RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. Blood. 2007;109(3): 980-6.
14. Miyake K, Utsugisawa T, Flygare J, Kiefer T, Hamaguchi I, Richter J, et al. Ribosomal protein S19 deficiency leads to reduced proliferation and increased apoptosis but does not affect terminal erythroid differentiation in a cell line model of Diamond-Blackfan anemia. Stem Cells. 2008;26(2): 323-9.
15. Gazda HT, Grabowska A, Merida-Long LB, Latawiec E, Schneider HE, Lipton JM, et al. Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. Am J Hum Genet. 2006;79(6):1110-8.
16. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. Hum Mutat. 2007;28(12): 1178-82.
17. Gazda HT, Sheen MR, Vlachos A, Choesmel V, O'Donohue MF, Schneider H, et al. Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. Am J Hum Genet. 2008;83(6):769-80.
18. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Petrtylova K, Mihal V, et al. Identification of mutations in the ribosomal protein L5 (RPL5) and ribosomal protein L11 (RPL11) genes in Czech patients with Diamond-Blackfan anemia. Hum Mutat. 2009;30(3): 321-7.
19. Farrar JE, Nater M, Caywood E, McDevitt MA, Kowalski J, Takemoto CM, et al. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. Blood. 2008;112(5):1582-92.
20. Ebert BL, Pretz J, Bosco J, Chang CY, Tamayo P, Galili N, et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. Nature. 2008;451 (7176):335-9.
21. Xu G, Nagano M, Kanezaki R, Toki T, Hayashi Y, Taketani T, et al. Frequent mutations in the GATA-1 gene in the transient myeloproliferative disorder of Down syndrome. Blood. 2003;102(8):2960-8.
22. Willig TN, Draptchinskaia N, Dianzani I, Ball S, Niemeyer C, Ramenghi U, et al. Mutations in ribosomal protein S19 gene and Diamond Blackfan anemia: wide variations in phenotypic expression. Blood. 1999;94(12):4294-306.
23. Ramenghi U, Campagnoli MF, Garelli E, Carando A, Brusco A, Bagnara GP, et al. Diamond-Blackfan anemia: report of seven further mutations in the RPS19 gene and evidence of mutation heterogeneity in the Italian population. Blood Cells Mol Dis. 2000;26(5):417-22.
24. Cmejla R, Blafkova J, Stopka T, Zavadil J, Pospisilova D, Mihal V, et al. Ribosomal protein S19 gene mutations in patients with Diamond-Blackfan anemia and identification of ribosomal protein S19 pseudogenes. Blood Cells Mol Dis. 2000;26(2):124-32.
25. Proust A, Da Costa L, Rince P, Landois A, Tamary H, Zaizov R, et al. Ten novel Diamond-Blackfan anemia mutations and three polymorphisms within the rps19 gene. Hematol J. 2003;4(2):132-6.
26. Campagnoli MF, Ramenghi U, Armiraglio M, Quarello P, Garelli E, Carando A, et al. RPS19 mutations in patients with Diamond-Blackfan anemia. Hum Mutat. 2008;29(7):911-20.
27. Gazda H, Lipton JM, Willig TN, Ball S, Niemeyer CM, Tchernia G, et al. Evidence for linkage of familial Diamond-Blackfan anemia to chromosome 8p23.3-p22 and for non-19q non-8p disease. Blood. 2001;97(7): 2145-50.
28. Lidral AC, Murray JC. Genetic approaches to identify disease genes for birth defects with cleft lip/palate as a model. Birth Defects Res A Clin Mol Teratol. 2004; 70(12):893-901.
29. Quarello P, Garelli E, Carando A, Brusco A, Calabrese R, Dufour C, et al. Diamond-Blackfan anemia: genotype-phenotype correlation in Italian patients with RPL5 and RPL11 mutations. Haematologica. 2010; 95(2):206-13.

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Roles of Porphyrin and Iron Metabolisms in the δ-Aminolevulinic Acid (ALA)-induced Accumulation of Protoporphyrin and Photodamage of Tumor Cells

Yoshiko Ohgari[1], Yoshinobu Miyata[1], Taeko Miyagi[1], Saki Gotoh[1], Takano Ohta[1], Takao Kataoka[1], Kazumichi Furuyama[2] and Shigeru Taketani*[1,3]

[1]Department of Biotechnology, Kyoto Institute of Technology, Kyoto, Japan
[2]Department of Molecular Biology and Applied Physiology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[3]Insect Biomedical Center, Kyoto Institute of Technology, Kyoto, Japan

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ABSTRACT

δ-Aminolevulinic acid (ALA)-induced porphyrin accumulation is widely used in the treatment of cancer, as photodynamic therapy. To clarify the mechanisms of the tumor-preferential accumulation of protoporphyrin, we examined the effect of the expression of heme-biosynthetic and -degradative enzymes on the ALA-induced accumulation of protoporphyrin as well as photodamage. The transient expression of heme-biosynthetic enzymes in HeLa cells caused variations of the ALA-induced accumulation of protoporphyrin. When ALA-treated cells were exposed to white light, the extent of photodamage of the cells was dependent on the accumulation of protoporphyrin. The decrease of the accumulation of protoporphyrin was observed in the cells treated with inducers of heme oxygenase (HO)-1. The ALA-dependent accumulation of protoporphyrin was decreased in HeLa cells by transfection with HO-1 and HO-2 cDNA. Conversely, knockdown of HO-1/-2 with siRNAs enhanced the ALA-induced protoporphyrin accumulation and photodamage. The ALA effect was decreased with HeLa cells expressing mitoferrin-2, a mitochondrial iron transporter, whereas it was enhanced by the mitoferrin-2 siRNA transfection. These results indicated that not only the production of porphyrin intermediates but also the reuse of iron from heme and mitochondrial iron utilization control the ALA-induced accumulation of protoporphyrin in cancerous cells.

INTRODUCTION

Photodynamic therapy (PDT) was developed as treatment of nonmelanoma skin tumors and preneoplastic skin lesions. PDT includes the activation of photosensitizer, which causes the release of singlet oxygen and other reactive oxygen species upon exposure to light, resulting in photodamage of cells, followed by tissue destruction (1). In tumor cells, *via* the heme biosynthesis pathway, photosynthesizer protoporphyrin is synthesized from a large amount of exogenous ALA and accumulates in a specific manner (2). The application of ALA following PDT treatment has been used in the treatment of skin diseases and has advantages over systemic administration in that the entire body does not face sensitization (3,4). ALA-induced PDT has been successfully applied in various medical fields, including urology, gastroenterology and dermatology (3–5). In heme biosynthesis, ALA is catalyzed by four cytosolic enzymes, ALA-dehydratase, porphobilinogen deaminase (PBGD), uroporphyrinogen synthase (UROS) and uroporphyrinogen decarboxylase and by two mitochondrial enzymes, coproporphyrinogen oxidase (CPOX) and protoporphyrinogen oxidase (PPOX), converting to protoporphyrin (6). Finally, ferrochelatase (FECH) catalyzes the insertion of ferrous ions into protoporphyrin to produce heme (7). Although there are reports that ALA-induced PDT can also be used as a fluorescence detection marker for the photodiagnosis of tumors (3,4,8), the mechanisms involved in the specific accumulation of protoporphyrin in cancerous tissues have not been clearly demonstrated. Previously, we (7,8) reported that protoporphyrin accumulates owing to limited capacity for the FECH reaction. In addition, we also reported an increase in the uptake of ALA by cancerous cells (8).

Heme oxygenase (HO) is the rate-limiting enzyme in the cellular catabolism of heme to biliverdin, carbon monoxide and free iron. Biliverdin is subsequently converted to bilirubin by biliverdin reductase (9,10). The enzyme is expressed in a variety of organisms. In mammals, two HO isoforms, HO-1 and HO-2, have been reported. The expression of HO-1 is induced by heme, a substrate of the enzyme and metal ions, such as arsenite and cadmium, whereas that of HO-2 is constant (10). Interestingly, most of the known HO-1 inducers stimulate the production of ROS or lead to a depletion of glutathione levels, indicating the involvement of the induction of HO-1 in cellular protection against oxidative stress (11,12). The induced HO-1 could have an advantage in cell growth, resulting in a protective effect against the photosensitivity of tumors, whereas the knockdown of HO-1 gives rise to suppression of cell growth with failure in the photosensitivity (13). In contrast, the reduced expression of HO-1 mRNA by siRNA increased cell death upon ALA-PDT (14). Thus, the effect of the expression of HO-1 on ALA-PDT is inconclusive. Furthermore, the contribution of HO-2 to the effectiveness of ALA-PDT is unclear.

