS19, CD79A, ARHGFF1, RABAC1, ATP1A3, GRIK5, ZNF574, POU2F2, DEDD2, ZNF526, GSK3A, ERF, CIC, PAFAH1B3, PRR19, TMEM145, MEGF8, CNFN, LIPE*

Table 5 Characteristics of DBA patients with large deletions

	Patients (Gender)	Inheritance	Age at diagnosis	Physical anamalies
RPS19	24 proband (F)	Sporadic	1 M	Short stature, SGA
	72 proband (M)	Sporadic	0 Y	Thumb anomaly, Flat thenar, testicular hypoplasia, Fetal dydrops
RPS17	14 proband (M)	sporadic	5 Y	Short stature, white spots
	60 proband (F)	sporadic	2 M	SGA
	62 proband (F)	sporadic	1 M	Small ASD, SGA, short stature
RPL5	3 proband (M)	Sporadic	0 Y	Short stature, thumb anomalies
RPL35a	71 proband (M)	Sporadic	0 Y	Thumb anomalies, synostosis of radius and ulna, Cohelia Lange-like face, cleft plate, underdescended testis, short stature, cerebellar hypoplasia, fetal dydrops

う．これまで報告されているDBAの原因遺伝子はすべてリボソーム蛋白遺伝子であったが，全エクソン解析を行った検体の中には，約80種類存在するリボソームタンパク遺伝子に変異のないものが多数みられた．この結果から，リボソームタンパク遺伝子以外のDBA原因遺伝子が存在することが強く示唆された．効率的に原因遺伝子を同定するために，まず，家族内に複数の罹患者がある症例を中心に検索を進める予定である．

8. バイオマーカーの発見

DBAの確定診断にはリボソームタンパク遺伝子変異の同定が有用だが，原因遺伝子が多数あること，遺伝子変異が同定される症例が未だ40～50%であることが大きな問題である．生化学的バイオマーカーとして，赤血球アデノシンデアミナーゼ（eADA）の上昇が知られているが，臨床像，血液学的所見でDBAが強く疑われる症例の10～20%ではeADAが基準値内であり，より高感度にDBAを診断出来る新規バイオマーカーが必要と考えられていた．今回，菅野らは赤血球内の分子XがDBA患者で有意に高値であることを発見し，eADAと併用することで，検討した9家系11例のDBA患者を100%診断し得た（未発表）．このことから，XはDBA診断における新規バイオマーカーとして，極めて有用と考えられた．

IV 結　語

日本のDBA患者で原因遺伝子の片アレル欠失解析を行った結果，DBAでは高頻度（27例中7例）で原因遺伝子の片アレルの欠失が認められた．この結果から，DBAの確定診断のためには，これまで行われてきたシーケンス解析に加えて，コピー数解析を行うことが重要であることが示された．また，遺伝子変異同定率が上昇することによって，日本のDBAの遺伝子型と貧血・奇形等の表現型との相関をより詳しく解析することができるようになると思われる．

DBAに対する治療は副腎皮質ステロイドが第一選択であるが，ステロイド依存や輸血依存症例が全体の約40%存在する．難治例には造血幹細胞移植が行われているが，

本疾患では家族内に発端者と同一の遺伝子異常をもっていても貧血のない軽症例が存在することが知られている．本症は悪性疾患を合併しやすいことから，造血幹細胞移植のドナーを選択する上で軽症例の診断は重要課題になっている．しかし，軽症例も含めたDBAの診断基準の作成は海外でも検討が始まったばかりであり，我が国でも利用できるような診断基準は存在しない．今回のバイオマーカーの発見は，骨髄移植ドナーの選定をする上で非常に重要な情報を与えてくれると思われる．

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The carboxyl-terminal region of erythroid-specific 5-aminolevulinate synthase acts as an intrinsic modifier for its catalytic activity and protein stability

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Erythroid-specific 5-aminolevulinate synthase (ALAS2) is essential for hemoglobin production, and a loss-of-function mutation of ALAS2 gene causes X-linked sideroblastic anemia. Human ALAS2 protein consists of 587 amino acids and its carboxyl(C)-terminal region of 33 amino acids is conserved in higher eukaryotes, but is not present in prokaryotic ALAS. We explored the role of this C-terminal region in the pathogenesis of X-linked sideroblastic anemia. In vitro enzymatic activity was measured using bacterially expressed recombinant proteins. In vivo catalytic activity was evaluated by comparing the accumulation of porphyrins in eukaryotic cells stably expressing each mutant ALAS2 tagged with FLAG, and the half-life of each FLAG-tagged ALAS2 protein was determined by Western blot analysis. Two novel mutations (Val562Ala and Met567Ile) were identified in patients with X-linked sideroblastic anemia. Val562Ala showed the higher catalytic activity in vitro, but a shorter half-life in vivo compared to those of wild-type ALAS2 (WT). In contrast, the in vitro activity of Met567Ile mutant was about 25% of WT, while its half-life was longer than that of WT. However, in vivo catalytic activity of each mutant was lower than that of WT. In addition, the deletion of 33 amino acids at C-terminal end resulted in higher catalytic activity both in vitro and in vivo with the longer half-life compared to WT. In conclusion, the C-terminal region of ALAS2 protein may function as an intrinsic modifier that suppresses catalytic activity and increases the degradation of its protein, each function of which is enhanced by the Met567Ile mutation and the Val562Ala mutation, respectively. © **2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

5-Aminolevulinate synthase (ALAS) is the first and rate-limiting enzyme in the heme biosynthetic pathway [1]. There are two isozymes of ALAS in higher eukaryotes, ALAS1 and ALAS2. ALAS1 (alternatively, ALAS-N) is expressed ubiquitously in all types of nucleated cells, and expression of ALAS2 (or ALAS-E) is restricted in erythroid cells and essential for hemoglobin production during erythroid differentiation [1]. Both ALAS1 and ALAS2, which are encoded by the distinct nuclear genes, function in mitochondria [2,3], and the amino-terminal region of each isozyme acts as a targeting signal for mitochondrial translocation [4–6]. The remaining regions of ALAS protein consist of a core catalytic region and a carboxyl terminal (C-terminal) region, and the catalytic region is conserved among several species [7]. In addition, the C-terminal region of 33 amino acids (positions 555–587), which is encoded by the 11th exon of the human ALAS2 gene, is well conserved in higher eukaryotes, but the equivalent region is not present in bacterial ALAS [7]. It is conceivable that the C-terminal region of mammalian ALAS2 protein might have an important regulatory role in heme biosynthesis.

The human ALAS2 gene that is mapped on X chromosome consists of 11 exons [8], and a genetic mutation of the ALAS2 gene causes X-linked sideroblastic anemia (XLSA) [9,10] or X-linked dominant protoporphyria [11]. To the best of our knowledge, >50 different mutations of

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the ALAS2 gene have been identified in about 100 pedigrees with XLSA [12–14]. Reported mutations in patients with XLSA are distributed from the 5th exon to the 11th exon of the human ALAS2 gene, but only four mutations were detected in 11th exon [14–17]. In the case of X-linked dominant protoporphyria, two different frame-shift mutations have been identified in the 11th exon of the ALAS2 gene in two independent probands [11]. These frame-shift mutations cause deletions of 19 and 21 amino acids at the C-terminal end of ALAS2, both of which are accompanied by replacement of the C-terminal end with one unrelated amino acid and an unrelated peptide of 23 amino acids, respectively. Using recombinant proteins expressed in *Escherichia coli*, those authors provided evidence that deletion of 19 or 21 amino acids at C-terminal end increased the catalytic activity of ALAS2, suggesting that the C-terminal region can inhibit the enzymatic activity of ALAS2 [11]. Recently, it was also reported that the substitution (Tyr586Phe) at the penultimate amino acid of the C-terminal of ALAS2 increased its catalytic activity in vitro, which might be related to the severe phenotype of congenital erythropoietic porphyria [18]. Interestingly, such gain-of-function mutations of the ALAS2 gene were solely identified within the C-terminal region of ALAS2 protein. However, it is still unclear how the C-terminal region of ALAS2 is involved in the regulation of ALAS2 function in vivo.

Here, we report novel missense mutations in the 11th exon of the ALAS2 gene in independent probands with XLSA. Based on in vitro and in vivo functional studies of these mutants, as well as a C-terminal deletion mutant, we provide evidence that the C-terminal region of human ALAS2 protein reduces its catalytic activity and protein stability in mitochondria.

Case reports

Case 1

Japanese male proband presented with microcytic hypochromic anemia (hemoglobin: 8.1 g/dL; mean corpuscular volume: 57.7 fL) at age 14 years. Serum ferritin, serum iron, and total iron binding capacity were 222.7 ng/mL, 242 μg/dL, and 279 μg/dL, respectively. Proband's mother and maternal uncles had mild anemia, but they did not receive any medication for anemia.

