(A)

(B)

(C)



Figure 4. Enhancement of the ALA-induced accumulation of proto-porphyrin and photodamage by knockdown of HO-1 and HO-2. (A) The cells (5 × 10⁵) transfected with HO-1 and HO-2 siRNAs were cultured for 48 h, followed by incubation with 1 mM ALA for 16 h. The cellular protein from the cells as above was analyzed by SDS-PAGE. Immunoblots of HO-1 and HO-2 were carried out; (B) Porphyrin was extracted and determined; (C) Effect of double knockdown of HO-1 and HO-2 on the photodamage. The cells treated with HO-1/-2 siRNAs in combination were irradiated. The survival of cells was examined by MTT assay. Data are the mean ± SD of three to four independent experiments.

control (Fig. 6C). Without irradiation, virtually no photo-damage was observed. These results indicated that the decrease of the supply of iron in mitochondria led to the enhancement of ALA-induced photodamage.
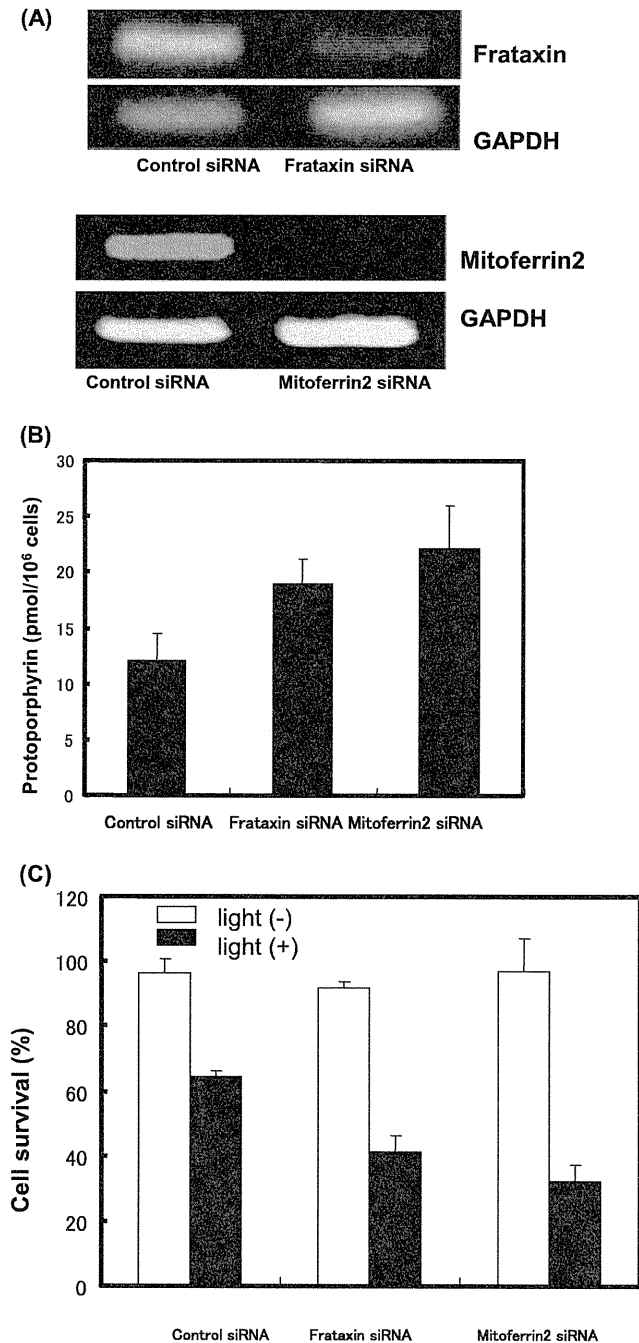
DISCUSSION

This study demonstrated that the increased expression of heme-biosynthetic enzymes, including PBGD, UROS and CPOX in HeLa cells increased the accumulation of ALA-induced protoporphyrin and photodamage. The increased expression of PPOX did not have any effect. In cells highly expressing FECH, the accumulation of protoporphyrin decreased, presumably due to insertion of ferrous ions into protoporphyrin, which was consistent with our previous findings (7,8) that the accumulation of protoporphyrin was inversely correlated with the expression of FECH. Recently,

(A)

(B)

(C)
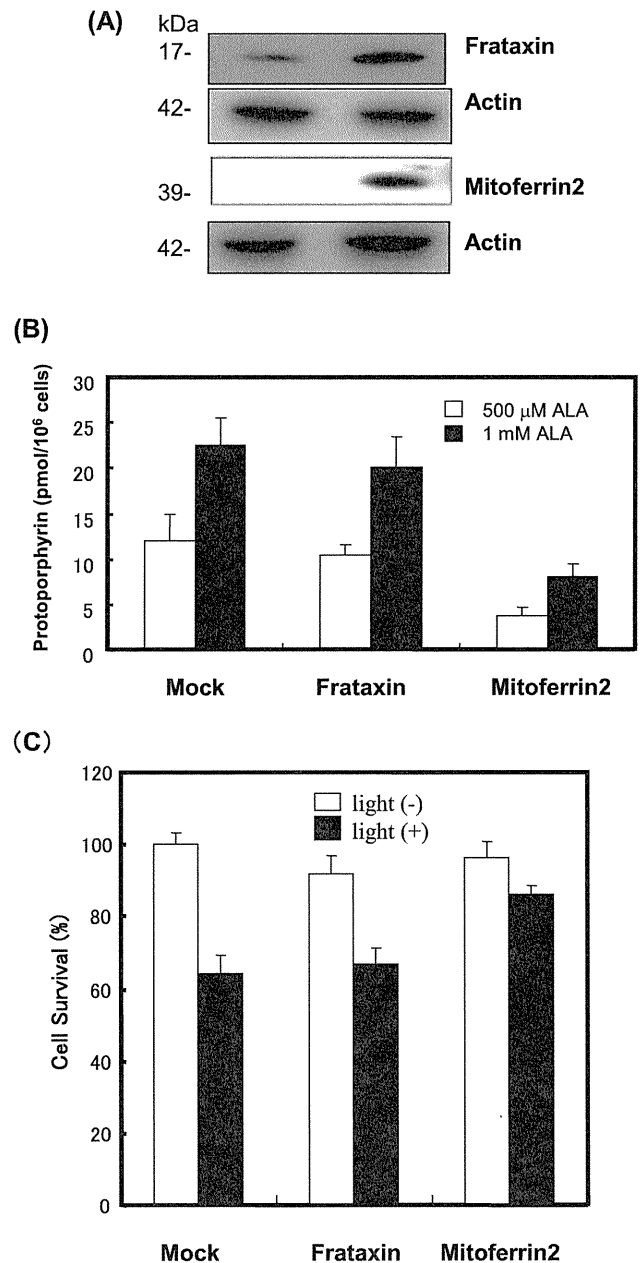
Figure 5. Effect of the expression of frataxin and mitoferrin-2 on the ALA-induced protoporphyrin and photodamage. HeLa cells (5 × 10⁵) were transfected with pcDNA3-frataxin or pCG-C-mitoferrin-2 and incubated for 16 h. The cells were then incubated with 500 μM and 1 mM ALA for 8 h. (A) Immunoblots. Cellular proteins from the cells as treated above were analyzed by SDS-PAGE, followed by immuno-blotting with anti-frataxin and anti-HA, as the primary antibodies; (B) The porphyrin was extracted and measured; (C) Photodamage. The cells treated as above were irradiated. The survival of cells was examined by MTT assay. Data are the mean ± SD of three to four independent experiments.