*Corresponding author email: taketani@kit.ac.jp (Shigeru Taketani)

Iron utilization in mitochondria in cancerous cells also remains poorly understood. It was shown that functions of the respiratory chain enzymes including iron- or heme-containing proteins were impaired in tumors (15). As for mitochondrial iron metabolism, mitoferrin, a mitochondrial iron importer, transports iron in mitochondria and can regulate iron-chelation into protoporphyrin by FECH (16). Mitoferrin-1 is synthesized in erythroid cells, whereas mitoferrin-2 is synthesized in various tissues (16). Otherwise, iron delivery to the iron-sulfur cluster biosynthetic machinery can be mediated by frataxin, a mitochondrial iron-chaperon (17). The reduction of the expression of frataxin causes Friedreich's ataxia, an inherited neurodegenerative disorder (17). Targeted disruption of frataxin in murine hepatocytes causes decreased life span and increased liver tumor formation, whereas the over expression of frataxin leads to inhibition of cell growth of cancer, by increasing oxidative phosphorylation (18). Thus, iron deficiency related to FECH in cancerous cells may be responsible for the ALA-induced accumulation of protoporphyrin. Previous studies (8,19) also showed that removal of iron from the cells with an iron chelator, desferrioxamine, markedly enhanced the ALA-induced accumulation of protoporphyrin. However, as desferrioxamine has a protective effect against phototoxicity *in vitro* and *in vivo*, it did not appear to confer additional benefit in ALA-PDT (20). Therefore, it is necessary to clarify the utilization of mitochondrial iron for heme production. In this study, we investigated the role of the utilization of iron and the recycling of iron from heme for the ALA-induced accumulation of protoporphyrin and photodamage. Down-regulation of the expression of iron-metabolizing molecules mitoferrin-2, frataxin and HO-1/-2 increased the ALA-induced photodamage, whereas up-regulated expression gave the reverse effect. The importance of increased expression of porphyrin-metabolizing enzymes on ALA-PDT was also shown.

MATERIALS AND METHODS

Materials. Protoporphyrin IX, cobalt-protoporphyrin (Co-PP) and tin-protoporphyrin (Sn-PP) were purchased from Frontier Scientific Co. (Logan, UT). The antibodies for HO-1, HO-2 and actin used were as previously described (10). Monoclonal antibodies for frataxin and HA were products of Millipore Co. (Billerica, MA) and MBL Laboratories (Tokyo, Japan), respectively. HO-1 (No. sc-44306), frataxin (No. sc-40580), mitoferrin-2 (No. sc-90800) and control siRNAs (No. sc-37007) were products of Santa Cruz Biotechnology (Santa Cruz, CA). HO-2 siRNA was synthesized by Sigma–Aldrich (Tokyo, Japan): sense r(CCACCACGGCACUUUACUUCA) and antisense r(AAGUAAAGUGCCGUGGUGGCC). All other chemicals used were of analytical grade.

Plasmids. Plasmids pcDNA3-HF (human FECH) (8), pcDNA3-HCPOX (human CPOX) (21), pCD-PPOX (human PPOX) (22), pCAG-HMBSu (human nonerythroid PBGD) (23) and pCAG-UROS (human nonerythroid UROS) (23) were used for the expression of enzymes in cells. Plasmids pHHO-1 and pHHO-2 carrying human HO-1 and HO-2 cDNAs, respectively, were kind gifts from Dr. Shibahara (24,25). To construct pcDNA3-frataxin, PCR was performed with mouse liver cDNA library. Primers 5′-AAGGATCCATGTGGACT CTCGGGCGC-3′ and 5′-AAGGATCCTCAAGCATCTTTTCCGG A-3′ were used. Amplified cDNAs were digested with *Bam*HI and ligated into *Bam*HI-digested pcDNA3. To obtain the full-length cDNA fragment of human mitoferrin-2, PCR reaction was performed with the following primers: 5′-AATCTAGAGAGTTGGAGGG GCGGGGT-3 and 5′-AAAAGCTTGCCAGCCCTCCACTCT-3′ for mitoferrin-2 and human kidney cDNA library as a template. Then, to make mammalian expression vector carrying mitoferrin-2 containing an HA-tag at the C-terminus, the amplified cDNA was ligated into the *Xba*I/*Hind*III site of the vector pCG-C-HA (26).

Cell cultures. Human epithelial cervical cancer HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (FCS) and antibiotics. The cells (1×10^5) in a 1.5-cm-diameter dish were transfected using Lipofectamine 2000 (Invitrogen Co., San Jose, CA) with the indicated plasmid then incubated in the presence of 10% FCS at 37°C for 16–24 h (26). The cells also transfected with siRNAs were cultured for 48 h. The cells were then incubated in the absence or presence of ALA (0.5–1 mM) for 16 h before being exposed to light, as described previously (7,8).

Exposure of the cells to light. The cells were incubated with ALA (1 mM) for 8–16 h and 1.0 mL of fresh drug-free medium was then added. Irradiation with visible light was carried out under sterile conditions, using a fluorescence lamp, in a CO_2 incubator, as described previously (8,27). Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Each experiment was carried out in triplicate or quadruplicate. Cell viability (cell survival) is expressed as a percentage of control cells. Porphyrins were extracted from the cells with 96% ethanol containing 0.5 M HCl (8). The amount of protoporphyrin was determined by fluorescence spectrophotometry, as previously described (8,27).

Immunoblotting. The lysates from HeLa cells were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). Immunoblotting was carried out with antibodies for HO-1, HO-2, HA, frataxin and actin, as the primary antibodies (8).

Reverse transcriptase (RT)-PCR analysis. Total RNA was isolated from the cells by the guanidium isothiocyanate method (26). Single-strand cDNA derived from the RNA was synthesized with the oligo (dT) primer, using ReveTra Ace (Toyobo, Co., Tokyo, Japan), followed by PCR, using the indicated primers. The amount of cDNA added to the reaction mixture was normalized by the intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon. The cDNAs obtained were analyzed using a 1% agarose gel and electrophoresed. The primers were 5′-CCGGGGCCGGGGGACCTTAG-3′ (forward) and 5′-GCGGGTACCCACGCGAATCAC-3′ for PBGD, 5′-CCCCATCGGAAATTGCTTAGG-3′ (forward) and 5′-CTTTCC CAGACTTCAGTTTTATTG-3′ for UROS, 5′-ATGTTGCCTAAGA GACCTC-3′ (forward) and 5′-ACAAAATGGCAATTTACC-3′ for CPOX, 5′-CCCACAGCCAGACTCAGC-3′ (forward) and 5′-GCTG TTAGGTTCTGTGCC-3′ for PPOX, 5′-GTGCAAAACCTCAAG TT-3′ (forward) and 5′-TCACAGCTCCTGGCTGGT-3′ for FECH, 5′-ATGTGGACTCTCGGGCGC-3′ (forward) and 5′-CTCAAGCA TCTTTTCCGGA-3′ for frataxin, 5′-GAGTTGGAGGGGCGGG GT-3′ (forward) and 5′-GCCAGCCCTCCACTCT-3′ for mitoferrin-2 and 5′-TGGGTGTGAACCACGAGA-3′ (forward) and 5′-TTACT CCTTGGAGGCCATG-3′ for GAPDH.

RESULTS

Effect of the expression of porphyrin-biosynthetic enzymes on the ALA-induced accumulation of protoporphyrin and photodamage in HeLa cells

Previously, we (8) reported that the decrease of the expression of FECH led to enhancement of the ALA-induced accumulation and photodamage. To examine whether other heme-biosynthetic enzymes are involved in the enhancement of ALA-induced accumulation of protoporphyrin, HeLa cells were transfected with pcDNA3-HF, pcDNA3-HCPOX, pCD-PPOX, pCAG-HMBSu and pCAG-UROS. The expression of these enzymes was not examined owing to lack of availability of the corresponding antibody, but RT-PCR analysis showed the increased expression of the corresponding transcript by transfection (Fig. 1A). The cells were incubated with 1 mM ALA and the accumulation of protoporphyrin was examined.