Bone marrow examination of the patient showed erythroid hyperplasia (myeroid to erythroid ratio [M:E] = 0.45), with ringed sideroblasts comprising > 10% of nucleated cells. Pyridoxine treatment (80 mg/d) was started, and the hemoglobin concentration gradually increased from 7.3 g/dL to 12.0 g/dL after 14 months.

Case 2

Japanese male proband was admitted to the hospital at age 36 years because of microcytic hypochromic anemia (hemoglobin: 6.5 g/dL; mean corpuscular volume: 64.4 fL) with systemic iron overload (ferritin: 2581.4 ng/mL). Anemia was pointed out before he was school age, but he did not receive any medication for anemia. Prussian blue staining of bone marrow cells revealed the presence of ring sideroblasts in the proband, and the diagnosis of sideroblastic anemia was established. Pyridoxine treatment (60 mg/d) was started when hemoglobin was 5.4 g/dL, then anemia was improved after 1 month to 9.9 g/dL hemoglobin. Although pyridoxine treatment was continued for an additional 4 months, the hemoglobin level did not exceed 10 g/dL.

Materials and methods

Reagents

Chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), Nacalai Tesque (Kyoto, Japan), or Wako Pure Chemicals (Osaka, Japan). Restriction enzymes and modifying enzymes used for construction of each plasmid were purchased from New England Biolabs (Ipswich, MA, USA), unless otherwise noted. ExTaq DNA polymerase and PrimeStar Max DNA polymerase were purchased from Takara Bio Inc. (Shiga, Japan) and were used for polymerase chain reaction (PCR) and site-directed mutagenesis, respectively. Protein concentration was measured with Bio-Rad Protein assay reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) or Pierce 660 nm Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as a standard. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and Western blot analysis were performed as described previously [19]. Prestained XL-ladder broad range (APRO Science, Tokushima, Japan) was loaded as a size marker for SDS-PAGE and Western blot analysis.

Identification of ALAS2 mutations

Genetic analyses performed in this project had been approved by the ethical committee of Tohoku University School of Medicine. Blood samples were drawn from the probands and the family members after informed consent. Genomic DNA was then extracted from them using QIAamp DNA Blood Midi Kit (Qiagen GmbH, Hilden, Germany). All exons including exon-intron boundaries, the proximal promoter region, and the erythroid enhancer in intron 8 of ALAS2 gene were amplified using ExTaq DNA polymerase. Sequences of primers and the condition for PCR were reported previously [20], except for an antisense primer for exon 5 and a primer pair for the erythroid-specific enhancer region in intron 8. The sequence of antisense primer for exon 5 used is (5′-TCATCTCCTCTGGCCACTGC-3′). For the amplification of the erythroid-specific enhancer in intron 8, the following primers were used: sense, 5′-GGTACCACTCGCATCCCACTGCA GAG-3′and antisense, 5′-GGTACCACACAGCCAAAGGCCTT GCC-3′. Each amplified DNA fragment was electrophoresed on 1% agarose gel in TAE buffer and stained with ethidium bromide. DNA fragment was excised from the gel for purification using QIAquick Gel Extraction Kit (Qiagen GmbH). Purified DNA fragment was directly sequenced using BigDye terminator v1.1 cycle sequencing kit and ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The same primers were used for PCR and direct sequencing analysis. Sequencing results were

analyzed using Lasergene software (DNASTAR Inc., Madison, WI, USA), and the mutation of ALAS2 gene was confirmed by repeated amplification and direct sequencing.

Expression and purification of recombinant ALAS2 proteins

Complementary DNA for human mature ALAS2 that lacks the amino-terminal region was amplified with PrimeStar Max DNA polymerase (Takara Bio Inc.) using the following primers (sense, 5′-GGTGGTCATATGATCCACCTTAAGGCAACAAAGG-3′; antisense, 5′-GGCATAGGTGGTGACATACTG-3′), each of which was phosphorylated at its 5′ end beforehand. Amplified complementary DNA (cDNA) was digested with NdeI restriction enzyme, and was cloned between NdeI site and blunt ended SapI site of pTXB1 expression vector (New England Biolabs). Resulting plasmid, named as pTXB1-AEm, expresses human mature ALAS2 in *E. coli* as a fusion protein with Intein tag and Chitin binding domain at its C-terminal end. Using pTXB1-AEm as a template, each mutation or deletion was introduced using PrimeStar Max site-directed mutagenesis kit (Takara Inc.). The sequences of primers used for mutagenesis are available upon request. After the amplification of cDNA or mutagenesis, the sequence of mature ALAS2 cDNA and the junction sequence for fusion protein was confirmed by DNA sequencing before use. These expression vectors were used for transformation of the *E. coli* strain, BL21(DE3). Expression and purification of recombinant proteins were performed according to manufacturer's instruction for Impact System (New England Biolabs), with minor modifications. Briefly, expression of recombinant proteins was induced in *E. coli* with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at 25°C for overnight. The isopropyl β-D-1-thiogalactopyranoside–treated cells were collected by centrifugation and resuspended with lysis buffer (20 mM Tris-HCl [pH 8.5], 300 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL antipain, 1 μg/mL pepstatin, and 1 μg/mL leupeptin). After sonication and centrifugation, cleared cell lysates were incubated with Chitin beads for 1 hour at 4°C, and then washed with wash buffer (20 mM Tris-HCl [pH 8.5], 500 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100). To obtain a tag-free recombinant mature ALAS2 protein, on-column cleavage was induced with 50 mM dithiothreitol in wash buffer at room temperature for 16 hours. After the elution from the column, each recombinant protein was dialyzed against wash buffer before use. Purity of each recombinant protein was examined using SDS-PAGE, followed by staining with Quick-CBB PLUS (Wako Pure Chemical). Enzymatic activity of each recombinant protein was measured according to the protocol described previously [21]. Student's *t* test was performed for statistical analysis.

Expression of wild-type or mutant ALAS2 protein in eukaryotic cells

The plasmid "pGEM-AET," which carries cDNA for full-length ALAS2 tagged with FLAG at its C-terminal, was described previously [22]. Site-directed mutagenesis was performed by PrimeStar Max mutagenesis kit (Takara Inc.) using pGEM-AET as a template to obtain cDNA encoding each FLAG-tagged mutant. In addition, cDNA encoding FLAG-tagged luciferase protein was constructed by replacing ALAS2 cDNA in pGEM-AET with amplified luciferase cDNA derived from pGL3 basic (Promega Corporation, Madison, WI, USA).

For establishing the stable transformants in which expression of FLAG-tagged ALAS2 protein or FLAG-tagged luciferase protein is inducible with tetracycline, cDNA for each protein was cloned into pcDNA5/FRT/TO vector (Invitrogen Corporation, Carlsbad, CA, USA). The resulting cDNA construct was then co-introduced with pOG44 vector into Flp-In T-REx 293 cells (Invitrogen), derived from human embryonic kidney cells (HEK293). After transfection, cells were incubated with 100 μg/mL Hygromycin B (Wako Pure Chemicals) and 15 μg/mL Blasticidin (Invitrogen). At least three independent clones, which were resistant to Hygromycin B and sensitive to Zeocin (Invitrogen), were selected and expanded for subsequent experiments. This phenotype of a given clone confirmed the integration of each cDNA expression cassette into the expected site in the genome of Flp-In T-REx 293 cell line.

For the determination of protein stability, expression of wild-type ALAS2 or mutant ALAS2 was induced by the addition of tetracycline into the culture medium (final concentration of 1 μg/mL) for 48 or 72 hours, and then the culture medium was replaced with fresh complete medium containing tetracycline with or without 10 μM cycloheximide. At 0, 3, 6, 9, and 12 hours after incubation, cells were harvested and lysed in RIPA buffer (10 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% TritonX-100, 1 mM sodium fluoride, 0.4 mM Na_3VO_4, 10 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL aprotinin). Cell lysates were centrifuged at 13,200*g* for 10 minutes at 4°C, and the supernatants were used for SDS-PAGE. Expression of FLAG-tagged ALAS2 protein was detected by Western blot analysis with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) as a first antibody. For normalization of loaded samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with anti-GAPDH monoclonal antibody (MAB374; Millipore Corporation, Billerica, MA, USA) as a first antibody. For a second antibody, horseradish peroxidase–conjugated anti-mouse IgG (NA931VI GE Healthcare, UK Limited, Buckinghamshire, UK) was used. Intensity of each band was measured using ImageJ software (available at http://rsb.info.nih.gov/ij/). The intensity of each band for FLAG-tagged ALAS2 was normalized with that of GAPDH, and the normalized intensity of FLAG-tagged ALAS2 at each time point was compared with that of the sample harvested at 0 hour. We repeated this series of experiments three times for each clone, and an average of these results was used for determination of the half-life of each protein.