other investigators applied FECH siRNA to enhance ALA-PDT in glioma of septum and found a high efficacy of ALA-PDT in vivo (30). On the base of the fact that FECH deficiency leads to the accumulation of protoporphyrin leading to the inherited disease erythropoietic protoporphyria (31), the decrease of FECH activity is closely related to ALA-PDT. In addition to the decrease of FECH activity, it is reported that

(A)

Frataxin

GAPDH

Control siRNA   Frataxin siRNA

Mitoferrin2

GAPDH

Control siRNA   Mitoferrin2 siRNA

(B)

Control siRNA Frataxin siRNA Mitoferrin2 siRNA

(C)

light (-)
light (+)

Control siRNA   Frataxin siRNA   Mitoferrin2 siRNA

Figure 6. Enhancement of the ALA-induced accumulation of protoporphyrin and photodamage by knockdown of frataxin and mitoferrin-2. (A) The cells (5×10^5) transfected with frataxin or mitoferrin-2 siRNA were cultured for 48 h, followed by incubation with 1 mM ALA for 16 h. RNA was isolated from the cells treated as above. RT-PCR was performed to estimate the levels of frataxin and mitoferrin-2 mRNAs; (B) Porphyrin was extracted and determined; (C) Photodamage. The cells treated as above were irradiated. Surviving cells were examined by MTT assay. Data are the mean $\pm$ SD of three independent experiments.

the treatment of prostate cancer cells with methotrexate, an anticancer reagent, resulted in an increase in ALA-induced PDT with concomitant elevation of CPOX (32). Sinha et al. (33) reported that up-regulation of CPOX enhanced ALA-PDT of prostate cancer cells. Our results support this effect of

CPOX expression on ALA-PDT because the transfection of the cells with CPOX-expression plasmid caused an increase in the ALA-induced photodamage. Hinnen et al. (34) and Krieg et al. (35) reported that an increase in the expression of PBGD in adenocarcinoma cells was related to the hypersensitivity of ALA-PDT, suggesting that the elevation of PBGD in cancerous cells might be a useful parameter for predicting the accumulation of protoporphyrin. We found that the augmentation of PBGD expression caused an increase in the ALA-induced accumulation of protoporphyrin and photodamage. Thus, the elevation of the level of heme-biosynthetic enzymes, including PBGD, UROS and CPOX could be responsible for the high accumulation of protoporphyrin in tumor cells. In addition, considering that silencing of ALA-dehydratase caused the decrease of ALA-induced accumulation of protoporphyrin (36), ALA-dehydratase seems to play a role for ALA-PDT.

The results in our study supported the findings of previous studies that the supply of iron and the reuse of iron from heme by HO reduced the ALA-induced accumulation of protoporphyrin (27). Here, we demonstrated that the induction of HO-1 by hemin and heavy metal ions decreased the accumulation. It is possible that the decrease of the accumulation can be due to the heavy metal toxicity as the intoxication by heavy metal ions caused the reduction of ALA-induced accumulation of protoporphyrin (37). On the other hand, Sn-PP, an inhibitor of HO but not Co-PP, a substrate of HO, increased the ALA-induced accumulation of protoporphyrin (Fig. 2A). Furthermore, the expression of HO-1 in HeLa cells was shown to be inversely related to the ALA-induced accumulation of protoporphyrin (Fig. 3B). The increased expression of HO-2 also decreased the accumulation, and knockdown of the expression of HO-1/-2 in HeLa cells resulted in a marked enhancement of the photodamage (Fig. 4C). Thus, iron generated by HO-1 as well as HO-2 is reused for the iron-chelating reaction by FECH. As such, challenge of HO-1 and HO-2 siRNA may facilitate for the enhancement of ALA-PDT for tumors.

Some researchers (13) showed that the ALA-induced phototoxicity was variable among cancer cell lines even when knockdown of HO-1 in several cells by siRNA was carried out, and suggested that the level of HO-1 was unrelated to ALA-PDT. However, the contribution of HO-2 in ALA-PDT was not examined. We found that expression of HO-2 as well as HO-1 decreased the accumulation of protoporphyrin in the presence of ALA, whereas deficiency of HO-2 or HO-1 in HeLa cells increased the accumulation. In addition to the decreased expression of FECH in tumor cells, the low expression of HO-1/-2 in cancer cells may be linked to hyperphotosensitivity derived from ALA. Therefore, the decrease of HO function can cause the ALA-induced accumulation of protoporphyrin. Alternatively, we have shown that HO-1 is markedly induced not only by chemicals that produce oxidative stress involving the generation of reactive oxygen species but also by the substrate heme (28,29), and that HO-1 in ALA-treated cells was induced in time- and dose-dependent manners, and the induction of HO-1 was seen in the protoporphyrin-accumulated cells (8). It is considered that uncommitted heme in the cells is very dangerous for the maintenance of living systems, and reutilization of iron, including degradation of heme, catalyzed by HO, is essential for the homeostasis of iron in cells (9). By the treatment of cells

with ALA, excess heme produced from ALA may induce HO-1. It was also possible that the accumulated protoporphyrin generates reactive oxygen species *via* autoxidation (38), which leads to the induction of HO-1. On the basis of the fact that HO degrades heme, producing iron, CO and biliverdin (12), the supply of iron for its reutilization reduced the protoporphyrin and high level of HO-1 in tumor cells may be responsible for their resistance to anticancer treatment. In contrast, the iron supply was stopped by the inhibition of the HO reaction with Sn-PP, leading to an increase in the production of protoporphyrin. The photosensitivity caused by the ALA-dependent accumulation of protoporphyrin was different among tumor cells. One of the reasons to explain the different photosensitivity may be the different rates for the production of heme and the degradation of heme in species of tumor cells.

It is well known that iron metabolism in mitochondria is different between normal and cancerous cells. Among molecules involved in mitochondrial iron metabolism, mitoferrin-2 functions in the import of mitochondrial iron in nonerythroid cells (16). Reduction of mitoferrin-1/2 levels by RNA interference resulted in the decrease of mitochondrial iron and heme synthesis (16). Mutation of erythroid-type mitoferrin in zebrafish caused defects in hemoglobinization (16). The present data revealed that knockdown of mitoferrin-2 in HeLa cells led to the increase in the ALA-induced accumulation of protoporphyrin and enhancement of photodamage. On the other hand, transient expression of mitoferrin-2 in HeLa cells decreased the ALA-induced accumulation of protoporphyrin, which showed the increased availability of iron for the reaction of FECH. Although no study on whether the expression of mitoferrin-2 in cancerous cells is reduced has been reported, it is possible that the function of mitoferrin-2 can be impaired in transformed cells.