As shown in Fig. 1B, the highest accumulation of protoporphyrin in PBGD-transiently expressing cells was observed

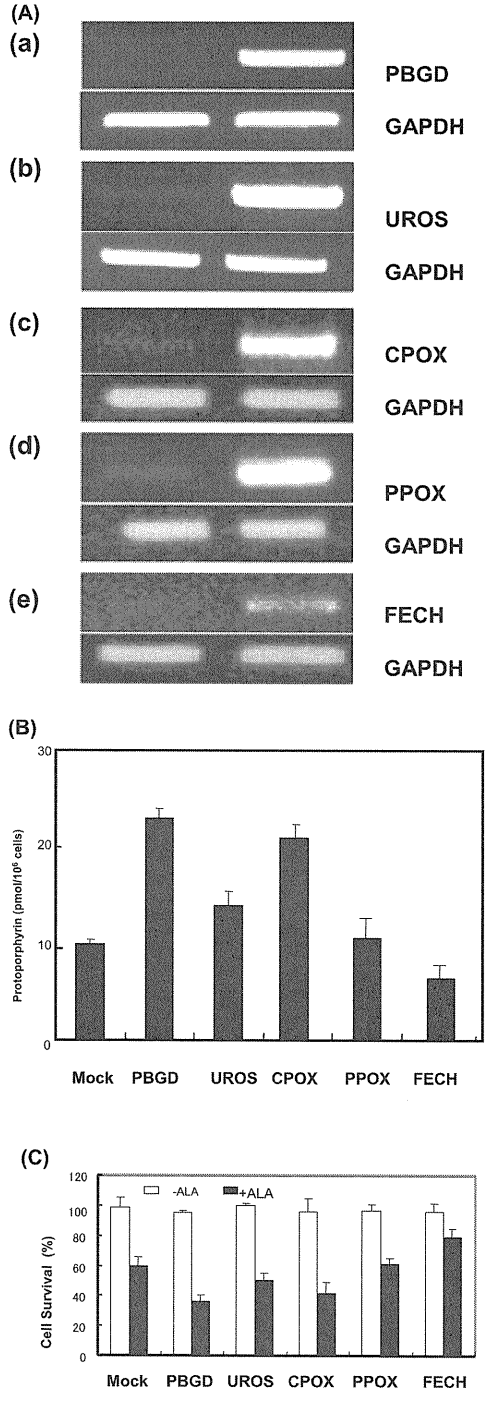

Figure 1. Effect of the expression of heme-biosynthetic enzymes on ALA-induced accumulation of protoporphyrin and photodamage in HeLa cells. (A) HeLa cells were transfected with pcDNA3-HF, pcDNA3-HCPOX, pCD-PPOX, pCAG-HMBSu and pCAG-UROS. After 16-h incubation, RNA was isolated and treated with DNase I. RT-PCR for PBGD (a), UROS (b), CPOX (c), PPOX (d) and FECH mRNA (e) was performed; (B) Effect of the expression of PBGD, UROS, CPOX, PPOX and FECH on the ALA-induced accumulation of protoporphyrin. HeLa cells (5×10^5) transfected with the indicated plasmids were incubated for 24 h and treated with 1 mM ALA for 16 h. The cells were washed twice with phosphate-buffered saline, then porphyrin was extracted and measured fluorospectrophotometrically; (C) Photosensitivity. Fresh DMEM was added to the cells treated as above, followed by exposure to white light; then surviving cells were assessed by MTT assay. Data are the mean ± SD of three independent experiments.

compared with that in control cells. The expression of UROS or CPOX also increased the accumulation. The accumulation in FECH-expressing cells was decreased, whereas that in PPOX-expressing cells was similar to that in the control. When enzyme-expressing cells were exposed to white light and photodamage was examined, the extent of cell survival was found to be related to low accumulation of protoporphyrin (Fig. 1C). No significant cell death was observed by irradiation minus ALA or by treatment with ALA minus light (data not shown). These results indicated that increase in the expression of heme-biosynthetic enzymes, including PBGD, UROS and CPOX led to high accumulation of protoporphyrin.

Involvement of iron reutilization from heme in ALA-induced accumulation of protoporphyrin and photodamage in HeLa cells

To examine if iron-containing compounds decrease the accumulation of protoporphyrin from ALA, HeLa cells were incubated with 0.5 mM ALA by the addition of 10 μM hemin or 50 μM Fe-NTA for 16 h. Porphyrins were extracted from the cells and determined. As shown in Fig. 2A, the

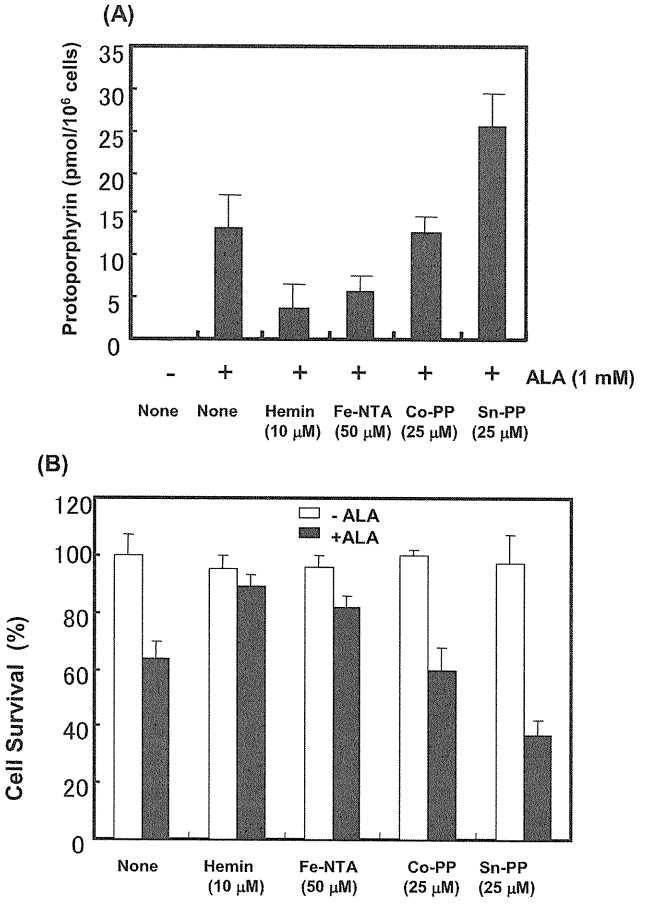

Figure 2. Effect of metalloporphyrins and Fe-NTA on ALA-induced accumulation of protoporphyrin and photodamage. (A) Effect of hemin, Fe-NTA, Co-PP and Sn-PP on the ALA-induced accumulation of protoporphyrin. HeLa cells (5×10^5) were incubated with 1 mM ALA plus the indicated concentration of chemicals for 16 h. Porphyrin was extracted from the cells and measured using a fluorospectrophotometer; (B) Effect of hemin, Fe-NTA, Co-PP and Sn-PP on ALA-induced photodamage. The cells treated as above were irradiated, and survival of the cells was analyzed by MTT assay. Data are the mean ± SD of three to four independent experiments.

accumulation of ALA-induced protoporphyrin in hemin- or ferric ion-nitrilotriacetate (FeNTA)-treated cells was decreased, compared with that in ALA-treated cells. Sn-PP, an inhibitor of HO, increased the accumulation of protoporphyrin. Co-PP, a substrate of HO, was without effect on the accumulation. When the ALA-induced photodamage was examined, hemin and Fe-NTA reduced the photodamage dependent on the decrease of protoporphyrin (Fig. 2B). Sn-PP but not Co-PP increased the photodamage. The above results suggest that the generation of iron from heme may decrease the photodamage and accumulation of protoporphyrin.