The catalytic activity of each mutant protein was also evaluated by comparing the accumulation of porphyrins in Flp-In T-Rex 293 cells that expressed wild-type or mutant ALAS2 cDNA in an inducible manner. For this assay, cells of low-passage numbers (between passage 5 and passage 15) were used for obtaining reproducible results. To induce expression of wild-type ALAS2 or mutant ALAS2 protein in isolated cell lines, cells were treated for 60 hours with tetracycline at a suitable concentration (12.5–50 ng/mL), depending on cell lines. Then, cells were washed with phosphate-buffered saline twice and collected in the sample tube. Flp-In T-REx 293 cells, which express FLAG-tagged luciferase protein in an inducible manner, were also treated with tetracycline as a negative control. Cells were separately collected for Western blot analysis and RNA preparation. Real-time PCR analysis was performed as described previously [23]. Remaining cells were collected by centrifugation and then packed cells were exposed to ultraviolet light for detection of porphyrins.

Table 1. Summarized features of recombinant ALAS2 proteins

Recombinant protein	In vitro enzymatic activity (nmol ALA/mg protein/h)			Half-life in HEK293 cells (h)	Porphyrin accumulation in HEK293 cells
	Without PLP (% of wild-type)	With 200 μM PLP (% of wild-type)	Ratio with/without PLP		
Wild-type	14,824 ± 754 (100%)	27,627 ± 713 (100%)	1.86	7.8	+++
Val562Ala	22,324 ± 1,555 (150.6%)	32,300 ± 709 (116.9%)	1.44	5.3	++
Met567Ile	5,653 ± 897 (38.1%)	6,975 ± 299 (25.2%)	1.23	>12	±
Ser568Gly*	(19.5%)*	(31.6%)*	2.51*	>12	±
delC33	15,769 ± 382 (106.4%)	53,066 ± 1,843 (192.1%)	3.37	>12	++++

*Data with GST-fused Ser568Gly protein taken from reference [15].

Results

Identification of novel mutations of ALAS2 gene

Analyzing the genomic DNA extracted from the proband of case 1, we identified the c.T1685C mutation in the 11th exon of ALAS2 gene (Supplementary Figure E1A, upper panel; online only, available at www.exphem.org). This transition results in an amino acid substitution at the 562nd residue of ALAS2 protein from valine to alanine (Val562Ala). The same mutation was identified in one allele of the proband's mother (Supplementary Figure E1A, middle panel; online only, available at www.exphem.org), and the proband's father does not carry this mutation (Supplementary Figure E1A, lower panel; online only, available at www.exphem.org), indicating the X-linked inheritance of this mutation. For the proband of case 2, the c.G1701C transversion was identified in exon 11 of ALAS2 gene (Supplementary Figure E1B; online only, available at www.exphem.org), the mutation of which results in an amino acid substitution at the 567th residue from methionine to isoleucine (Met567Ile).

To exclude the possibility that these mutations represent single nucleotide polymorphisms, we examined the 11th exon of ALAS2 gene in 96 Japanese healthy volunteers (57 male and 39 female subjects, with the total allele number of 135) using PCR followed by direct sequencing. As a result, no base change was found in the 11th exon of ALAS2 gene in these subjects, suggesting that the mutation found in each proband might not represent single nucleotide polymorphism. It is therefore conceivable that either the c.T1685C or c.G1701C mutation might be responsible for XLSA.

Enzymatic activities of mutant ALAS2 proteins in vitro

Wild-type ALAS2 or each mutant ALAS2 protein was expressed in *E. coli* and purified as a tag-free protein. The combination of pTXB1 expression vector and IMPACT system allowed us to obtain a tag-free/C-terminal intact recombinant protein. Indeed, modified Coomassie Brilliant Blue staining of the gel after SDS-PAGE revealed that the purity of each prepared protein was >95% (data not shown). These recombinant proteins were suitable for determination of the catalytic activity of each mutant protein that carries the amino acid substitution near the C-terminal end.

We measured the catalytic activity of each recombinant ALAS2 protein with or without pyridoxal 5-phosphate (PLP). Data are summarized in Table 1. Unexpectedly, the catalytic activity of Val562Ala protein was significantly higher than that of wild-type protein ($p = 0.0046$), when the activity was measured without PLP in the assay mixture. In addition, in the presence of 200 μM PLP, Val562Ala mutant showed significantly higher activity than that of wild-type ALAS2 ($p = 0.0087$). In contrast, the catalytic activity of Met567Ile protein was lower than that of wild-type protein, irrespective of without PLP ($p = 0.0011$) or with PLP ($p = 0.0003$). It is also noteworthy that the PLP-associated increases in enzymatic activities were 86%, 44%, and 23% for wild-type, Val562Ala, and Met567Ile proteins, respectively, suggesting that Val562Ala and Met567Ile mutations decreased the responsiveness to PLP (Table 1). The lowest PLP responsiveness of Met567Ile mutant protein might account for the clinical course of the proband in case 2; that is, the anemia of this proband was improved only marginally, despite pyridoxine treatment.

Because we previously reported on the Ser568Gly mutation [15], which is also located in the C-terminal region of human ALAS2 protein, the reported data for the Ser568Gly mutation were included as a reference in Table 1. In vitro enzymatic activity of glutathione *S*-transferase (GST)–fused Ser568Gly was significantly lower than that of the GST–fused wild-type ALAS2 with or without PLP [15]. Therefore, the functional consequence of amino acid substitution at Ser568 was similar to that of Met567Ile (Table 1). In addition, the degree of PLP-mediated increase in Ser568Gly activity, indicated as "ratio with/without PLP" in Table 1, was larger than that with wild-type protein, although the possibility remains that the GST tag might have influenced the PLP responsiveness of a recombinant ALAS2 protein. We, therefore, included Ser568Gly mutant in subsequent analyses.

The higher catalytic activity of Val562Ala protein prompted us to examine the function of the C-terminal region of ALAS2 protein. We measured the enzymatic

activity of the deletion mutant that lacks the 33 amino acids at the C-terminal end (positions 555–587) of human ALAS2 (delC33 mutant), the region of which was conserved among mammalian ALAS2 proteins, including Val562. As shown in Table 1, the enzymatic activity of the delC33 mutant was higher by two times in the presence of PLP than that of wild-type ALAS2 ($p = 0.002$), whereas they showed similar enzymatic activity in the absence of PLP. These results suggest that the 33 amino acids at the C-terminal end of human ALAS2 protein might repress the enzymatic activity, probably by interfering with the access of PLP cofactor to the catalytic site.

Stability of mutant ALAS2 proteins in vivo

We were interested in studying how the Val562Ala mutation is associated with XLSA, despite higher enzymatic activity. We examined the stability of the Val562Ala mutant protein and other C-terminal mutant proteins in vivo. When human ALAS2 protein is expressed as a FLAG-tagged protein in eukaryotic cells, the precursor and mature proteins should be detected as 65.7-kDa and 60.5-kDa proteins, respectively. As shown in Figure 1B (upper panel) and Figure 2 (middle panel), FLAG-tagged wild-type ALAS2 and mutant ALAS2 proteins, except for delC33 mutant, were detected as bands at about 60 kDa, an expected size of the mature protein. These results suggest that the leader peptide at the N-terminal end was cleaved after translocation of the precursor protein into mitochondria [4]. In fact, the precursor protein was detected at an expected size, when HeLa cells were transfected with FLAG-ALAS2 expression vector, and then incubated with hemin (Supplementary Figure E2; online only, available at www.exphem.org), which is known to inhibit mitochondrial translocation of ALAS precursor protein into mitochondria [4]. Based on our experiments (Fig. 1A–C), the half-lives of wild-type and Val562Ala mature proteins in mitochondria were calculated as 7.8 hours and 5.3 hours, respectively. The half-life of the Val562Ala mutant protein (Fig. 1C) is shorter than that of wild-type ALAS2 protein (Fig. 1B). In contrast, the half-life of Met567Ile (Fig. 1D) or Ser568Gly (Fig. 1E) mutant was not measurable by our experiments because the 50% reduction of the protein level was not observed within 12 hours for these mutants. Thus, the half-lives of Met567Ile and Ser568Gly mutants were longer than 12 hours. Importantly, the amino acid substitutions in the C-terminal region influenced the stability of the mature ALAS2 protein in mitochondria in different manners. Namely, Val562Ala mutation results in destabilization of the mature protein, and either Met567Ile or Ser568Gly mutation stabilizes the mature protein in mitochondria.