The overexpression of mitochondrial frataxin in cancer cells decreased ROS production and induced mitochondrial functions, including respiratory, membrane potential and ATP content (39). It is reported that several cancer cells do not express detectable frataxin, but untransformed cells produce frataxin (40). Thus, the reduction of the function of frataxin in cancerous cells lead to the decrease of mitochondrial function and may contribute to enhancement of cancer-specific ALA-PDT. HeLa cells used in this study produced detectable frataxin and transient overexpression of frataxin did not affect ALA-induced accumulation of protoporphyrin, suggesting that the expression of frataxin in control HeLa cells can be enough to maintain iron metabolism in mitochondria. The expression of frataxin in frataxin-deficient tumor cells may reduce ALA-PDT. On the other hand, knockdown of frataxin led to an increase in the ALA-induced photodamage with the accumulation of protoporphyrin. Frataxin is an iron-chaperon and plays an essential role in Fe-S cluster biogenesis in mitochondria (39). Considering that FECH is an Fe-S cluster-containing protein and the expression level of FECH is dependent on the intracellular level of iron (41), a loss of function of frataxin decreases the level of FECH, leading to enhancement of ALA-PDT. In contrast, Schoenfeld *et al.* (17) reported that lymphoblasts of frataxin-knockout mice were protected from ALA-induced phototoxicity by the reduced expression of CPOX. The different effect of frataxin deficiency on ALA-induced photodamage can be due to different

metabolic regulations of mitochondrial iron utilization between normal and cancerous cells. Thus, the present study revealed important roles of multiple factors such as porphyrin synthesis, iron reutilization and mitochondrial iron metabolism for characteristics of tumor-specific ALA-dependent accumulation of protoporphyrin. Further systematic studies should shed light on the mechanism of resistance against PDT and overcome the limitation in clinical application for various carcinoma cells.

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Original Articles

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 μg/m² 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
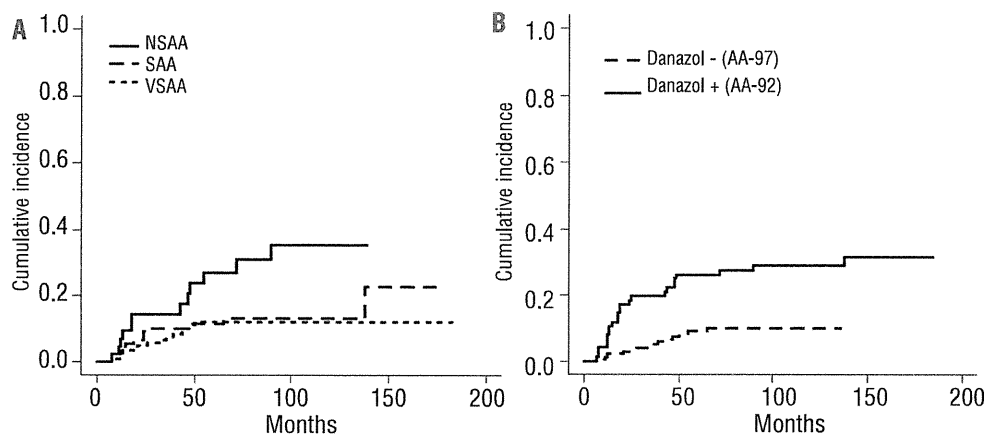
Figure 1. Cumulative incidence of relapse after immunosuppressive therapy in children with aplastic anemia. (A) The cumulative relapse rate of patients with non-severe aplastic anemia (NSAA) was significantly higher than that of patients with severe aplastic anemia (SAA) (*P*=0.025) and very severe aplastic 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 ana-

bolic steroid (oxymetholone) to antithymocyte globulin treatment improved the response rate of patients with treated AA.[14] 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

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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Letter to the Editor

CD7-positive acute myelomonocytic leukemia with trisomy 21 as a sole acquired chromosomal abnormality in two adolescents

1. Introduction

Trisomy 21 is one of the most common acquired chromosomal abnormalities in myeloid malignancies, but is rarely found as a sole anomaly. It has been reported that only 0.3% of adult patients with acute myeloblastic leukemia (AML) or myelodysplastic syndrome have trisomy 21 as a sole acquired abnormality [1]. Although several reports indicate that this karyotypic abnormality is associated with the French–American–British (FAB) AML subtypes M2, M4 and M5 [2,3] and with a poor prognosis [3,4], its clinical and prognostic implications have not been fully evaluated. Some investigators have focused on the association between this chromosomal abnormality and CD7 expression, and 4 adult patients with CD7-positive AML have been reported so far [5–8]. Although one report includes 2 adolescent AML cases with trisomy 21 as a sole acquired abnormality [3], there have been no reports on pediatric CD7-positive AML patients with this anomaly. Here, we report 2 unique adolescent cases of CD7-positive acute myelomonocytic leukemia with trisomy 21 as a minor clone and as a sole acquired chromosomal abnormality.

2. Case reports

2.1. Case 1

A 15-year-old boy was admitted to our hospital in November 2006 because of fever and gingival swelling and bleeding. Findings on physical examination included petechiae over the body and hepatomegaly (5 cm below the costal margin). Examination of the peripheral blood revealed the following: hemoglobin 8.3 g/dL, platelets 15×10^9/L, and leukocytes 227×10^9/L with 40% blasts and 8% monocytes (18.2×10^9/L). The bone marrow was hypercellular with 96% blasts, which were positive for myeloperoxidase but negative for non-specific esterase staining. Immunophenotypic analysis by flow cytometry showed that the blasts were positive for CD7, CD13, CD33, CD34, and HLA-DR expression and negative for lymphoid markers. Chromosome analysis of bone marrow cells revealed 47,XY,+21[2]/46,XY[38]. The patient had no clinical findings of Down syndrome. Interphase fluorescence in situ hybridization (FISH) analysis using a specific probe for the RUNX1 gene labeled with Spectrum-Green plus a set of 3 probes for 21q22 (D21S259, D21S341 and D21S342) labeled with Spectrum-Orange, showed that 26 out of 1000 bone marrow cells had 3 green and 3 red signals (2.6%). Serum and urine lysozyme levels were 43.4 µg/mL (reference range, 5.0–10.2 µg/mL) and 8.6 µg/mL (undetectable in normal subjects), respectively. Although morphological analysis of bone marrow cells was suggestive of a diagnosis of AML-M2 based on the FAB classification, the final diagnosis was AML-M4 because

of the increased number of peripheral blood monocytes and elevated serum and urine lysozyme levels [9]. The patient was treated with the AML-99 protocol [10] and achieved complete remission after the first course of chemotherapy. Chromosome analysis of the bone marrow cells showed a normal karyotype, 46,XY in all 20 cells analyzed. He received 5 additional courses of consolidation chemotherapy, which was finished in June 2007. Trisomy 21 was never found on chromosome analysis performed after each course of chemotherapy. He has remained in continuous complete remission for 4 years after diagnosis.