Our previous studies (28,29) showed that treatment of the cells with hemin and metal ions resulted in the induction of HO-1. When the cells were treated with hemin, arsenite or cadmium ions for 16 h, HO-1 was markedly induced (Fig. 3A). The ALA-induced accumulation of protoporphyrin in arsenite and cadmium ion-treated cells was decreased compared with that in the control, but the extent was less than that in the case of hemin (Fig. 3A). These results suggest that reutilization of iron generated from heme by HO led to the decrease of the accumulation of protoporphyrin. Then, the HeLa cells were transfected with pHHO-1 or pHHO-2. As shown in Fig. 3B, the expression of HO-1 and HO-2 by the transfection was increased. When the cells were then treated with ALA, the accumulation of protoporphyrin in HO-1 or HO-2-expressing cells was decreased, indicating that the increase in the expression of HOs can facilitate the recycling of iron from heme. These observations led us to examine if knockdown of the HO-1/-2 expression affects the accumulation of protoporphyrin. When HeLa cells were transfected with HO-1/-2 siRNAs and incubated for 48 h, the levels of HO-1 and HO-2 proteins were markedly decreased (Fig. 4A). After the subsequent 16-h incubation with ALA, the content of porphyrin was measured. As expected, the accumulation of protoporphyrin was increased by knockdown of HO-1 and HO-2 (Fig. 4B). The photodamage by HO-1/-2 double knockdown was much greater than that by transfection of control RNA (Fig. 4C). These results indicate that cessation of the recycling of iron from heme enhances the ALA-induced photodamage.

Involvement of mitochondrial iron-metabolizing proteins in regulating the ALA-induced accumulation of protoporphyrin and photodamage

Recently, some researchers (16) reported that mitochondrial iron-metabolizing proteins including mitoferrin-2 and frataxin regulate heme and Fe-S cluster biosynthesis. To clarify the involvement of these proteins in the ALA-induced accumulation of protoporphyrin, HeLa cells transiently expressing frataxin or nonerythroid type mitoferrin-2 were made (Fig. 5A). After these cells were incubated with ALA for 16 h, porphyrin in the cells was examined. The expression of mitoferrin-2, but not frataxin, decreased the ALA-induced accumulation of protoporphyrin in a dose-dependent manner (Fig. 5B). The ALA-induced photodamage with these cells was also examined. The light-resistant cells were increased dependent on the decrease of protoporphyrin (Fig. 5C). Finally, knockdown of the expression of frataxin and mitoferrin-2 using siRNA was carried out (Fig. 6A). The ALA-induced accumulation of protoporphyrin in frataxin- or mitoferrin-2-deficient cells was more than that of control cells (Fig. 6B). Upon exposure of the cells to light, photodamage of frataxin- or mitoferrin-2-deficient cells was greater than that of the

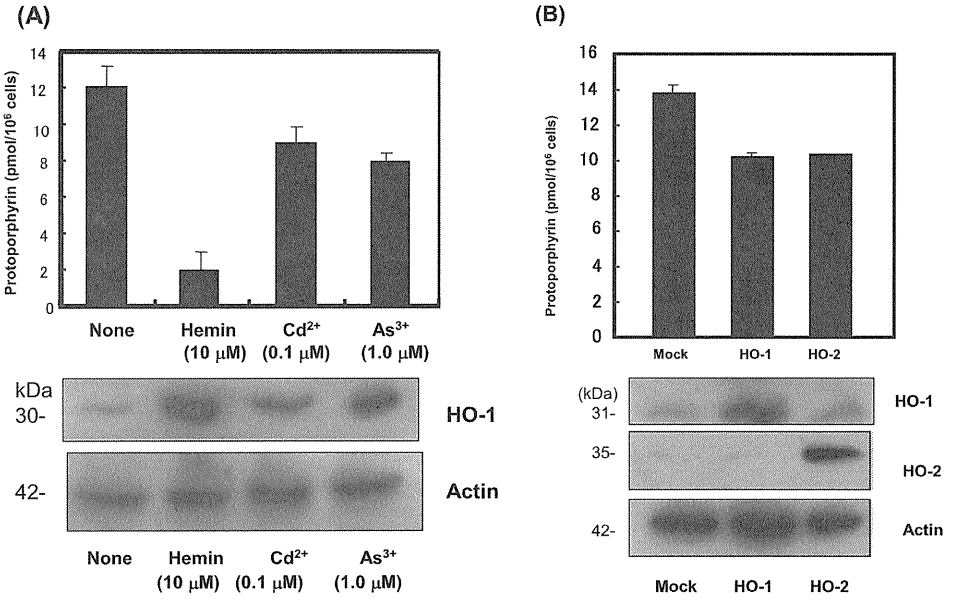

Figure 3. Reduction of ALA-induced accumulation of protoporphyrin and photodamage by the expression of HO-1,-2. (A) Effect of hemin, sodium arsenite and cadmium chloride on the ALA-dependent accumulation of protoporphyrin. Upper panel: HeLa cells (5×10^5) were treated with the above chemicals at the indicated concentration plus 1 mM ALA for 16 h. The accumulated protoporphyrin was measured. Data are the mean ± SD of three independent experiments. Lower panels: Immunoblots of HO-1. Cell lysates from cells treated with hemin, sodium arsenite and cadmium chloride for 16 h were analyzed by SDS-PAGE, followed by immunoblotting; (B) Effect of over expression of HO-1 and HO-2 on the accumulation of protoporphyrin. The cells transfected with pHHO-1 and pHHO-2 were cultured for 16 h, followed by incubation with 1 mM ALA for 8 h. Upper panel: Porphyrin was extracted and determined. Lower panel: Immunoblots of HO-1 and HO-2.

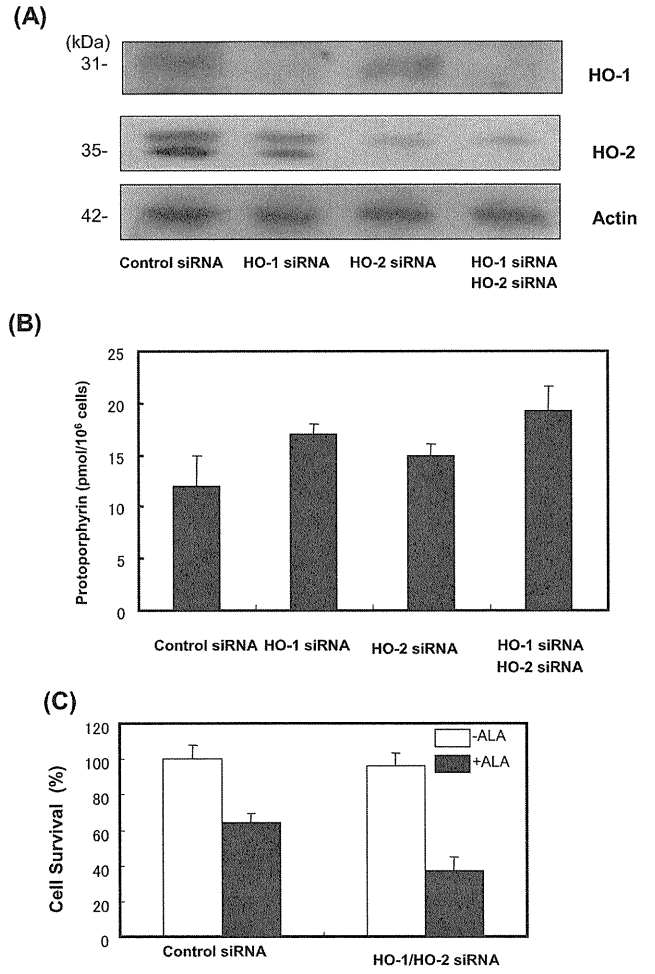

Figure 4. Enhancement of the ALA-induced accumulation of protoporphyrin and photodamage by knockdown of HO-1 and HO-2. (A) The cells (5×10^5) transfected with HO-1 and HO-2 siRNAs were cultured for 48 h, followed by incubation with 1 mM ALA for 16 h. The cellular protein from the cells as above was analyzed 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.