In addition, we measured the half-life of delC33 mutant in HEK293-derived cells (Fig. 1F), showing that the 50% reduction was not observed within 12 hours, which was similar to Met567Ile and Ser568Gly mutants. These results suggested that the 33 amino acids at C-terminal region of ALAS2 protein suppressed the catalytic activity in vitro, as well as protein stability in mitochondria. Our data also indicate that Val562Ala mutation might enhance the destabilization function of the C-terminal region, whereas Met567Ile and Ser568Gly mutations might enhance the suppressive function for enzymatic activity.

Enzymatic activity of each ALAS2 mutant protein in vivo

Val562Ala mutant showed higher enzymatic activity in vitro (Table 1), but it was less stable in mitochondria (Fig. 1A) compared with wild-type ALAS2. On the other hand, Met567Ile and Ser568Gly mutants showed lower enzymatic activities in vitro (Table 1), with prolonged half-lives in mitochondria (Fig. 1A). We, therefore, determined the catalytic activity of each mutant protein in vivo. For this purpose, we compared the accumulation of porphyrins in HEK293-derived cells that expressed wild-type protein or a mutant protein, as we described previously [20]; that is, the accumulation of porphyrins was evaluated by comparing the intensity of the fluorescence under ultraviolet light (Fig. 2, upper panel). The accumulation of porphyrins was detected in cells expressing wild-type ALAS2, but not in cells expressing tagged luciferase. These results indicate that FLAG-tagged ALAS2 is catalytically active in mitochondria. In contrast, the accumulation of porphyrins was decreased in cells expressing Val562Ala, Met567Ile, or Ser568Gly protein, compared to cells expressing wild-type ALAS2. Among these three missense mutations, Val562Ala mutant showed higher catalytic activity than did Met567Ile or Ser568Gly mutant (Fig. 2, upper panel). In addition, the highest porphyrin accumulation was observed in cells expressing delC33. Of note, the expression level of Val562Ala mutant protein was much lower than that of any other mutant or wild-type ALAS2, as judged by Western blot analysis (Fig. 2, middle panel), although there was no significant difference in relative expression level of each mutant ALAS2 messenger RNA (Fig. 2, lower panel). These results suggest that Val562Ala mutant protein is catalytically hyperactive but unstable in mitochondria, which is consistent in part with the higher enzymatic activity detected in vitro (Table 1) and with the short-half life in vivo (Fig. 1A).

In conclusion, Val562Ala, Met567Ile, or Ser568Gly ALAS2 has lower enzymatic activity in mitochondria compared with the activity of wild-type ALAS2. Therefore, these three mutations are categorized as a loss-of-function mutation and are responsible for sideroblastic anemia.

Discussion

It is well known that a loss-of-function mutation of the ALAS2 gene causes XLSA. In addition to the ALAS2 gene, other genes (e.g., SLC25A38 [24], GLRX5 [25], ABCB7 [26], PUS1 [27], SLC19A2 [28], and mitochondrial DNA [29]) were reported to be responsible for

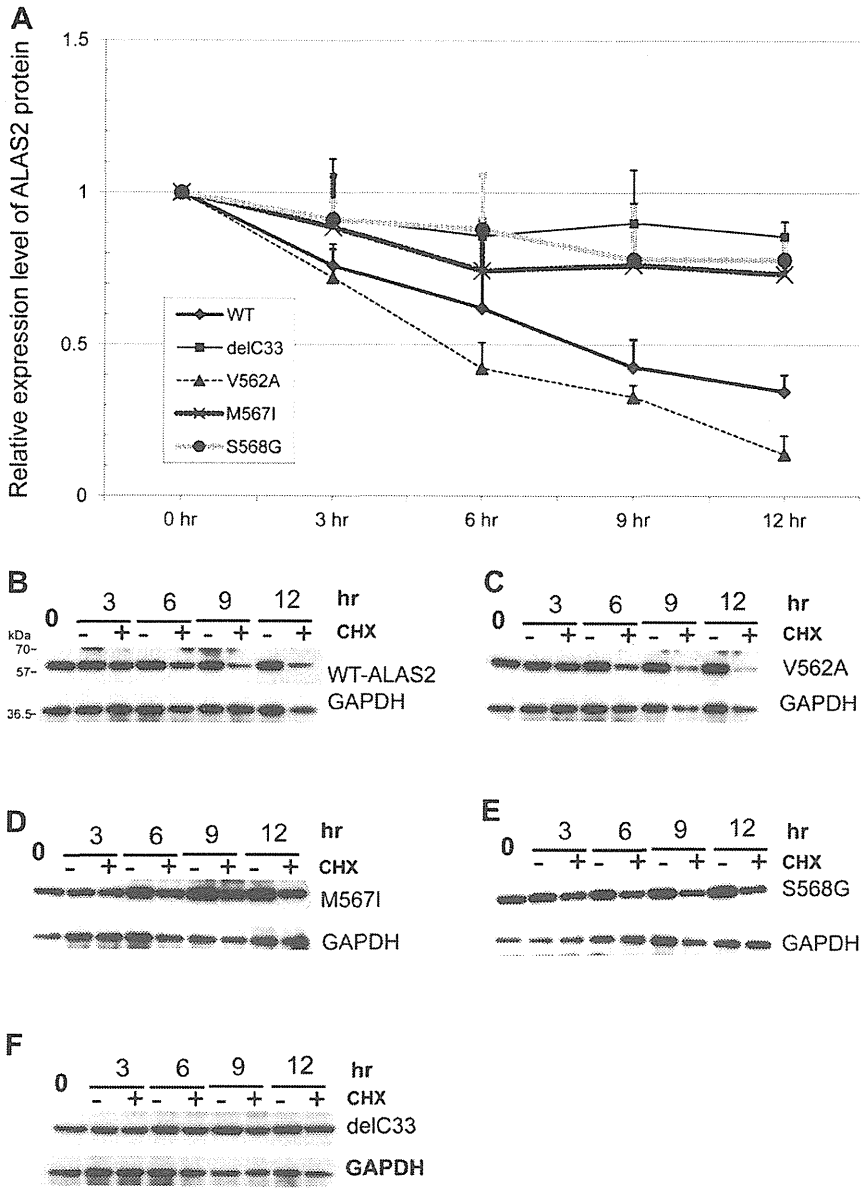

Figure 1. Effect of cycloheximide on FLAG-tagged ALAS2 protein level in eukaryotic cells. Expression of each FLAG-tagged protein was induced with tetracycline (1 μg/mL) in HEK293-derived cells for 48 hours, and then cells were treated with 10 μg/mL cycloheximide (CHX) for the indicated hours. Cells were collected and lysed in RIPA buffer, and FLAG-tagged proteins were detected by Western blot analysis (**B**–**F**). The intensity of the FLAG-tagged protein was normalized with the intensity of GAPDH for each time point. In (**A**), the relative intensity representing FLAG-tagged protein at 0 hours was considered to be 100%. The half-life of each protein was calculated on the basis of 50% reduction of each protein expression from the relative expression curves obtained from the samples with CHX. Average value of three independent experiments was used for preparing (**A**). Representative data of each ALAS2 protein are shown (**B**–**F**): (**B**) wild-type (WT) ALAS2; (**C**) Val562Ala; (**D**) Met567Ile; (**E**) Ser568Gly; and (**F**) delC33.

hereditary or congenital sideroblastic anemia. Among these candidate genes, mutations in ALAS2 gene are most frequently identified in patients with sideroblastic anemia [30], but characterization of each mutant ALAS2 protein was not fully performed. To the best of our knowledge, 24 of 56 mutations of the ALAS2 gene were characterized in vitro using recombinant proteins with or without a peptide-tag [9,10,14,15,20,21,31–36]. In the 11th exon of the ALAS2 gene, Ser568Gly [15], Arg559His [17], Arg560His [16], and Arg572His [14] mutations have been reported; however, only Ser568Gly and Arg572His mutants were characterized using recombinant proteins. Concerning the Ser568Gly mutation [15], we confirmed that Ser568Gly mutation resulted in decreased enzymatic activity in vitro (about 30% of wild-type with PLP in the assay mixture), as shown in Table 1. In contrast, Ducamp et al. [14] were