2.2. Case 2

A 14-year-old girl was referred to our hospital in April 2007 because of fever, nasal bleeding, and gingival swelling and bleeding. On admission, petechiae were observed over the body, and the liver and spleen were palpable 8 cm and 4 cm below the costal margin, respectively. Examination of the peripheral blood revealed the following: hemoglobin 5.1 g/dl, platelets 4×10^9/L, and leukocytes 73.1×10^9/L with 56% blasts and 15% monocytes (11.0×10^9/L). The bone marrow was hypercellular with 61% blasts, which were positive for myeloperoxidase but negative for non-specific esterase staining. Surface marker analysis showed that the blasts expressed CD7, CD13, CD33, CD34, and HLA-DR. Cytogenetic analysis of bone marrow cells revealed 47,XX,+21[2]/46,XX[18]. The patient did not manifest characteristics associated with Down syndrome. Interphase FISH analysis using a probe for the RUNX1 gene showed that 41 out of 1000 bone marrow cells had trisomy 21 (4.1%). The serum lysozyme level was elevated at 43.1 µg/mL, but lysozyme was undetectable in urine. A diagnosis of AML-M2 was suggested by the morphological findings of the bone marrow cells, but peripheral blood monocytosis and an elevated serum lysozyme level led to a final diagnosis of AML-M4. The patient underwent induction chemotherapy using the AML-99 protocol and achieved complete remission after a single course of chemotherapy. Cytogenetic analysis of bone marrow cells showed a normal female karyotype in all 20 metaphases analyzed. She was subsequently treated with 5 courses of consolidation chemotherapy and completed therapy in November 2007. Trisomy 21 was never detected on chromosome analysis performed after each course of chemotherapy. She has remained in continuous complete remission for 3 years after diagnosis.

The clinical characteristics and laboratory data of the 2 patients are summarized in Table 1. No mutations were detected in the RUNX1 or GATA1 genes in DNA from the patient's bone marrow cells (data not shown).

3. Discussion

We describe the first 2 pediatric CD7-positive AML cases with trisomy 21 as a sole acquired abnormality. These cases shared several unique clinical features. First, both patients were diagnosed

Table 1
Clinical characteristics and laboratory data of the patients.

	Case 1	Case 2
Age	15y	14y
Sex	Male	Female
WBC count ($\times 10^9$/L)	227	73.1
FAB classification	M4	M4
Non-specific esterase staining	Negative	Negative
Monocyte count ($\times 10^9$/L)	18.2	11.0
Serum lysozyme level[a] (μg/ml)	43.4	43.1
Urine lysozyme level[b] (μg/ml)	8.6	Not detected
Immunophenotype	CD7, 13, 33, 34, HLA-DR	CD7, 13, 33, 34, HLA-DR
Karyotype	47,XY,+21[2]/46,XY[38]	47,XX,+21[2]/46,XX[18]
Trisomy 21-positive cells on FISH analysis	26/1000 cells	41/1000 cells
CR after 1st course of chemotherapy	Yes	Yes
Outcome	CR, 4y after diagnosis	CR, 3y after diagnosis

WBC, white blood cell; FAB, French–American–British; FISH, fluorescence in situ hybridization; CR, complete remission.
[a] Reference range, 5.0–10.2 μg/mL.
[b] Undetectable in normal subjects.

with AML-M4 based on the FAB classification because of their peripheral blood monocytosis ($\geq 5 \times 10^9$/L) and elevated serum and/or urine lysozyme levels (>3 $\times$ normal values), although blasts in the bone marrow were negative for non-specific esterase and were morphologically classified as AML-M2 [9]. Some reports have suggested that AML with trisomy 21 as a single abnormality is associated with AML-M2, -M4 and -M5 [2,3], but monocyte counts and lysozyme levels were not discussed. Our findings indicate that evaluation of monocyte counts and serum/urine lysozyme levels are important for correct FAB classification of AML with this karyotypic abnormality.

Second, trisomy 21 was observed in only 2 out of the 40 or 20 bone marrow cells examined in Case 1 and 2, respectively, which were much lower percentages than the percentages of morphologically detected blast cells (96% and 61%, respectively). These low fractions of trisomy 21-positive cells were confirmed by interphase FISH analysis (2.6% and 4.1% in Case 1 and 2, respectively). We think that a constitutional mosaic trisomy 21 (mosaic Down syndrome) is not likely, because trisomy 21 was never found on serial cytogenetic analyses performed after remission in either patient (a total of 100 bone marrow cells were analyzed in each patient). These results clearly show that most of the blast cells had normal karyotypes and the blasts that had acquired trisomy 21 were minor clones in both patients.

Third, both patients achieved complete remission after the first course of chemotherapy and have remained in continuous complete remission for 3–4 years after diagnosis. Although several reports have indicated that this chromosomal abnormality is associated with poor prognosis [3,4], the prognostic implications have not been fully evaluated, because there are so few patients with this anomaly. To confirm that pediatric CD7-positive AML with this karyotypic abnormality has a favorable outcome, more patients need to be studied.

In conclusion, we report the first 2 pediatric cases of CD7-positive AML with trisomy 21 as a sole acquired chromosomal abnormality. The patients shared some clinical features including AML-M4 subtype, the presence of minor clones with trisomy 21, and favorable outcomes, and they might have had a distinct subtype of AML.

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Ⅶ 先天性骨髄不全症候群

資料 6. 遺伝性鉄芽球性貧血／診療の参照ガイド

1. 緒 言

鉄芽球性貧血は，骨髄における環状鉄芽球の出現を特徴とする貧血であり，環状鉄芽球はミトコンドリアへの鉄の異常蓄積により形成される．鉄芽球性貧血は，遺伝性鉄芽球性貧血と，骨髄異形成症候群（MDS）およびアルコールや薬剤による二次性鉄芽球性貧血からなる後天性鉄芽球性貧血に大別される．遺伝性鉄芽球性貧血は稀な疾患で，ヘム合成不全，鉄-硫黄クラスター形成不全などにより，ミトコンドリアにおける鉄代謝に異常が生じ発症する難治性貧血である．1945 年に Cooley が X 連鎖性小球性低色素性貧血を呈する家族性貧血症を報告したが，1946 年に Rundles と Falls がこの家系を含む 2 家系を報告したことで，この X 連鎖性小球性低色素性貧血は Rundles and Falls 症候群と名づけられた[1]．のちにこの貧血は赤血球におけるヘム合成系の初発酵素である δ-アミノレブリン酸合成酵素（ALAS2）の変異による X 連鎖性鉄芽球性貧血（XLSA）であることが証明された[2]．現在，遺伝性鉄芽球性貧血の原因としてこの ALAS2 の変異が最も多く報告されているが，その他にも鉄-硫黄クラスター合成・輸送にかかわる遺伝子，ミトコンドリア DNA 遺伝子，ミトコンドリアトランスポーター遺伝子，ミトコンドリア tRNA 関連遺伝子など複数の遺伝子の変異が報告されている．さらに，原因遺伝子が同定されない遺伝性鉄芽球性貧血も多く，既報の遺伝子以外にも原因となる遺伝子が存在すると考えられている．遺伝性鉄芽球性貧血は，原因遺伝子の機能の多様性から，貧血以外に神経・筋などほかの臓器に異常を認める場合が多く，また貧血の重症度も様々である．多くの遺伝性鉄芽球性貧血では特異的治療法がないものの，XLSA のように適切な診断・治療がなされれば，貧血の改善が期待できるみられるタイプも存在するため，遺伝子診断による確定診断が重要である．