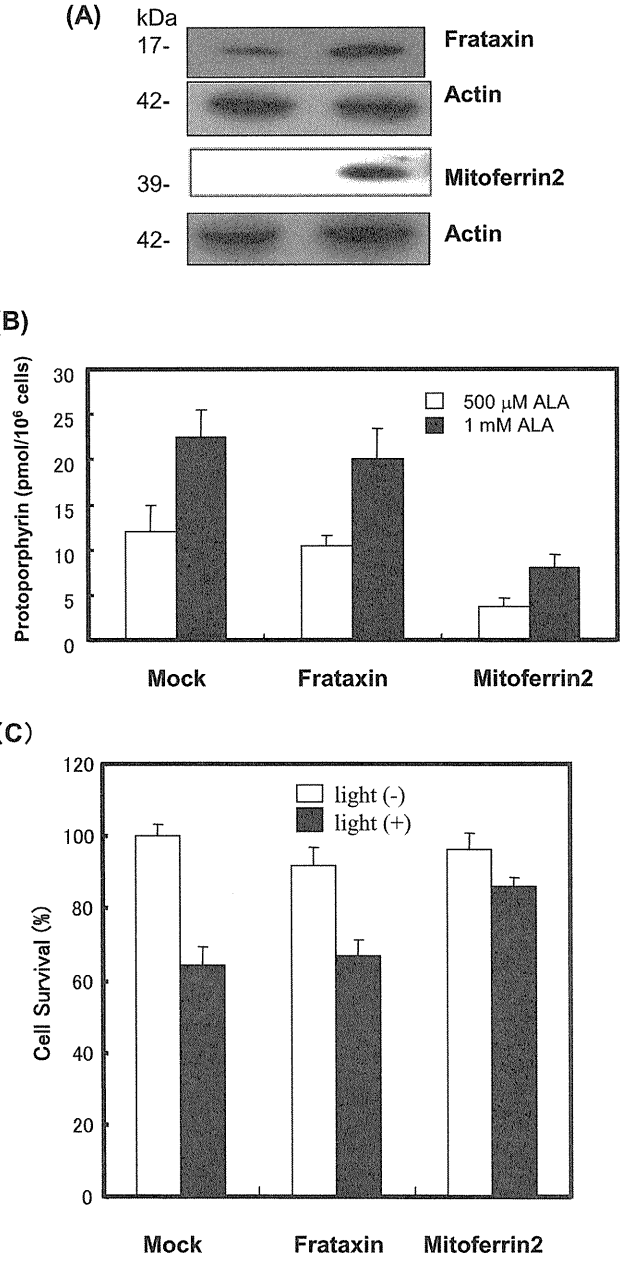

Figure 5. Effect of the expression of frataxin and mitoferrin-2 on the ALA-induced protoporphyrin and photodamage. HeLa cells (5×10^5) were transfected with pcDNA3-frataxin or pCG-C-mitoferrin-2 and incubated for 16 h. The cells were then incubated with 500 μM and 1 mM ALA for 8 h. (A) Immunoblots. Cellular proteins from the cells as treated above were analyzed by SDS-PAGE, followed by immunoblotting 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.

control (Fig. 6C). Without irradiation, virtually no photodamage 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, 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

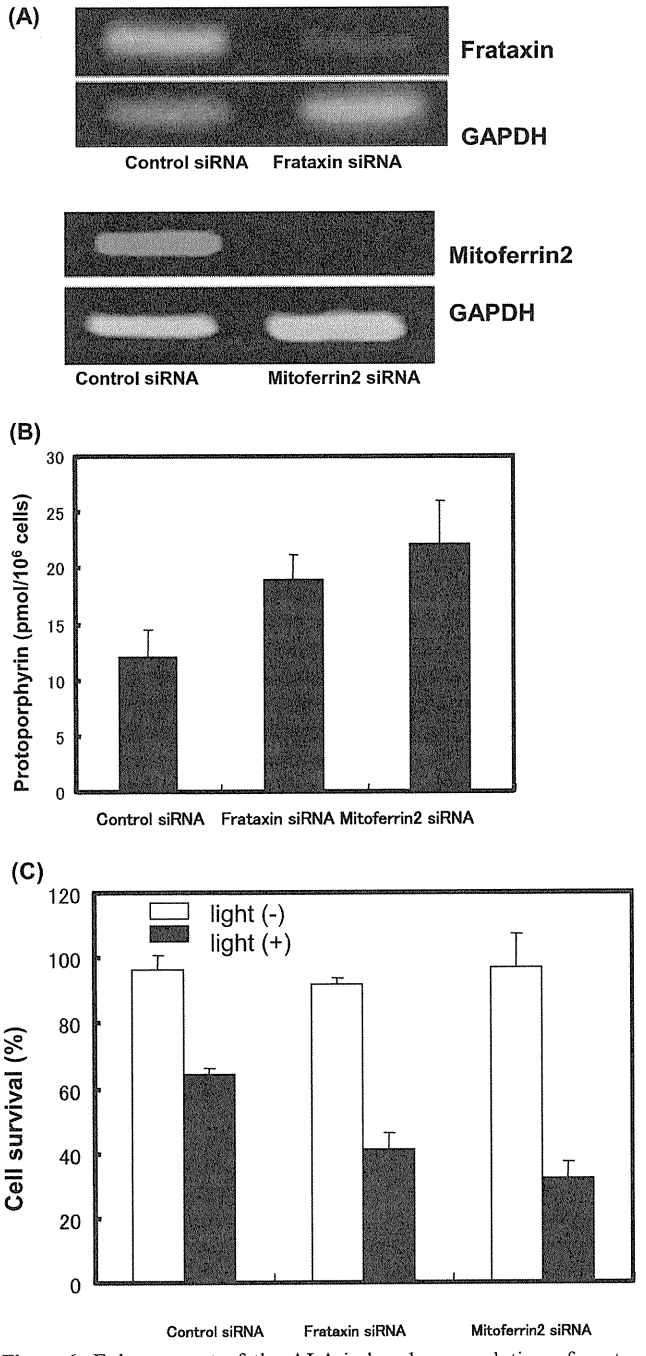

Figure 6. Enhancement of the ALA-induced accumulation of protoporphyrin and photodamage by knockdown of frataxin and mitoferrin-2. (A) The cells (5×10^5) transfected with frataxin or mitoferrin-2 siRNA were cultured for 48 h, followed by incubation with 1 mM ALA for 16 h. RNA was isolated from the cells 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 ± 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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REFERENCES

1. Dougherty, T. J., C. J. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan and Q. Peng (1998) Photodynamic therapy. *J. Natl. Cancer Inst.* **90**, 889–905.
2. De Rosa, F. S., R. F. Lopez, J. A. Thomazine, A. C. Tedesco, N. Lange and M. Bentley (2004) *In vitro* metabolism of 5-ALA esters derivatives in hairless mice skin homogenate and *in vivo* PpIX accumulation studies. *Pharm. Res.* **21**, 2247–2252.
3. Fischer, F., E. F. Dickson, J. C. Kennedy and R. H. Pottier (2001) An affordable, portable fluorescence imaging device for skin lesion detection using a dual wavelength approach for image contrast enhancement and aminolaevulinic acid-induced protoporphyrin IX. Part II. *In vivo* testing. *Lasers Med. Sci.* **16**, 207–212.
4. Fischer, F., E. F. Dickson, R. H. Pottier and H. Wieland (2001) An affordable, portable fluorescence imaging device for skin lesion detection using a dual wavelength approach for image contrast enhancement and aminolaevulinic acid-induced protoporphyrin IX. Part I. Design, spectral and spatial characteristics. *Lasers Med. Sci.* **16**, 199–206.
5. Hirschberg, H., F. A. Uzal, D. Chighvinadze, M. J. Zhang, Q. Peng and S. J. Madsen (2008) Disruption of the blood–brain barrier following ALA-mediated photodynamic therapy. *Lasers Surg. Med.* **40**, 535–542.
6. Furuyama, K., K. Kaneko and P. D. Vargas (2007) Heme as a magnificent molecule with multiple missions: heme determines its own fate and governs cellular homeostasis. *Tohoku J. Exp. Med.* **213**, 1–16.
7. Yamamoto, F., Y. Ohgari, N. Yamaki, S. Kitajima, O. Shimokawa, H. Matsui and S. Taketani (2007) The role of nitric oxide in delta-aminolevulinic acid (ALA)-induced photosensitivity of cancerous cells. *Biochem. Biophys. Res. Commun.* **353**, 541–546.
8. Ohgari, Y., Y. Nakayasu, S. Kitajima, M. Sawamoto, H. Mori, O. Shimokawa, H. Matsui and S. Taketani (2005) Mechanisms involved in delta-aminolevulinic acid (ALA)-induced photosensitivity of tumor cells: relation of ferrochelatase and uptake of ALA to the accumulation of protoporphyrin. *Biochem. Pharmacol.* **71**, 42–49.
9. Taketani, S. (2005) Aquisition, mobilization and utilization of cellular iron and heme: endless findings and growing evidence of tight regulation. *Tohoku J. Exp. Med.* **205**, 297–318.
10. Andoh, Y., H. Suzuki, M. Araki, A. Mizutani, T. Ohashi, T. Okumura, Y. Adachi, S. Ikehara and S. Taketani (2004) Low- and high-level expressions of heme oxygenase-1 in cultured cells under uninduced conditions. *Biochem. Biophys. Res. Commun.* **320**, 722–729.
11. Ryter, S. W. and A. M. Choi (2009) Heme oxygenase-1/carbon monoxide: from metabolism to molecular therapy. *Am. J. Respir. Cell Mol. Biol.* **41**, 251–260.
12. Jansen, T., M. Hortmann, M. Oelze, B. Opitz, S. Steven, R. Schell, M. Knorr, S. Karbach, S. Schuhmacher, P. Wenzel, T. Münzel and A. Daiber (2010) Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection by heme oxygenase-1-evidence for direct and indirect antioxidant actions of bilirubin. *J. Mol. Cell. Cardiol.* **49**, 186–195.