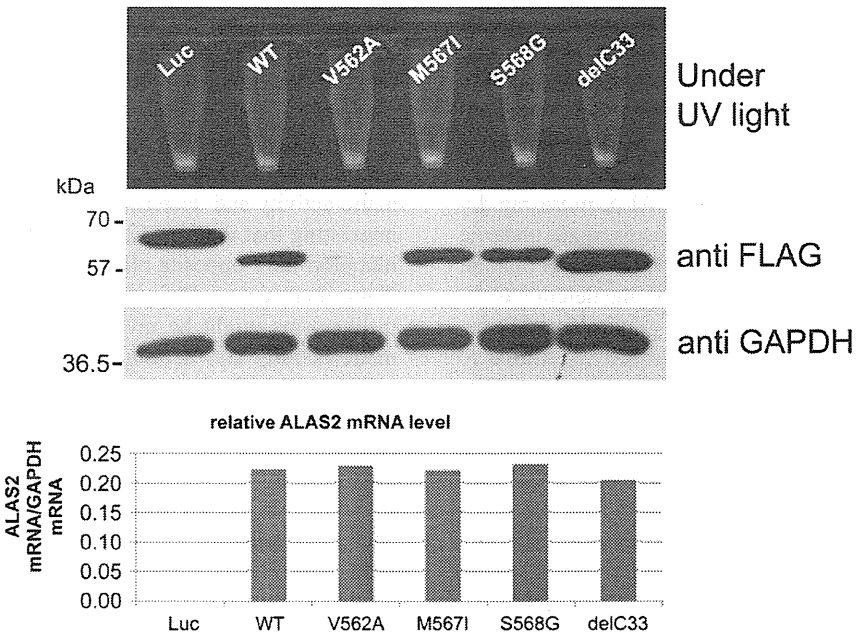

Figure 2. Evaluation of porphyrin production in cells expressing each ALAS2 mutant protein. Each FLAG-tagged ALAS2 protein or FLAG-tagged luciferase protein as a control was expressed in Flp-In T-REx 293 cells. Accumulation of porphyrins in each cell line was visualized by ultraviolet light exposure (upper panel). Expression levels of each FLAG-tagged protein and GAPDH (loading control) were detected by Western blot analysis (middle panels). Expression level of ALAS2 messenger RNA (mRNA) was measured by real-time PCR, and it was normalized with the expression level of GAPDH mRNA. Note that the data confirm the similar mRNA level of each ALAS2 protein (lower panel).

unable to determine the defect of the Arg572His mutant using an in vitro system because the mutant protein showed the enzymatic activity similar to that of wild-type ALAS2.

Measurement of enzymatic activity of every recombinant protein in vitro is one of the most useful techniques to characterize a mutant ALAS2 protein. Met567Ile mutant showed lower enzymatic activity than did wild-type protein (see Fig. 2), suggesting that this mutation causes sideroblastic anemia. In contrast, we were unable to uncover the pathogenesis of Val562Ala mutant protein using this in vitro assay system, indicating a limitation of the in vitro assay system with a bacterially expressed recombinant protein. In fact, using the in vivo system (Fig. 2), we have successfully demonstrated that the Val562Ala mutant protein showed lower porphyrin accumulation due to enzyme instability compared to wild-type ALAS2. In addition, the half-life of mature Val562Ala protein (5.3 hours) was shorter than that of wild-type ALAS2 (7.8 hours) (Fig. 1), suggesting that Val562Ala mutation altered the protein stability in mitochondria. These in vivo methods seem to be useful to characterize a mutant protein that does not show decreased enzyme activity in the in vitro assay system.

It is of particular interest that the Val562Ala and Met567Ile mutants exerted opposite effects on the enzymatic activity in vitro (Table 1) and on the protein stability in mitochondria (Fig. 1). In this connection, the deletion of 33 amino acids at C-terminal end of ALAS2 protein, the region of which contains Val562 and Met567 residues, resulted in higher enzymatic activity in vitro and in vivo (Table 1) and stable protein with a longer half-life in mitochondria (Fig. 1). The C-terminal region has a suppressive function on enzymatic activity, as well as protein stability in mitochondria. Because this region is conserved in eukaryotic ALAS2 but is absent in prokaryotic ALAS, the suppressor domain might be involved in the functional regulation of ALAS2 in mitochondria. In fact, in the C-terminal region, two frame-shift mutations of the ALAS2 gene were reported to cause X-linked dominant protoporphyria [11], and six (including present two cases) missense mutations were identified in patients with XLSA. In addition, it was recently reported that the Tyr586Phe mutation of ALAS2 protein increased the enzymatic activity, which can contribute to the severe clinical phenotype of the patient with congenital erythropoietic porphyria [18]. These results suggest that the C-terminal region of ALAS2 functions as an intrinsic suppressor for protoporpyrin production in erythroid cells.

It is still unclear how this C-terminal region suppresses the enzymatic activity of ALAS2 protein in mitochondria. It has been reported that certain amino acids are essential for catalytic activity of mouse Alas2 [18,37–45]. However, only limited information is available concerning the role of the C-terminal region in the catalytic activity of ALAS2. To-Figueras et al. [18] performed the stoichiometric analysis of the mature ALAS2 protein to characterize Tyr586-Phe mutant, which was reported as a gain-of-function

mutation at the penultimate C-terminal amino acid of ALAS2 protein. Steady-state kinetic analyses revealed that Tyr586Phe mutant showed higher catalytic activity with greater catalytic efficiency for glycine and succinyl-CoA than those of wild-type ALAS2. In addition, these authors provided evidence that the Tyr586Phe mutant enzyme was able to form and release ALA more rapidly than wild-type enzyme. Similar mechanisms might underlie the increased activity of every C-terminal deletion mutant, including the mutant ALAS2 protein with the deletion of 19 or 21 amino acids [11] and the delC33 mutant. In addition, the delC33 mutant expressed enzymatic activity similar to wild-type ALAS2 without PLP in assay mixture, but its enzymatic activity was increased about twofold compared to the wild-type with PLP (Table 1). These results suggest that this region might be involved in efficient use of PLP or accessibility of PLP to the catalytic site.

Crystal structure analysis of homodimeric ALAS from Rhodobactor capsulatus ($ALAS_{RC}$) revealed that $ALAS_{RC}$ showed open or closed structure, which was related to the conformational change of the active site loop [17]. This active site loop consists of evolutionally conserved structure at the C-terminal region of $ALAS_{RC}$, and seems to cover the catalytic site, which is located at the homodimer interface of ALAS protein. It should be noted that $ALAS_{Rc}$ does not contain the C-terminal region equivalent to that of mammalian ALAS2 [17]. The open conformation was observed only in the substrate-free $ALAS_{RC}$ protein, and the closed conformation was observed in $ALAS_{RC}$ protein that bound glycine and succinyl-CoA. To clarify the functional consequence of the conformational change of this active site loop, Lendrihas et al. introduced a mutation into nonconserved amino acid at this active site loop in mouse Alas2 protein and obtained several hyperactive variants [46]. Pre–steady-state kinetic analysis revealed that release of ALA from the catalytic site of the enzyme, which is coincident with opening of the active site loop [45], was accelerated in these hyperactive variants. Because the release of ALA from catalytic site is the rate-limiting step of enzymatic reaction of ALAS [47], these results suggest that the dynamic conformational change of this active site loop might control the rate of the reaction. Importantly, the accelerated release of ALA from the enzyme was also proposed in Tyr586Phe mutant [18]. It is therefore possible that the C-terminal domain of human ALAS2 protein is involved in the regulation of the conformational change of the active site loop.