2. 診 断

1）疾患概念

骨髄における環状鉄芽球の出現を特徴とする貧血である．

2）診断基準

環状鉄芽球が骨髄総赤芽球の 15% を超える（FAB 分類）．

血清フェリチンの増加，不飽和鉄結合能減少を認める．

上記に加えて遺伝子変異が確認できたものが，遺伝性鉄芽球性貧血の確定診断となる．家族歴は遺伝性鉄芽球性貧血を強く疑う所見である．

遺伝性で最も頻度の高い XLSA は小球性低色素性の貧血で男児発症を特徴とする．

環状鉄芽球の定義：核周囲 1/3 以上にわたって 10 個以上の鉄顆粒が存在（新 WHO 分類）

3）診断のフローチャート

遺伝性鉄芽球性貧血の診断は，まず環状鉄芽球の存在，遺伝性を確認し，確定診断は遺伝子解析である．家系のなかでの遺伝性が明らかでない場合は，造血細胞以外の組織で遺伝子の変異を確認する．遺伝性鉄芽球性貧血のなかでは ALAS2 変異による XLSA の頻度が最も高いため，特に男児で家族歴を認める場合，また，診療過程でビタミン B6 に反応性を認めた場合は積極的に遺伝子解析を行う．XLSA の場合は変異 ALAS2 蛋白質の活性低下を in vitro で確認することも可能である．現在報告されている遺伝子変異を表 1 に示す．

4）鑑別診断

以下にあげる後天性鉄芽球性貧血を除外する必要がある．

後天性鉄芽球性貧血
　○薬剤性，中毒性：抗結核薬，鉛など
　○アルコール性：ヘム合成酵素障害，ビタミン B6 欠乏

表1 遺伝性鉄芽球性貧血の責任遺伝子

	遺伝形式	遺伝子座	遺伝子	治療
XLSA*	X連鎖性	Xp11.21	ALAS2	ビタミンB_6
XLSA/A**	X連鎖性	Xq13.1	ABC7	－
SA/GLRX5	常染色体劣勢？	14q32.13	GLRX5	？
SA/SCL25A38	常染色体劣勢？	3p22.1	SCL25A38	？
PMPS***	母性	ミトコンドリア	ミトコンドリア	－
TRMA****	常染色体劣勢？	1q23.3	SCL19A2	ビタミンB_1
MLASA*****	常染色体劣勢？	12q24.33 ・	PUS1	－

*：X-連鎖性鉄芽球性貧血. **：小脳失調を伴うX-連鎖性鉄芽球性貧血. ***：Pearson marrow-pancreas 症候群.
****：チアミン反応性巨赤芽球性貧血. *****：ミトコンドリア筋症を伴う鉄芽球性貧血

○骨髄異形成症候群

　通常，後天性鉄芽球性貧血は発症年齢，遺伝性から鑑別が可能であるが，成年発症の XLSA 症例も報告されていることから[3]，ときに遺伝性との鑑別を必要とする．アルコール性，薬剤性の後天性鉄芽球性貧血については，生活歴，治療歴から鑑別する．薬剤性はビタミンB_6に対する拮抗作用を原因として発症することが多い．ビタミンB_6は ALAS2 の補酵素であるため，その欠乏により，ALAS2 活性が低下し鉄芽球性貧血の発症に至る．抗結核薬の INH はその代表的な薬剤である．骨髄異形成症候群の場合，多系統の血球に異常が認められる場合，染色体異常が認められる場合は除外できるが，貧血のみで，染色体異常がなく，ビタミンB_6に反応する場合は，遺伝子解析を考慮する．

3. 疫　学

1）発生頻度

　発症頻度は極めて稀で詳細な疫学データはない．最も頻度の高い遺伝性鉄芽球性貧血は XLSA で，現在までに74家系，48種類の ALAS2 の変異が報告されている（未発表を含める）．83例の遺伝性鉄芽球性貧血症例を解析した米国の報告では，ALAS2，SLC25A38, mitochondria DNA, PUS1 に変異を認めた頻度はそれぞれ37%，15%，2.5%，2.5%であった[4]．現在，厚生労働省研究班にて遺伝性鉄芽球性貧血の実態を調査中であるが，日本において診断されている遺伝性鉄芽球性貧血は ALAS2 変異によるものがほとんどであり，SLC25A38, PUS1, ABCB7, GLRX5, SLC19A2遺伝子の変異は認められていない．

2）自然歴・予後

　極めて稀な疾患のため，疫学，病態解析に関してまとまった報告がなく，不明である．

4. 病因・病態

　遺伝性鉄芽球性貧血の原因となる遺伝子は複数あり，それぞれの機能は異なっている．ヘム合成はミトコンドリアにおいてグリシンとスクシニル CoA が重合し，δ-アミノレブリン酸が合成されるステップから始まるが，ALAS2 は赤血球において特異的にこの重合を触媒する酵素であり，本遺伝子の変異によりヘム合成が不全となり，ミトコンドリアでの鉄利用障害が起こるものと考えられている．SLC25A38 はミトコンドリア内膜に存在するトランスポーターであり，グリシンの輸送に関与していると考えられており，鉄芽球性貧血の発症機序は ALAS2 変異と同様であることが予想される[5]．一方，チアミンtransporter である SLC19A2 遺伝子の変異によるミトコンドリア鉄沈着は，チアミン欠乏によるスクシニル CoA の不足が原因と考えられている[6]．ただし，SLC19A2 の変異による鉄芽球性貧血は XLSA と異なり，血中プロトポルフィリンレベルの低下が認められず，また大球性であるため，XLSA 同様のヘム合成障害が原因であるかどうか疑問である．Pearson marrow-pancreas 症候群はミトコンドリア DNA の欠失によるものであり，神経・筋・外分泌機能に障害が認められ，多くは乳児期に死亡する[7]．鉄芽球の形成機序は明らかとなっていないが，呼吸鎖遺伝子の異常によって鉄の還元障害が起こり，フェロケラテーゼによるプロトポルフィリンへの鉄挿入が不全となっている可能性が考えられる．GLRX5 はヘムと