13. Miyake, M., M. Ishii, K. Kawashima, T. Kodama, K. Sugano, K. Fujimoto and Y. Hirao (2009) siRNA-mediated knockdown of the heme synthesis and degradation pathways: modulation of treatment effect of 5-aminolevulinic acid-based photodynamic therapy in urothelial cancer cell lines. *Photochem. Photobiol.* **85**, 1020–1027.
14. Frank, J., M. R. Lornejad-Schäfer, H. Schöffl, A. Flaccus, C. Lambert and H. K. Biesalski (2007) Inhibition of heme oxygenase-1 increases responsiveness of melanoma cells to ALA-based photodynamic therapy. *Int. J. Oncol.* **31**, 1539–1545.
15. Modica-Napolitano, J. S., M. Kulawiec and K. K. Singh (2007) Mitochondria and human cancer. *Curr. Mol. Med.* **7**, 121–131.
16. Paradkar, P. N., K. B. Zumbrennen, B. H. Paw, D. M. Ward and J. Kaplan (2009) Regulation of mitochondrial iron import through differential turnover of mitoferrin 1 and mitoferrin 2. *Mol. Cell. Biol.* **29**, 1007–1016.
17. Schoenfeld, R. A., E. Napoli, A. Wong, S. Zhan, L. Reutenauer, D. Morin, A. R. Buckpitt, F. Taroni, B. Lonnerdal, M. Ristow, H. Puccio and G. A. Cortopassi (2005) Frataxin deficiency alters heme pathway transcripts and decreases mitochondrial heme metabolites in mammalian cells. *Hum. Mol. Genet.* **15**, 3787–3799.
18. Thierbach, R., T. J. Schulz, F. Isken, A. Voigt, B. Mietzner, G. Drewes, J. C. von Kleist-Retzow, R. J. Wiesner, M. A. Magnuson, H. Puccio, A. F. Pfeiffer, P. Steinberg and M. Ristow (2005) Targeted disruption of hepatic frataxin expression causes impaired mitochondrial function, decreased life span and tumor growth in mice. *Hum. Mol. Genet.* **14**, 3857–3864.
19. Inoue, K., T. Karashima, M. Kamada, T. Shuin, A. Kurabayashi, M. Furihata, H. Fujita, K. Utsumi and J. Sasaki (2009) Regulation of 5-aminolevulinic acid-mediated protoporphyrin IX accumulation in human urothelial carcinomas. *Pathobiology* **76**, 303–314.
20. Choudry, K., R. C. Brooke, W. Farrar and L. E. Rhodes (2003) The effect of an iron chelating agent on protoporphyrin IX levels and phototoxicity in topical 5-aminolaevulinic acid photodynamic therapy. *Br. J. Dermatol.* **149**, 124–130.
21. Taketani, S., T. Furukawa and K. Furuyama (2001) Expression of coproporphyrinogen oxidase and synthesis of hemoglobin in human erythroleukemia K562 cells. *Eur. J. Biochem.* **268**, 1705–1711.
22. Nishimura, K., S. Taketani and H. Inokuchi (1995) Cloning of a human cDNA for protoporphyrinogen oxidase by complementation *in vivo* of a hemG mutant of *Escherichia coli*. *J. Biol. Chem.* **270**, 8076–8080.
23. Vargas, P. D., K. Furuyama, S. Sassa and S. Shibahara (2008) Hypoxia decreases the expression of the two enzymes responsible for producing linear and cyclic tetrapyrroles in the heme biosynthetic pathway. *FEBS J.* **275**, 5947–5959.
24. Yoshida, T., P. Biro, T. Cohen, R. M. Müller and S. Shibahara (1988) Human heme oxygenase cDNA and induction of its mRNA by hemin. *Eur. J. Biochem.* **171**, 457–461.
25. Shibahara, S., M. Yoshizawa, H. Suzuki, K. Takeda, K. Meguro and K. Endo (1993) Functional analysis of cDNAs for two types of human heme oxygenase and evidence for their separate regulation. *J. Biochem.* **113**, 214–218.
26. Sakaino, M., T. Kataoka and S. Taketani (2009) Post-transcriptional regulation of the expression of ferrochelatase by its variant mRNA. *J. Biochem.* **145**, 733–738.
27. Ohgari, Y., Y. Miyata, T. T. Chau, S. Kitajima, Y. Adachi and S. Taketani (2011) Quinolone compounds enhance delta-aminolevulinic acid-induced accumulation of protoporphyrin IX and photosensitivity of tumour cells. *J. Biochem.* **149**, 153–160.
28. Masuya, Y., K. Hioki, R. Tokunaga and S. Taketani (1998) Involvement of the tyrosine phosphorylation pathway in induction of human heme oxygenase-1 by hemin, sodium arsenite, and cadmium chloride. *J. Biochem.* **124**, 628–633.
29. Harada, H., R. Sugimoto, A. Watanabe, S. Taketani, K. Okada, E. Warabi, R. Siow, K. Itoh, M. Yamamoto and T. Ishii (2008) Differential roles for Nrf2 and AP-1 in upregulation of HO-1 expression by arsenite in murine embryonic fibroblasts. *Free Radic. Res.* **42**, 297–304.
30. Teng, L., M. Nakada, S. G. Zhao, Y. Endo, N. Furuyama, E. Nambu, I. V. Pyko, Y. Hayashi and J. I. Hamada (2011) Silencing of ferrochelatase enhances 5-aminolevulinic acid-based fluorescence and photodynamic therapy efficacy. *Br. J. Cancer* **104**, 798–807.
31. Yasui, Y., S. Muranaka, T. Tahara, R. Shimizu, S. Watanabe, Y. Horie, E. Nanba, H. Uezato, A. Takamiyagi, S. Taketani and R. Akagi (2002) A new ferrochelatase mutation combined with low expression alleles in a Japanese patient with erythropoietic protoporphyria. *Clin. Sci. (Lond).* **102**, 501–506.
32. Anand, S., G. Honari, T. Hasan, P. Elson and E. V. Maytin (2009) Low-dose methotrexate enhances aminolevulinate-based photodynamic therapy in skin carcinoma cells *in vitro* and *in vivo*. *Clin. Cancer Res.* **15**, 3333–3343.
33. Sinha, A. K., S. Anand, B. J. Ortel, Y. Chang, Z. Mai, T. Hasan and E. V. Maytin (2006) Methotrexate used in combination with aminolaevulinic acid for photodynamic killing of prostate cancer cells. *Br. J. Cancer* **95**, 485–495.
34. Hinnen, P., F. W. de Rooij, E. M. Terlouw, A. Edixhoven, H. van Dekken, R. van Hillegersberg, H. W. Tilanus, J. H. Wilson and P. D. Siersema (2000) Porphyrin biosynthesis in human Barrett's oesophagus and adenocarcinoma after ingestion of 5-aminolaevulinic acid. *Br. J. Cancer* **83**, 539–543.
35. Krieg, R. C., H. Messmann, J. Rauch, S. Seeger and R. Knuechel (2002) Metabolic characterization of tumor cell-specific protoporphyrin IX accumulation after exposure to 5-aminolevulinic acid in human colonic cells. *Photochem. Photobiol.* **76**, 518–525.
36. Grinblat, B., N. Pour and Z. Malik (2006) Regulation of porphyrin synthesis and photodynamic therapy in heavy metal intoxication. *J. Environ. Pathol. Toxicol. Oncol.* **25**, 145–158.
37. Feuerstein, T., A. Schauder and Z. Malik (2009) Silencing of ALA dehydratase affects ALA-photodynamic therapy efficacy in K562 erythroleukemic cells. *Photochem. Photobiol. Sci.* **8**, 1461–1466.
38. Krieg, R. C., S. Fickweiler, O. S. Wolfbeis and R. Knuechel (2000) Cell-type specific protoporphyrin IX metabolism in human bladder cancer *in vitro*. *Photochem. Photobiol.* **72**, 226–233.
39. Stemmler, T. L., E. Lesuisse, D. Pain and A. Dancis (2010) Frataxin and mitochondrial FeS cluster biogenesis. *J. Biol. Chem.* **285**, 26737–26743.
40. Schulz, T. J., R. Thierbach, A. Voigt, G. Drewes, B. Mietzner, P. Steinberg, A. F. Pfeiffer and M. Ristow (2006) Induction of oxidative metabolism by mitochondrial frataxin inhibits cancer growth: Otto Warburg revisited. *J. Biol. Chem.* **281**, 977–978.
41. Taketani, S., Y. Adachi and Y. Nakahashi (2000) Regulation of the expression of human ferrochelatase by intracellular iron levels. *Eur. J. Biochem.* **267**, 4685–4692.