In the present study, we determined the stability of ALAS2 protein in vivo, although the protein was tagged with a small peptide and expressed in HEK293-derived cells. Based on our assay condition, the half-life of human ALAS2 mature protein is 7.8 hours; however, it is not clear whether this result is comparable with that of the native ALAS2 protein in erythroid mitochondria, which has never been reported. On the other hand, this assay revealed that the stability of the Val562Ala mutant protein was decreased in mitochondria (Fig. 1), although the in vitro assay using purified recombinant protein failed to detect the unstable property of this mutant. In addition, our in vivo assay system clearly showed that the C-terminal region of 33 amino acids of human ALAS2 protein suppressed the enzymatic activity and decreased the protein stability. It is also interesting that the Val562Ala mutation and the Met567Ile mutation have opposite effects on the two functions of the C-terminal region. These results suggest that independent mechanisms might be involved in the reduction of enzymatic activity and destabilization in mitochondria. Taken together, the C-terminal region of ALAS2 protein can decrease catalytic activity by altering the open or closed structure of the catalytic site, while the post-translational modification of the C-terminal region, which is induced by a certain intracellular condition (e.g., increased or decreased oxidative stress) or by the association with other molecules, can enhance the disappearance of ALAS2 protein from mitochondria. The crystal structure of ALAS from $ALAS_{RC}$ provided critical information about the mechanisms for catalytic reaction of ALAS [45,46]. However, determination of the crystal structure of mammalian ALAS2 should await additional investigation on the function of the C-terminal region of ALAS2 protein.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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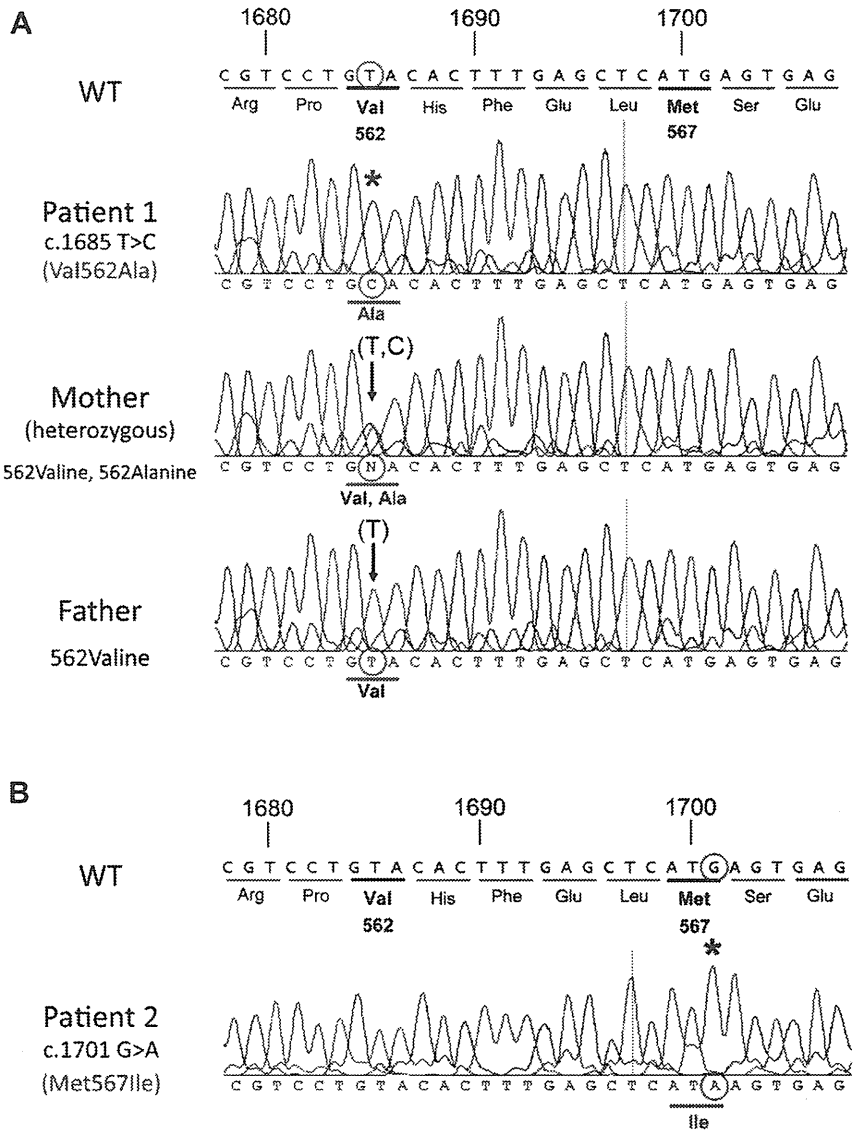
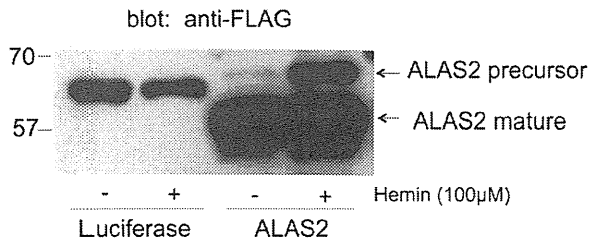


Supplementary Figure E1. Direct sequencing of 11th exon of ALAS2 gene in patients with sideroblastic anemia. Exon 11 of ALAS2 gene from each proband was amplified by PCR, and the amplicon was sequenced directly. Numbers shown at top indicate the positions of cDNA sequence, which is started from the first nucleotide of the ATG-translation initiation codon. Second and third lines indicate wild-type DNA sequence and amino acid sequence, respectively. Identified mutations are indicated with asterisks, and the expected amino acid substitution is shown under each mutation. (**A**) The c.1685T > C mutation of ALAS2 gene in case 1. The heterozygous condition of proband's mother and the wild-type allele of proband's father are shown. (**B**) The c.1701G > A mutation of ALAS2 gene in case 2.



Supplementary Figure E2. Transient expression of FLAG-ALAS2 and FLAG-Luciferase in HeLa cells. HeLa human cervical cancer cells were transfected with FLAG-ALAS2 or FLAG-luciferase expression vector, then treated with 100 μM hemin. Cell lysates were subjected to the Western blot analysis with anti-FLAG antibody. Shown are the representative data.

【第53回日本小児血液・がん学会学術集会】シンポジウム1：本邦における骨髄不全症候群の現状

リバージョン・モザイク型Fanconi貧血の診断と臨床

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要　旨

Fanconi貧血（FA）は種々の身体異常と小児期に発症する骨髄不全，白血化や高発がんを特徴とするまれな遺伝性疾患で，*FANCA*から*FANCP*に到る15個という多数の遺伝子異常が報告されている．細胞生物学的にはマイトマイシン（mitomycin C: MMC）などのDNA架橋剤に対する高い感受性を特徴とする．FA患者の中にはこうしたDNA架橋剤の感受性が正常化したリンパ球の混在する体細胞モザイク状態が観察され，その分子機構として，遺伝子の変異配列の野生型配列への復帰や，代償性変異により蛋白機能が回復した造血細胞クローンが増大する状態，すなわち「リバージョン・モザイク（reversion mosaicism）」で説明されている．リバージョンを起こしたクローンの増大がリンパ球で著しいと，通常のリンパ球の染色体断裂などの異常がみられず，皮膚などの線維芽細胞を用いた遺伝子検索で初めて確定診断にいたることが多く，FAの診断を極めて困難にしている．

キーワード：Fanconi貧血，染色体不安定性，リバージョン・モザイク，造血細胞移植
Key words: fanconi anemia, chromose instability, reversion mosaicism, hematopoietic stem cell transplantation

はじめに

スイスの小児科医Fanconiは1927年に家族性の貧血と身体奇形を特徴とする兄弟例を報告し，ファンコニ貧血（Fanconi anemia: FA）と命名した[1]．1964年，Schroederらは，FAの患者リンパ球に染色体異常がみられることを発見し[2]，さらに，佐々木らは，この染色体異常が，MMCなどのDNA架橋剤によって，著しく増加することを見いだし，本疾患の原因が染色体不安定性にあることを明らかにした[3]．FAは遺伝的にも多様な疾患であり，既知の13の責任遺伝子の他に，2010年のFA国際シンポジウムで認められた*FANCO*に加え，*FANCP*に到る15個という多数の遺伝子異常が報告されている．日本小児血液学会の1988年～2005年の全国登録データによれば，1,411例の再生不良性貧血のうち89例がFAで，先天性骨髄不全症の中ではDiamond-Blackfan貧血（98例）に次いで多く，全登録例の約6%を占めていた[4]．しかしながら，一部のFA患者においては，DNA架橋剤への感受性が正常化し，全く断裂が認められないリンパ球の混在する体細胞モザイク状態がみられ，FAの診断が極めて困難である．このようなリバージョンを生じたリンパ球はアルキル化剤などの化学療法剤にも抵抗性であるため，前処置を弱めたFAの造血細胞移植に際しては，拒絶やキメラとなる可能性がある．リンパ球にリバージョン・モザイクを認めた症例を中心に，FAの診断，造血細胞移植を含めた臨床像につき紹介する．

I　Fanconi貧血蛋白群が形成するBRCA経路

FAは13の責任遺伝子に加え[5] FANCO，FANCPに到る15個という多数の遺伝子異常が報告されている．FANCD1，FANCJ，FANCNはそれぞれ家族性乳がん遺伝子タンパク群のBRCA2，BRIP1，PALB2と同一であり，ヘテロ接合体ではFAを発症しないが，発がんリスクの増加がみられる．これらのFA蛋白群は図1に示すように共通のネットワー