並ぶ鉄利用分子である鉄-硫黄クラスターの合成にかかわる遺伝子であり[8]，ABCB7 はこの鉄-硫黄クラスターのミトコンドリアからの排出を担うトランスポーターである[9]．いずれも，鉄-硫黄クラスターの障害を通じてミトコンドリアにおける鉄の利用障害を誘導すると考えられているが，その機序は共通でない．すなわち，GLRX5 の変異による鉄着は IRP を介した ALAS2 活性低下によるものと考えられているが，ABCB7 の変異においては，これらの所見は確認されていない．PUS1 は tRNA の修飾に関与する遺伝子であり，本遺伝子の変異により，ミトコンドリアでの蛋白質の翻訳に障害が生じるものと考えられているが，鉄利用障害に至る直接的な関与については明らかとなっていない[10]．いずれにおいても，ミトコンドリアでの鉄利用障害により，過剰な鉄がミトコンドリアに沈着し，環状鉄芽球が認められるようになる．この鉄過剰状態は細胞内の酸化還元反応を障害し，アポトーシスを誘導し貧血の発症に至ると考えられている[11]．

5. 臨床症状，検査所見

1）貧　血
病型により軽度〜中等度まで認められる．原因遺伝子が同じであっても，変異によって重症度が異なる．

2）ヘモクロマトーシス
病型と輸血量によりその程度は異なる．
HFE 遺伝子に変異を認めるとヘモクロマトーシスの進行速度が速いが，日本人ではその遺伝子の変異の頻度は少ないといわれている．

3）その他の合併症
病型により，造血不全以外の臓器障害（ataxia，代謝性アシドーシス，膵外分泌不全，インスリン依存性糖尿病，神経症状など）を認めることがある（各病型の特徴を参照）．

4）各病型の特徴
(1) XLSA
小球性低色素性貧血，全身の鉄過剰状態を認める．XLSA の多くの症例において，ALAS2 蛋白質の構造変化により，補酵素であるビタミン B_6 との親和性が低下することが貧血の原因となっていると考えられており，実際に半数以上でビタミン B_6 の投与にて貧血の改善を認める．

(2) GLRX5 変異による遺伝性鉄芽球性貧血
glutaredoxin5 の変異で Fe-S clusters 合成が障害される結果，ミトコンドリアに鉄が沈着する．骨髄での環状鉄芽球は少ないが，中等度の貧血，肝脾腫，鉄過剰を認める．

(3) ataxia を伴う XLSA（XLSA/A）
早期より（通常1歳以内より）ataxia を認める．ataxia は進行しないか，進行しても緩徐である．貧血は小球性低色素性である．貧血は軽度でピリドキシンに反応しない．ミトコンドリアの膜輸送蛋白である ABCB7 遺伝子の変異が原因である．

(4) SLC25A38 変異による遺伝性鉄芽球性貧血
SLC25A38 は glycine を輸送するミトコンドリアの膜蛋白遺伝子と考えられている．常染色体劣性遺伝で，前述のとおり，ALAS2 に次いで，頻度が高い遺伝性鉄芽球性貧血と考えられている．多くは重度の小球性低色素性貧血を呈し，鉄過剰状態にあり，XLSA と同様の臨床症状を呈するため，XLSA を疑う症状を呈するものの ALAS2 の変異が認められない場合，本遺伝子の変異検索が必要である．

(5) Pearson marrow-pancreas syndrome
代謝性アシドーシス，ataxia，膵外分泌不全を伴う．通常乳児期に死亡する．貧血は正球性で好中球減少と血小板減少をときに伴う．ミトコンドリア DNA の欠損が原因で，通常孤発性で de novo の発症例が多い．

(6) thiamine-responsive megaloblastic anemia（TRMA）
インスリン依存性糖尿病，神経性難聴を伴う全身性の疾患．稀な常染色体劣性遺伝で通常幼少期に診断される．貧血は巨赤芽球を伴う大球性の貧血である．チアミンの投与に反応するが，葉酸やビタミン B_{12}，ピリドキシンには反応しない．thiamine transporter である SCL19A2 遺伝子の異常が原因である．

(7) mitochondrial myopathy and sideroblastic anemia（MLASA）
極めて稀な常染色体劣性遺伝疾患．筋症，乳酸アシドーシス，鉄芽球性貧血を特徴とする．pseudouridylate synthase 1 gene（PUS1）の欠損により発症する．

6. 治療法

1）薬物療法
(1) ビタミン補充療法
a. ピリドキシン投与
XLSA では半分以上の患者がピリドキシンの経口投与に反応する（50〜100 mg／日）．表2に XLSA に

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表2 XLSA における遺伝子変異 (pyridoxine に反応する変異は網掛けで示す)

Ex.	substitution		No. of pedigree	
4	L107P		1	
5	M154I		1	
	K156E		1	
	D159	N	1	2
		Y	1	
	T161A		1	
	F165L		2	
	R170	S	1	7
		C	2 (1)	
		L	3 (2)	
		H	1	
	A172T		1	
	D190V		1	
	Y199H		1	
	R204	Q	1	
		stop	1	

Ex.	substitution	No. of pedigree
6	R227C	1
	S251P	1
	D263N	1
7	C276W	1
	G291S	1
	K299Q	1
	V301A	1
8	D351R	1
	T388S	1
	C395Y	1
	G398D	1
9	R411C	4 (1)
	G416D	1
	M426V	1
	R436W	1

Ex.	substitution		No. of pedigree	
	R448Q		3	
9	R452	C	6 (3)	16
		S	2	
		H	8 (2)	
	R458H		1	
	I476N		1	
	Y506-fs		1	
	T508S		1	
10	R517	C	1	2
		G	1	
	P520L		2	
	H524D		1	
	R559H		1	
	R560H		3	
11	V562A		1	
	M567I		1	
	S568G		2 (1)	

おける遺伝子変異を示す. ピリドキシンに反応する変異は網掛けで示す.

b. チアミン投与

TRMA でビタミン B₁ $(25 \sim 75\,mg/日)$ の投与で反応を示す.

その他の疾患では特異的な薬物療法はない.

(2) 鉄キレート療法

特に輸血依存状態となった症例では, 鉄過剰症によるヘモクロマトーシスのリスクが高く, フェリチン値, 臓器障害の有無により, 鉄キレート療法を行う.

2) 輸血療法

必要に応じて施行する.

3) 造血幹細胞移植

これまでに3例の報告がある[12]. いずれも造血能の回復を認めており, 造血幹細胞移植は効果があると考えられる. ただし, ヘモクロマトーシスを伴っ ている症例が多く, その他の合併症が致命的となる可能性もあるため, 前処置などに配慮が必要と考えられる.

7. 問題点・将来展望

遺伝性鉄芽球性貧血は, ビタミン B₆ などで治療が可能なことがあり, 遺伝子の変異の同定が重要である. しかしながら, 稀少疾患であるため, 症例の把握と, 遺伝子解析のセンター化が必要である. さらに, 今後は既知の遺伝子変異を有さない症例における変異遺伝子の同定が課題であり, 同様の課題を持つほかの遺伝性造血不全グループと共同で新規遺伝子同定システムを構築する必要がある.