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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

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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 × 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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Conflict of interest: All authors have no conflicts of interest to declare.

References

[1] Cortes JE, Kantarjian H, O'Brien S, Keating M, Pierce S, Freireich EJ, et al. Clinical and prognostic significance of trisomy 21 in adult patients with acute myelogenous leukemia and myelodysplastic syndromes. Leukemia 1995;9:115–7.
[2] Gallego M, Solé F, Acín P, Florensa L, Sans-Sabrafèn J, Woessner S. A new case of trisomy 21 in a patient with an acute nonlymphocytic leukemia (M5b). Cancer Genet Cytogenet 1996;95:213–4.
[3] Chang Y, Wang H, Wang H, Chen Z, Jin J. Trisomy 21 in patients with acute leukemia. Am J Hematol 2008;84:193–4.
[4] Wan TSK, Au WY, Chan JCW, Chan LC, Ma SK. Trisomy 21 as the sole acquired karyotypic abnormality in acute myeloid leukemia and myelodysplastic syndrome. Leuk Res 1999;23:1079–83.
[5] Kondo H, Kobayashi A, Iwasaki H. Trisomy 21 as the sole acquired karyotypic abnormality in an adult patient with CD7-positive acute myeloid leukemia. Cancer Genet Cytogenet 2001;127:77–9.
[6] Yamamoto K, Nagata K, Hamaguchi H. A new case of CD7-positive acute myeloblastic leukemia with trisomy 21 as a sole acquired abnormality. Cancer Genet Cytogenet 2002;133:183–4.
[7] Udayakumar AM, Pathare AV, Muralitharan S, Alghzaly AA, Alkindi S, Raeburn JA. Trisomy 21 as a sole acquired abnormality in an adult Omani patient with CD7- and CD9-positive acute myeloid leukemia. Arch Med Res 2007;38:797–802.
[8] Naithani R, Singhal D, Kumar R, Raina V, Saxena R, Anand H. Sole acquired trisomy 21 in a case of CD7 and CD10 positive acute myeloid leukemia. Indian J Cancer 2008;45:132–4.
[9] Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukaemia. A report of the French–American–British co-operative group. Ann Intern Med 1985;103:626–9.
[10] Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, et al. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: the AML99 trial from the Japanese childhood AML cooperative study group. J Clin Oncol 2009;27:4007–13.

Ko Kudo
Kiminori Terui*
Shinya Sasaki
Takuya Kamio
Tomohiko Sato
Etsuro Ito
Department of Pediatrics, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori, 036-8562, Japan

* Corresponding author. Tel.: +81 172 39 5070; fax: +81 172 39 5071.
E-mail address: teru@cc.hirosaki-u.ac.jp (K. Terui)

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

Takuya Kamio,[1] Etsuro Ito,[1] Akira Ohara,[2] Yoshiyuki Kosaka,[3] Masahiro Tsuchida,[4] Hiroshi Yagasaki,[5] Hideo Mugishima,[5] Hiromasa Yabe,[6] Akira Morimoto,[7] Shouichi Ohga,[8] Hideki Muramatsu,[9] Asahito Hama,[9] Takashi Kaneko,[10] Masayuki Nagasawa,[11] Atsushi Kikuta,[12] Yuko Osugi,[13] Fumio Bessho,[14] Tatsutoshi Nakahata,[15] Ichiro Tsukimoto,[2] and Seiji Kojima,[9] on behalf of the Japan Childhood Aplastic Anemia Study Group

[1]Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki; [2]Department of Pediatrics, Toho University, Tokyo; [3]Department of Hematology and Oncology, Hyogo Children's Hospital; [4]Department of Pediatrics, Ibaraki Children's Hospital, Mito; [5]Department of Pediatrics, Nihon University School of Medicine, Tokyo; [6]Department of Cell Transplantation, Tokai University School of Medicine, Isehara; [7]Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto; [8]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [9]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya; [10]Department of Hematology/Oncology, Tokyo Metropolitan Children's Medical Center, Tokyo; [11]Department of Pediatrics, Graduate Medical School, Tokyo Medical and Dental University, Tokyo; [12]Department of Pediatrics, Fukushima Medical University, Fukushima, [13]Department of Pediatric Hematology/Oncology, Osaka City General Hospital, Osaka; [14]Department of Pediatrics, Kyorin University School of Medicine, Tokyo; [15]Department of Pediatrics, Graduate School of Medicine, Kyoto University, Kyoto, Japan

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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Correspondence:
Seiji Kojima, MD, PhD,
Department of Pediatrics,
Nagoya University Graduate School of Medicine, 65 Tsurumaicho, Showa-ku, Nagoya, 466-8550, Japan.
E-mail: kojimas@med.nagoya-u.ac.jp.

Introduction

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

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

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

Design and Methods

Patients

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

Treatment

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

Assessments

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

The hematologic response was evaluated 6 months after the

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

Statistical analysis

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

Results

Patients' characteristics

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

Risk factors for relapse

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

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

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

Table 1. Patients' pretreatment characteristics.

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

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

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

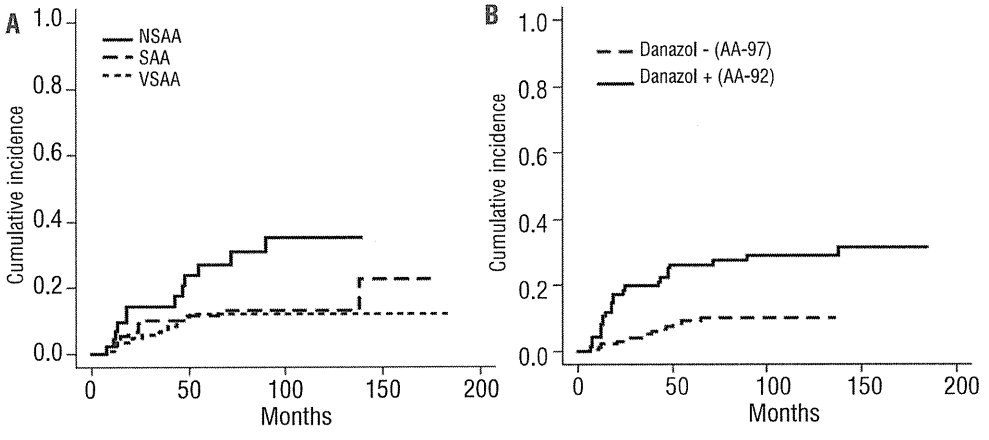

Figure 1. Cumulative incidence of relapse after immunosuppressive therapy in children with aplastic anemia. (A) The cumulative relapse rate of patients with non-severe aplastic anemia (NSAA) was significantly higher than that of patients with severe aplastic anemia (SAA) (P=0.025) and very severe aplastic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