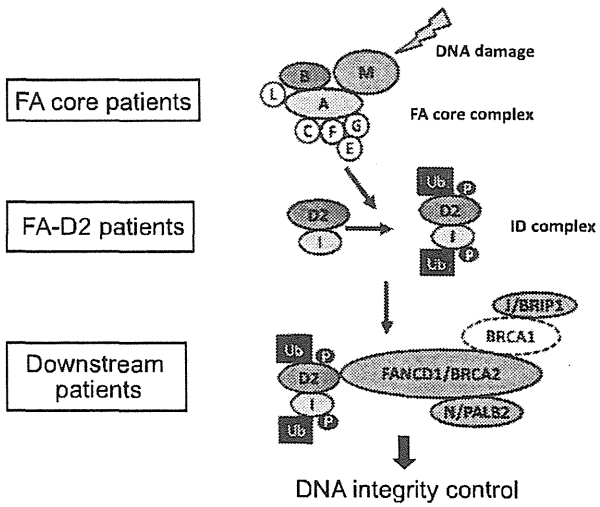

Figure 1 Fanconi anemia-BRCA pathway
The FA proteins represented by A, B, C, D ... N. Ub: ubiqutin
P: phosphoric acid

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クにおいて働き，どれか1つの遺伝子産物が先天的に欠損すると，FA経路の機能不全のためFAとして発症する．FA蛋白群のうち，FANCA, -B, -C, -E, -F, -G, -L, -Mは FANCD2のモノユビキチン化に必要なFA core complexを構成する．FANCD2，FANCIはFANCD2のモノユビキチン化に必要なID complexの構成因子である．FANCD1/BRCA2, FANCJ/ BRIP1，FANCN/PALB2はFANCD2のモノユビキチン化には不必要な因子群である．ID complexは家族性乳がん遺伝子産物であるBRCA1，BRCA2/FANCD1をはじめとする蛋白と相互作用し，DNA二重鎖架橋を修復する．日本人における解析では13の遺伝子群中，ほとんどはA, G, C群に含まれ，A群の頻度が最も高い[5]．従ってFANCD2産物に対する抗体を用い，ウェスタンブロット法でモノユビキチン化を確認する方法はスクリーニングに有用である[6]．

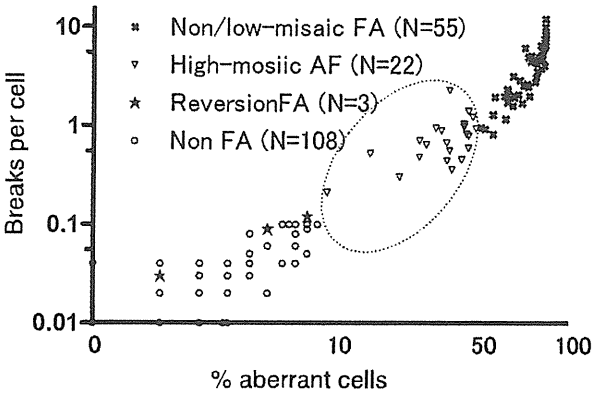

Figure 2 Diepoxybutane-induced chromosome fragility in non/low-mosaic Fanconi anemia (FA), high-mosaic FA, reversion FA and non-FA groups
The two-dimensional fragility intensity distribution was analyzed: percentage aberrant cells and breaks per cell.

II　リバージョン・モザイク型Fanconi貧血の病態

低濃度のMMCやdiepoxybutane（DEB）などのDNA架橋剤とともにリンパ球を培養すると，多数の染色体断裂や交換などの形成がみられる[3]．FA患者の中にはこうしたDNA架橋剤の感受性が正常化したリンパ球の混在する体細胞モザイク状態が，欧米では10–20%に観察されている．DEB添加で正常なリンパ球が50%以上を占める高レベルの体細胞モザイクの頻度は，図2の三角印に示すように本邦では30%近くに認め，欧米諸国の10%に比べて約3倍である[7]．星印で示されるリバージョン例では丸印の非FA患者群の中に組み込まれ，リンパ球における染色体断裂によるFAの診断ができない．その分子機構として，遺伝子の変異配列の野生型配列への復帰や，代償性変異により蛋白機能が回復した造血細胞クローンが増大する状態，すなわち「リバージョン・モザイク」で説明されている．リバージョンを起こしたクローンの増大がリンパ球で生じた場合，通常のリンパ球の染色体断裂や先に述べたFANCD2モノユビキチン化の異常が検出不能となり，造血細胞以外の皮膚の線維芽細胞などを用いた検査で初めて確定診断が得られる[8,9]．多くの症例ではリバージョンが造血幹細胞やリンパ球に生じることで骨髄不全の軽症化や自然寛解が報告されている．リバージョンが造血幹細胞で生じれば，「natural gene therapy」として正常造血を回復させる可能性がある．Mankadらは一卵性双生児において，造血細胞に同じ復帰変異が認められ，20年以上にわたり両姉妹とも造血障害を発症していない症例を報告した．これは，胎児期にどちらかの造血幹細胞に復帰変異を生じ，それが胎盤内循環により，もう一方の個体にも生着したためと考えられている[10]．造血幹細胞でのリバージョンはGregoryらによっても報告されているが，この症例ではリバージョンは骨髄不全の進行を抑制することはできなかった[11]．一部の患者では顆粒球にリバージョンがみられ，山下らのグループは，長期間にわたり骨髄不全の進行がみられない症例で，末梢血顆粒球に選択的にリバージョン・モザイクを見いだしている[12]．同論文で山下らは，日本人に頻度の高いFANCAの変異2546delCの復帰変異を非血縁の2例で検出しており，この変異はリバージョンを起こしやすい可能性を示唆している．これまでに報告されたFAのリバージョン・モザイクの報告例を表1に示した．

III　FAリバージョン・モザイクの自験例と造血細胞移植の経過

リバージョンが証明された3症例の臨床症状および生物学的特徴を表2に示す．遺伝子検査時年齢はそれぞれ7, 16, 29歳で，FAに特徴的な皮膚所見と低身長，指の異常などを認めた．症例2では骨髄不全発症時に前医でMMC検査がされており，FAが充分に示唆された．当院での染色体脆弱試験の再検査までに12年の経過があり，低レベルのモザイク状態から高レベルのモザイク状態へと移行したと考えられる．検査時病型は血液学的には重症再生不良性貧血が1例，2例がMDSと進行期であり，症例3では口腔底癌を合併していた．染色体脆弱検査では先に述べたように，DEB添加における断裂細胞の割合，1細胞あたりの断裂数ともに少なく，非FAあるいはコントロールとの区別が困難であった．末梢リンパ球でのFANCD2のモノユビキチン化はいずれも正常であり，症例1では頬粘膜細胞で，症例2では骨髄線維芽細胞でモノユビキチン化の障害がみられた．症例1ではリンパ球においてはもともとframeshiftである変異が，frameが正常化してほぼ正常なFANCA蛋白が形成され，一つのアミノ酸のみが正常では

Table 1 Fanconi anemia patients with genetic reversion

Patient code	Gene	Inherited mutation	Reversion	Revertant cells	Effect on phenotype	References
FA67	*FANCA*	2546delC	2546C>T	Gran, LCL	Mild pancytopenia	12)
FA98	*FANCA*	2546delC	2546C>T	PBLs	Progressive pancytopenia	**Case 1** 12)
EUFA704	*FANCA*	1615delG	1615delG+1637delA+1641delT	PBLs, LCL	—	21)
EUFA393	*FANCA*	3559insG	3559insG+3580insCGCTG	PBLs, LCL	—	21)
IFAR557-2	*FANCA*	2815-2816ins19	Wild type	PBLs, LCL, stem cells	Mild pancytopenia → clonal evolution	11)
URD	*FANCA*	856C>T	Wild type	PBLs, LCL	Mild pancytopenia	22)
STT	*FANCA*	862G>T	Wild type	PBLs, LCL	Mild pancytopenia	22)
MRB	*FANCA*	971T>G	Wild type	PBLs, LCL	Mild pancytopenia	22)
EUFA173	*FANCA*	2852G>A	Wild type	PBLs, LCL	Mild pancytopenia	22)
EUFA506	*FANCC*	1749T>G	1748C>T+ 1749T>G	PBLs, LCL	—	21)
EUFA806	*FANCC*	67delG/1806insA	Wild type	PBLs, LCL	Normal CBC	23)
EUFA449	*FANCC*	67delG	Wild type	PBLs, LCL	Mild pancytopenia	23)
—	*FANCD1*	8732C>A	8731T>G+ 8732C>A	Leukemic cells	Resistance to AML?	24)
—	*FANCA*	2670G>A	2927G>A+ 2670G>A	Stem cells	Normal CBC	10)

FA: Fanconi anemia, Gran: granulocytes, PBLs: peripheral blood lymphocytes, LCL: lymphoblastoid cell line. (Adapted with modifications from Table III in Hamanoue S et al: Br J Haematol, 132: 630–636, 2005[12].)