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総　説

遺伝性鉄芽球性貧血の病態と診断

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Pathophysiology and Diagnosis of Inherited Sideroblastic Anemia

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Abstract　Sideroblastic anemia is characterized by anemia with ring sideroblasts, which are formed by impaired iron utilization in mitochondria. Inherited sideroblastic anemia (ISA) is a rare and heterogeneous disease caused by mutations of genes involved in heme biosynthesis, iron-sulfur (Fe-S) cluster biogenesis, Fe-S cluster transport, and mitochondrial metabolism. There are two-types of ISA, i.e., the syndromic type and non-syndromic type. The most common ISA is X-linked sideroblastic anemia (XLSA), caused by mutations of the erythroid-specific δ-aminolevulinate synthase gene (*ALAS2*), which is the first enzyme of heme biosynthesis in erythroid cells. Reflecting the function of *ALAS2*, XLSA is the non-syndromic type. Sideroblastic anemia due to *SLC25A38* gene mutations, which is speculated to be a mitochondrial transporter for glycine, is the next most common ISA, and its phenotype is similar to that of XLSA. Other forms of inherited sideroblastic anemia are very rare and most of them are the syndromic type, accompanied by symptoms of the nervous system, muscle, or exocrine glands. Molecular analysis of ISA will contribute to the understanding of mitochondrial iron metabolism.

要　旨　鉄芽球性貧血は，環状鉄芽球の出現を特徴とする貧血である．環状鉄芽球はミトコンドリアにおける鉄の利用障害により形成される．遺伝性鉄芽球性貧血は，ヘム合成の異常，鉄-硫黄クラスターの合成・輸送異常にかかわる遺伝子の変異や，ミトコンドリア DNA の変異・欠失などにより発症する先天性疾患である．もっとも頻度が高い遺伝性鉄芽球性貧血は，赤芽球におけるヘム生合成系の初発酵素である赤血球型 5-アミノレブリン酸合成酵素（erythroid-specific 5-aminolevulinate synthase: *ALAS2*）の変異を原因とする X 連鎖性鉄芽球性貧血であり，この貧血の症例の多くは ALAS2 の補酵素であるビタミン B6 の投与により貧血が改善する．遺伝性鉄芽球性貧血はこのほか，複数の遺伝子異常により発症する多様な疾患であるが，いずれも発症頻度は低い．しかしながら，遺伝性鉄芽球性貧血の原因遺伝子の同定および機能解析は，ミトコンドリア，細胞内鉄代謝の生理機構を理解するうえで，医学的にきわめて重要である．

I.　はじめに

鉄芽球性貧血（sideroblastic anemia）は骨髄に環状鉄芽球が出現することを特徴とする疾患であるが，その原因として，遺伝的にいずれかの遺伝子に変異を有することにより発症する遺伝性鉄芽球性貧血と，後天的な要因

による後天性鉄芽球性貧血の2つに大きく分類される[1]．遺伝性鉄芽球性貧血の中でももっとも頻度が高い遺伝性鉄芽球性貧血は，赤芽球におけるヘム生合成系の初発酵素である赤血球型 5-アミノレブリン酸合成酵素（erythroid-specific 5-aminolevulinate synthase: *ALAS2*）の変異を原因とする貧血である．このほかにも，遺伝性鉄芽球性貧血は，ミトコンドリアにおける鉄-硫黄クラスターの合成・輸送，ヘム合成などに関わる遺伝子の変異により発症する．これらの原因遺伝子の多くが，ミトコンドリアの鉄代謝に関わる遺伝子であることから，遺伝性鉄芽球性貧血は造血以外の異常を伴うことが少なくない．さらに，軽症例の中には成人後に診断される例も存在するため，遺伝性鉄芽球性貧血の病態は多様であると

いえる．現在，遺伝性鉄芽球性貧血の疫学，臨床的特徴を明らかにするために，全国的調査研究（厚生労働省難治性疾患克服研究事業；遺伝性鉄芽球性貧血の診断分類と治療法の確立）が進められており，今後，日本における遺伝性鉄芽球性貧血の実態が明らかになっていくものと思われる．本稿では，まず，生体内における鉄代謝を概説し，引き続き，これまでに明らかとなっている遺伝性鉄芽球性貧血の原因遺伝子と，その臨床的特徴を紹介する．

II. 鉄代謝と赤血球造血

鉄は，酸素運搬だけでなく細胞の分裂・増殖や呼吸などに必須の金属元素である．一方で，その反応性からきわめて毒性の高い元素であるため，過剰にならないように吸収・代謝が厳密に制御されている[2]．体内の鉄の総量は 3～4 g であり，70%は赤血球に含まれるヘモグロビン鉄として利用されている．赤血球の寿命は 120 日であることから，毎日全体の 1/120 の赤血球が処理され，この赤血球に含まれる 25 mg の鉄が新たな材料鉄として再利用される．食餌から吸収される外来性の鉄は一日 1～2 mg であるため，利用される鉄は圧倒的に再利用鉄のほうが多く，生体は鉄利用をリサイクリングシステムに頼っているということが理解できる[2]．食餌鉄は主として十二指腸から吸収されるが，非ヘム鉄は，金属トランスポーターである divalent metal transporter 1（DMT1）によって腸上皮細胞内に取り込まれ，ヘム鉄は腸管内腔側細胞膜に存在する heme carrier protein-1（HCP-1）によって細胞内に取り込まれる[3]．非ヘム鉄，ヘム由来鉄ともに，血管腔側トランスポーターであるフェロポルチンにより上皮細胞から排出され[2]，トランスフェリンと結合し，赤血球でのヘモグロビン合成に利用される．マクロファージにて処理された赤血球由来の鉄も同様にフェロポルチンを介して，細胞外へ排出され，トランスフェリンにより運搬され，ヘモグロビン合成に利用される．生体内鉄量は，鉄飽和度，炎症，低酸素などさまざまな要因で調節されているが，その調節は肝臓から分泌されるペプチドホルモンであるヘプシジンによりなされている[4]．ヘプシジンは，前述の腸上皮細胞やマクロファージにおける鉄トランスポーターであるフェロポルチンの発現を調節することにより，生体内の鉄吸収・再利用を制御している．

トランスフェリンに結合した鉄はトランスフェリンとともにトランスフェリンレセプターと結合し細胞内に取り込まれた後，pH の低いエンドソームで結合が解かれる[3]．腸上皮細胞において非ヘム鉄のトランスポーター

として機能している DMT1 は，エンドソームにおいてもトランスポーターとして機能し，鉄はこの DMT1 を介して細胞質へと排出される．排出後，ミトコンドリアまでの鉄の輸送経路に関してはいまだ明らかではない．ミトコンドリアに到達した鉄は，ミトコンドリア内膜に存在するトランスポーターであるミトフェリンを介してミトコンドリアに取り込まれる．ミトコンドリアに移行後，鉄はヘム合成，鉄-硫黄クラスター合成に利用される．