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

Discussion

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

Multivariate analysis of the data from this retrospective multicenter study shows that the use of danazol was the most statistically significant risk factor for relapse. From 1992 to 2007, 441 children with newly diagnosed AA were treated with immunosuppressive therapy consisting of antithymocyte globulin and cyclosporine with (the AA-92 study) or without danazol (the AA-97 study). There are several reports of the efficacy of anabolic steroids in the treatment of AA. A randomized trial from the EBMT SAA working party demonstrated that the addition of an anabolic steroid (oxymetholone) to antithymocyte globulin treatment improved the response rate of patients with treated AA.[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

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References

1. Storb R. Bone marrow transplantation for aplastic anemia. Cell Transplant. 1993;2(5):365-79.
2. Horowitz MM. Current status of allogeneic bone marrow transplantation in acquired aplastic anemia. Semin Hematol. 2000;37(1):30-42.
3. Ades L, Mary JY, Robin M, Ferry C, Porcher R, Esperou H, et al. Long-term outcome after bone marrow transplantation for severe aplastic anemia. Blood. 2004;103(7):2490-7.
4. Davies JK, Guinan EC. An update on the management of severe idiopathic aplastic anaemia in children. Br J Haematol. 2007;136(4):549-64.
5. Kojima S, Hibi S, Kosaka Y, Yamamoto M, Tsuchida M, Mugishima H, et al. Immunosuppressive therapy using antithymocyte globulin, cyclosporine, and danazol with or without human granulocyte colony-stimulating factor in children with acquired aplastic anemia. Blood. 2000;96(6):2049-54.
6. Bacigalupo A, Bruno B, Saracco P, Di Bona E, Locasciulli A, Locatelli F, et al. Antilymphocyte globulin, cyclosporine, prednisolone, and granulocyte colony-stimulating factor for severe aplastic anemia: an update of the GITMO/EBMT study on 100 patients. European Group for Blood and Marrow Transplantation (EBMT) Working Party on Severe Aplastic Anemia and the Gruppo Italiano Trapianti di Midollo Osseo (GITMO). Blood. 2000;95(6):1931-4.
7. Führer M, Rampf U, Baumann I, Faldum A, Niemeyer C, Janka-Schaub G, et al. Immunosuppressive therapy for aplastic anemia in children: a more severe disease predicts better survival. Blood. 2005;106(6): 2102-4.
8. Kojima S, Ohara A, Tsuchida M, Kudoh T, Hanada R, Okimoto Y, et al. Risk factors for evolution of acquired aplastic anemia into myelodysplastic syndrome and acute myeloid leukemia after immunosuppressive therapy in children. Blood. 2002;100(3):786-90.
9. Schrezenmeier H, Marin P, Raghavachar A, McCann S, Hows J, Gluckman E, et al. Relapse of aplastic anaemia after immunosuppressive treatment: a report from the European Bone Marrow Transplantation Group SAA Working Party. Br J Haematol. 1993;85(2):371-7.
10. Saracco P, Quarello P, Iori AP, Zecca M, Longoni D, Svahn J, et al. Cyclosporin A response and dependence in children with acquired aplastic anaemia: a multicentre retrospective study with long-term observation follow-up. Br J Haematol. 2008;140(2):197-205.
11. Scheinberg P, Wu CO, Nunez O, Young NS. Long-term outcome of pediatric patients with severe aplastic anemia treated with antithymocyte globulin and cyclosporine. J Pediatr. 2008;153(6):814-9.
12. Kosaka Y, Yagasaki H, Sano K, Kobayashi R, Ayukawa H, Kaneko T, et al. Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia. Blood. 2008;111(3):1054-9.
13. Camitta BM, Thomas ED, Nathan DG, Gale RP, Kopecky KJ, Rappeport JM, et al. A prospective study of androgens and bone marrow transplantation for treatment of

severe aplastic anemia. Blood. 1979;53(3): 504-14.
14. Bacigalupo A, Chaple M, Hows J, Van Lint MT, McCann S, Milligan D, et al. Treatment of aplastic anaemia (AA) with antilymphocyte globulin (ALG) and methylprednisolone (MPred) with or without androgens: a randomized trial from the EBMT SAA working party. Br J Haematol. 1993; 83(1):145-51.
15. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc. 1999;94(446): 496-509.
16. Schrezenmeier H, Marin P, Raghavachar A, McCann S, Hows J, Gluckman E, et al. Relapse of aplastic anaemia after immunosuppressive treatment: a report from the European Bone Marrow Transplantation Group SAA Working Party. Br J Haematol. 1993;85(2):371-7.
17. Frickhofen N, Heimpel H, Kaltwasser JP, Schrezenmeier H. Antithymocyte globulin with or without cyclosporin A: 11-year follow-up of a randomized trial comparing treatments of aplastic anemia. Blood. 2003;101(4):1236-42.
18. Rosenfeld S, Follmann D, Nunez O, Young NS. Antithymocyte globulin and cyclosporine for severe aplastic anemia; association between hematologic response and long-term outcome. JAMA. 2003;289 (3): 1130-5.
19. Vulliamy T, Marrone A, Dokal I, Mason PJ. Association between aplastic anaemia and mutations in telomerase RNA. Lancet. 2002;359(9324):2168-70.
20. Yamaguchi H, Baerlocher GM, Lansdorp PM, Chanock SJ, Nunez O, Sloand E, et al. Mutations of the human telomerase RNA gene (TERC) in aplastic anemia and myelodysplastic syndrome. Blood. 2003;102 (3):916-8.
21. Yamaguchi H, Calado RT, Ly H, Kajigaya S, Baerlocher GM, Chanock SJ, et al. Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia. N Engl J Med. 2005;352(14):1413-24.
22. Fogarty PF, Yamaguchi H, Wiestner A, Baerlocher GM, Sloand E, Zeng WS, et al. Late presentation of dyskeratosis congenita as apparently acquired aplastic anaemia due to mutations in telomerase RNA. Lancet. 2003;362(9396):1628-30.
23. Liang J, Yagasaki H, Kamachi Y, Hama A, Matsumoto K, Kato K, et al. Mutations in telomerase catalytic protein in Japanese children with aplastic anemia. Haematologica. 2006;91(5):656-8.
24. Howard SC, Naidu PE, Hu XJ, Jeng MR, Rodriguez-Galindo C, Rieman MD, et al. Natural history of moderate aplastic anemia in children. Pediatr Blood Cancer. 2004; 43(5):545-51.
25. Teramura M, Kimura A, Iwase S, Yonemura Y, Nakao S, Urabe A, et al. Treatment of severe aplastic anemia with antithymocyte globulin and cyclosporin A with or without G-CSF in adults: a multicenter randomized study in Japan. Blood. 2007;110(6):1756-61.
26. Gurion R, Gafter-Gvili A, Paul M, Vidal L, Ben-Bassat I, Yeshurun M, et al. Hematopoietic growth factors in aplastic anemia patients treated with immunosuppressive therapy-systematic review and meta-analysis. Haematologica. 2009;94(5): 712-9.
27. Scheinberg P, Nunez O, Young NS. Retreatment with rabbit anti-thymocyte globulin and cyclosporin for patients with relapsed or refractory severe aplastic anaemia. Br J Haematol. 2006;133(6):622-7.

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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

Senkottuvelan Kadirvel[a], Kazumichi Furuyama[a], Hideo Harigae[b], Kiriko Kaneko[c], Yoshiko Tamai[d], Yoji Ishida[e], and Shigeki Shibahara[a]

[a]*Department of Molecular Biology and Applied Physiology;* [b]*Department of Hematology and Rheumatology;* [c]*Endocrinology and Applied Medical Science, Tohoku University School of Medicine, Sendai, Japan;* [d]*Division of Transfusion Medicine, Hirosaki University Hospital, Hirosaki, Japan;* [e]*Hematology and Oncology, Internal Medicine, Iwate Medical University School of Medicine, Morioka, Japan*

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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

Offprint requests to: Kazumichi Furuyama, M.D., Ph.D., Department of Molecular Biology and Applied Physiology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan; E-mail: furuyama@med.tohoku.ac.jp

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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 (NA931Vl 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.