Table 2 Clinical and biological characteristics of the 3 Fanconi anemia patients with lymphocytic reversion

Case	Age (y)	Bone marrow failure presentation	Positive findings in physical examination or history	Biological diagnosis
1	7	Progressive pancytopenia Diagnosed at age 6 years of pancytopenia	PE: skin pigmentation, short stature, abnormality of metacarpal bones	PBL: both breaks and FANCD2 normal. Buccal mucosa: abnormal FANCD2 Molecular diagnosis: FANCA mutations: c.2546delC/c.2546delC Reversion: c.2546C>T
2	16	Diagnosed at age 4 years of pancytopenia Slowly progressive cytopenia over a period of 12 years	History: breaks by MMC test in PBL at age 4 years PE: skin pigmentation, short stature, thumb anomaly, strabismus, esophageal atresia Bone marrow: RAEB	PBL: both breaks and FANCD2 normal. Bone marrow fibroblast: both FANCD2 and MMC-sensitivity abnormal Molecular diagnosis: FANCA mutations: c.2546delC/c.3295C>T Reversion: absence of c.3295C>T
3	29	Diagnosed at age 9 years of pancytopenia	PE: skin pigmentation, short stature, thumb anomaly Bone marrow: RA Follow-up: oral cavity carcinoma diagnosed at the age of 29 years	PBL: both breaks and FANCD2 normal. Bone marrow fibroblast: FANCA mutations Molecular diagnosis: FANCA mutations: c.2170T>C/c.44_69del Reversion: absence of c.44_69del

PE: physical examination, PBL: peripheral blood lymphocytes, MMC: mitomycin C, RAEB: refractory anemia with excess blasts, RA: refractory anemia.

ないものの，蛋白質の構造に問題無く，コア複合体が正常に機能していると思われた．頬粘膜あるいは好中球においては両アレルの変異が確認された[12]．症例2，3ともに骨髄線維芽細胞で検出された片アレルの異常がリンパ球では消失し，変異配列の野生型配列への復帰がみられた．

FA患者にとって，造血幹細胞移植のみが造血障害に対して唯一根治が期待できる治療法である．通常の再生不良性貧血で用いられる放射線照射や大量シクロフォスファミド（cyclophosphamide; CY）の投与では，粘膜障害や移植片対宿主病（graft-versus-host disease; GVHD）が重症化し，成績不良であった．少量のCYと局所放射線照射の併用が標準的な前処置法として用いられ，GVHDに対するシクロスポリンA（cyclosporine A; CyA）の投与により，ヒト白血球抗原（human leukocyte antigen; HLA）一致同胞間移植の成績は向上した[13]．HLA一致同胞ドナーが得られる確率は低いため，HLA一致同胞以外の代替ドナーからの移植もおこなわれてきたが，従来の前処置法では高い生着不全と急性GVHDのため満足すべき治療成績は得られなかった[14]．最近FAの患者に対して，フルダラビン（fludarabine; Flu）を含む移植前処置が開発され，Fluをベースに CY量は一定とした少量の胸腹部照射（thoraco-abdominal irradiation; TAI）にFlu, CY, 抗胸腺グロブリン（antithymocyte globulin: ATG）を用いることにより，飛躍的に移植成績が向上した[15,16]．本邦でもHLA一致同胞ドナーからの移植では，Fluを含む非照射レジメンで移植されたFA患者の7例全例が生存中である[17]．また非血縁やHLA不一致血縁などの代替ドナー

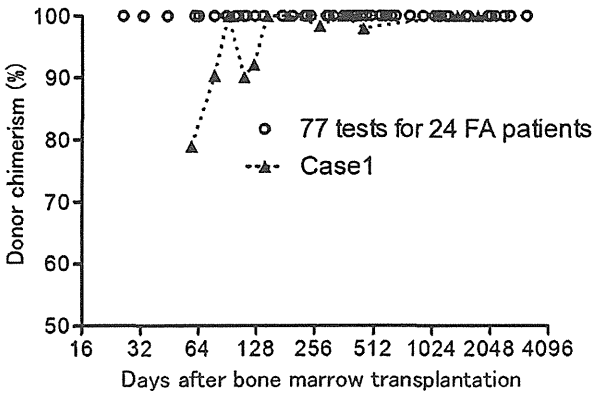

Figure 3 Donor chimerism of peripheral blood after alternative donor bone marrow transplantation

からの移植でも27例中26例が生存中であり[18]，造血細胞移植学会の一元化登録例の解析ではFluを含む前処置では約90%の5年生存率が得られている[19]．また，急性骨髄性白血病（acute myelogenous leukemia: AML）や進行期の骨髄異形成症候群（myelodysplastic syndrome: MDS）に対しては4.5 Gyの全身照射（totsl body irrsdistion: TBI）を用いることにより，成績の向上が得られている[18,20]．しかしながら，リバージョンを生じたリンパ球はアルキル化剤などの化学療法剤にも抵抗性であるため，前処置を弱めたFAの造血細胞移植に際しては，拒絶やキメラとなる可能性がある．TAI/Flu/CY/ATGレジメンを用いて代替ドナーから移植を行った24例のFA患者の移植後3週から約11年間に渡る末梢血全血のキメリズム解析では全例がドナータイプ100%であったが，リバージョンが証明された症例1（三角印）では移植後2か月から2年にわたり，混合キメラが観察された（図3）．症例1の造血機能は安定しているが，本人タイプのリンパ球の残存，増大は拒絶の可能性や，放射線やアルキル化剤の投与を受けた造血細胞はFA患者ではより白血化の可能性も高くなるため完全キメラに変わることが望まれる．症例2はTBI 4.5 Gyを用いて代替ドナーからの移植が行われたが，完全キメラが維持されている．

IV　FA患者における診断のフローチャート

FAの臨床像としては，1）汎血球減少，2）皮膚の色素沈着，3）身体奇形，4）低身長，5）性腺機能不全をともなうが，その表現型は多様で，汎血球減少のみで，身体異常を伴わない場合もある．また，血球減少が先行することなく，MDSや白血病あるいは固形癌を初発症状とすることもある．従って，臨床像のみで本疾患を確定診断するのは困難なため，小児や青年期に発症した再生不良性貧血患者や若年発症の頭頸部，婦人科領域の固形癌の発生がみられた場合には，全例にDNA架橋剤添加による染色体断裂試験を行い，FAを除外することが望まれる．リバージョン・モザイクの症例では末梢血リンパ球の染色体脆弱試験がスクリーニング検査としては指標にならないため，理学所見，家族歴，既往歴を詳細に検討し，造血細胞以外での遺伝子解析を考慮すべきである．FAの診断のフローチャートを図4に示す．

日本小児血液学会の中央診断システムが2009年2月から導入され，先天性骨髄不全症候群が疑われる際には診断依頼施設に各々の先天性骨髄不全症の専門家による遺伝子

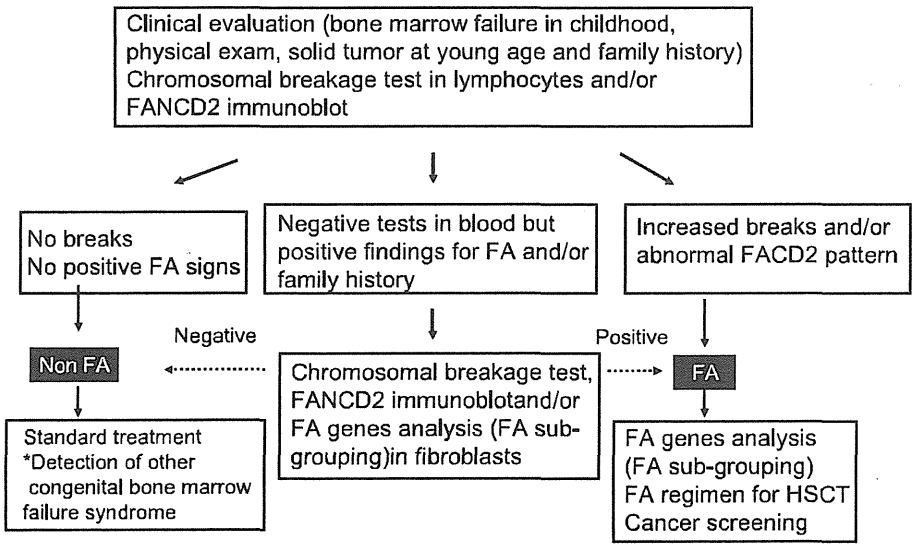

Figure 4 Diagnostic strategy for Fanconi anemia

* Shwachman-Diamond syndrome, Dyskeratosis congenita, congenital amegakaryocytic thrombocytopenia, Pearson syndrome and Diamond-Blackfan anemia. (Adapted with modifications from Figure 4. in Pinto FO et al: Haematologica, 94: 487–495, 2009[9].)