III. 遺伝性鉄芽球性貧血

これまでに，遺伝性鉄芽球性貧血においては複数の遺伝子変異が同定されているが，最近報告された米国における遺伝性鉄芽球性貧血の解析では，ALAS2, SLC25A38, mitochondria DNA, PUS1 に変異を認めた症例がそれぞれ 37%, 15%, 2.5%, 2.5%であった[5]．43%においては既報の遺伝子変異が認められず，これ以外の未知の遺伝子変異により発症する遺伝性鉄芽球性貧血が多く存在するものと推測される．現時点での日本における遺伝性鉄芽球性貧血の調査研究において，変異が認められている遺伝子は ALAS2 のみであるが，米国同様に変異遺伝子が同定されていない例も多く存在している．遺伝性鉄芽球性貧血は，貧血以外の症状を認めない non-syndromic type と，貧血以外に神経・筋などの臓器障害を認める syndromic type に大きく分けられる（表）．Non-syndromic type の代表は，ALAS2 の変異による X 連鎖性鉄芽球性貧血であり，syndromic type には，ミトコンドリア DNA の欠損による Pearson 症候群などが含まれる．以下，もっとも頻度が高い遺伝性鉄芽球性貧血である XLSA を中心に，おもな遺伝性鉄芽球性貧血とその原因遺伝子について紹介する．

1. 赤血球型 5-アミノレブリン酸合成酵素（ALAS2）の変異による X 連鎖性鉄芽球性貧血（X-linked sideroblastic anemia: XLSA）

ヘム合成を触媒する酵素は 8 種類からなり，最初の酵素と最後の 3 つの酵素はミトコンドリア内に存在し，中間の 4 つの酵素は細胞質に存在する．赤血球における最初のステップは赤血球型 5-アミノレブリン酸合成酵素（ALAS2）による 5-アミノレブリン酸の合成である．その後，順次ポルフィリン環が合成され，最終的に出来上がったポルフィリン環にフェロケラターゼにより鉄が挿入され，ヘムが完成する．ALAS2 はグリシンとスクシニル CoA を重合しアミノレブリン酸を合成する，赤血球におけるヘム合成の初発酵素である（図）．この ALAS2 遺伝子の変異によって発症する鉄芽球性貧血が X 連鎖性鉄芽球性貧血（XLSA）である．XLSA は遺伝性

表　遺伝性鉄芽球性貧血

	Non-syndromic			Syndromic			
	XLSA	SA/SCL25A38	SA/GLRX5	XLSA/A	PMPS	MLSA	TRMA
遺伝形式	X連鎖性	常染色体劣性?	常染色体劣性?	X連鎖性	不定	常染色体劣性?	常染色体劣性?
遺伝子座	Xp11.21	3番染色体	14q32.13	Xq13.1	ミトコンドリア	12q24	1q23.3
遺伝子	ALAS2	SCL25A38	GLRX5	ABCB7	ミトコンドリア	PUS1	SLC19A2
変異	ミスセンス	ミスセンス	ミスセンス	ミスセンス	欠失	ミスセンス	ミスセンス
治療	Vit B6	?	?	—	—	—	Vit B1
病変部位（造血以外）	—	—	—	神経	膵, 肝, 腎筋, 神経	筋, 乳酸アシドーシス	膵（糖尿病）難聴

XLSA: X-linked sideroblastic anemia, XLSA/A: X-linked sideroblastic anemia with ataxia, PMPS: Pearson marrow-pancreas syndrome, MLSA: mitochondrial myopathy and sideroblastic anemia, TRMA: thiamine-responsive megaloblastic anemia.
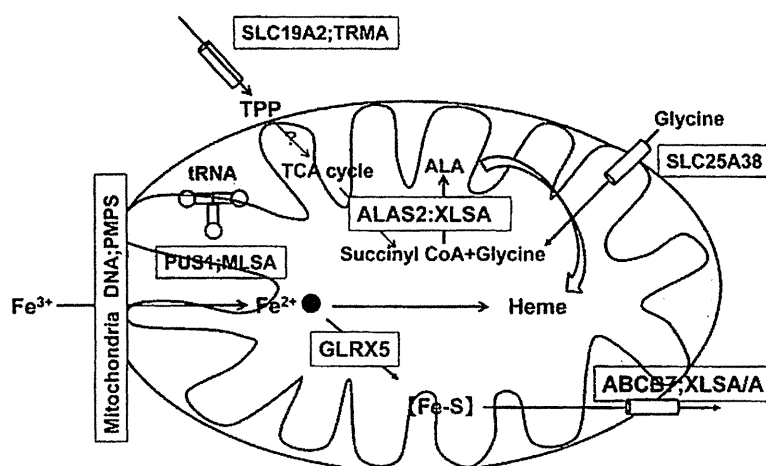

図　遺伝性鉄芽球性貧血の原因遺伝子とミトコンドリア鉄代謝
遺伝性鉄芽球性貧血の原因遺伝子の機能を示す. ALAS2はスクシニルCoAとグリシンを重合しALAを合成するヘム合成の初発酵素である. SCL25A38はALASにグリシンを供給するトランスポーターと考えられている. ABCB7は鉄-硫黄クラスターの輸送に, GLRX5は鉄-硫黄クラスターの合成に機能していると考えられている. PUS1はtRNAのウリジン修飾に機能している. SCL19A3はチアミン（ビタミンB1）のトランスポーターである. ミトコンドリアDNAの欠損は, 三価鉄の還元障害をきたし, その結果, 鉄の利用不全が起こると考えられている.

鉄芽球性貧血の中でもっとも発症頻度が高い. 赤血球型アイソザイムである *ALAS2* の遺伝子座がX染色体（Xp11.21）であることが1992年に明らかとなり[9], さらにXLSAで *ALAS2* 変異が報告されたことで[10], *ALAS2* がXLSAの原因遺伝子であることが確認された. ちなみに, 1946年に米国のRundlesとFallsにより, 遺伝性貧血として報告されたRundles-Falls syndromeは, 1994年に当該家系の遺伝子・臨床病態解析がなされた結果, XLSAであることが明らかとなっている[11,12]. *ALAS2* 遺伝子の変異による鉄芽球形成の機序は, ヘム合成不全に起因するミトコンドリアでの鉄利用障害と考えられている. われわれは *ALAS2* 変異と鉄芽球性発症をより明確に関連づけるためにALAS2欠損マウスを作製した[13].

このマウスは貧血のため, 胎生11.5日までに死亡し, その赤血球には鉄の沈着が認められ, *ALAS2* の変異が赤芽球における鉄代謝異常を引き起こすことが明らかとなった. さらに, XLSAの臨床病態を再現するために, 中島らは, ALAS2欠損マウスをヒトALAS2で部分的にレスキューしたトランスジェニックマウスを作製し, このマウスで環状鉄芽球が観察されることを報告している[14]. これまでに40家系以上のXLSAが報告されているが, XLSAの臨床的特徴は, 半数以上の症例がALAS2の補酵素であるビタミンB6の投与に反応することである. ビタミンB6の結合部位は *ALAS2* 遺伝子の第9エクソンに存在するが, 反応症例の変異部位はエクソン5からエクソン11にわたり幅広く同定されている.