American Journal of Hematology 62:112–114 (1999)

A Novel Mutation of the Erythroid-Specific δ-Aminolevulinate Synthase Gene in a Patient With Non-Inherited Pyridoxine-Responsive Sideroblastic Anemia

Hideo Harigae,[1] Kazumichi Furuyama,[2] Kazuko Kudo,[3] Norio Hayashi,[4] Masayuki Yamamoto,[5] Shigeru Sassa,[2]* and Takeshi Sasaki[6]

[1]Department of Clinical and Laboratory Medicine, Tohoku University School of Medicine, Sendai, Japan
[2]The Rockefeller University, New York, New York
[3]Department of Pediatrics, Nagoya University, Nagoya, Japan
[4]Department of Biochemistry, Tohoku University School of Medicine, Sendai, Japan
[5]Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan
[6]Department of Immunology and Hematology, Tohoku University School of Medicine, Sendai, Japan

A novel missense mutation, G663A, in exon 5 of the erythroid-specific δ-aminolevulinate synthase gene (ALAS2) was identified in a Japanese male with pyridoxine-responsive sideroblastic anemia. Activity of the mutant δ-aminolevulinate synthase protein expressed in vitro was 15.1% compared with the normal control, but was increased up to 34.5% by the addition of pyridoxal 5′-phosphate, consistent with the clinical response of the patient to pyridoxine treatment. The same mutation was also detected in genomic DNA from the oral mucosal membrane of the patient; however, it was not detected in other family members. These findings suggest that this G663A mutation is responsible for sideroblastic anemia in the proband, and may be an index mutation in this pedigree. Am. J. Hematol. 62:112–114, 1999.

Key words: X-linked sideroblastic anemia; ALAS

INTRODUCTION

The erythroid–specific δ-aminolevulinate synthase (ALAS2) gene, which is located at Xp11.21 [1], is thought to be responsible for X-linked sideroblastic anemia (SA). To date, ~20 different mutations of the ALAS2 gene have been reported in various pedigrees of X-linked SA [2–4]. However, no mutation of the ALAS2 gene in noninherited SA has been reported. We report here a novel mutation of the ALAS2 gene in a patient with pyridoxine-responsive SA, which is likely an index mutation in this pedigree.

MATERIALS AND METHODS

Case Report

Proband was an eight-month-old Japanese boy who visited Kamo Hospital, Kamo, Japan, in December, 1998, with chief complaints of pallor and reduced physical activity. Laboratory findings showed the presence of severe hypochromic microcytic anemia (red blood cell 1.56×10^{12}/l, Hb 3.5 g/dl, MCV 66.0 fl). Serum iron (433 μg/dl) was significantly increased. Bone marrow was normocellular with a decreased M/E ratio (0.6) accompanied by numerous ringed sideroblasts (18%). Chromosomal analysis of bone marrow cells showed no abnor-

Contract grant sponsor: Ministry of Health & Welfare, Japan (H.H.); Contract grant sponsor: Grants-in-Aid for Promotion of Science; Contract grant sponsor: Ministry of Culture, Sport & Sciences, Japan (N.H., M.Y., T.S.); Contract grant sponsor: Yamanouchi Molecular Medicine Research Fund (K.F.); Contract grant sponsor: U.S. Public Health Services (S.S.); Contract grant number: DK-32890.

*Correspondence to: Shigeru Sassa, M.D., Ph.D., Laboratory of Biochemical Hematology, The Rockefeller University, New York, NY 10021-6399. E-mail: sassa@rockvax.rockefeller.edu

Received for publication 22 March 1999; Accepted 2 June 1999

mality. His family members showed normal complete blood cell count (CBC), including normal RDW. The anemia of the patient was restored almost completely to normal (Hb 11.4 g/dl) by oral pyridoxine treatment for 2 months (50 mg/day).

Sequence Analysis of the ALAS2 Gene of the Proband

Genomic DNA was extracted by using the genomic DNA extraction system (DNA extraction WB kit, Wako Corp., Osaka, Japan). Each exon of the ALAS2 gene was amplified by using the specific primers as described previously [5]. Polymerase chain reaction (PCR) products were subcloned into pGEM-T vector (Promega, Madison, WI), and sequence analysis was performed.

ALAS Activity of the Mutant Protein

Mutation was introduced into human ALAS2 cDNA by site-directed mutagenesis. A cDNA fragment encoding the catalytic domain of ALAS2 was fused to the 3′-end of glutathione S-transferase gene. The bacterially expressed fusion protein was purified by using a Glutathione Sepharose 4B affinity column (Pharmacia Biotech). Enzymatic activity of the purified protein was determined as described previously [5].

Allele-Specific Oligonucleotide Hybridization (ASO) Analysis

Amplicons from exon 5 were spotted onto a nylon membrane and hybridized with the end-labelled mutant oligonucleotide; 5′-GCATGAGCCAACACCCTC, or the normal oligonucleotide; 5′-GCATGAGCCGACACCCTC. A membrane was then washed, dried, and exposed to an X-ray film [6].

Restriction Analysis

Mismatch PCR was performed by using genomic DNA of peripheral blood cells, or the oral mucosal membrane cells of the proband, as well as peripheral blood cells of his family members with 5′-AGAAACAGGATCACACCTAC, and 5′-TCACTGTGTGGCTTGCAAGACCTGAGGGAG as the sense and antisense primer, respectively. After digestion with *Hinf*I, amplicons were electrophoresed on a 2.0% agarose gel and stained with ethidium bromide.

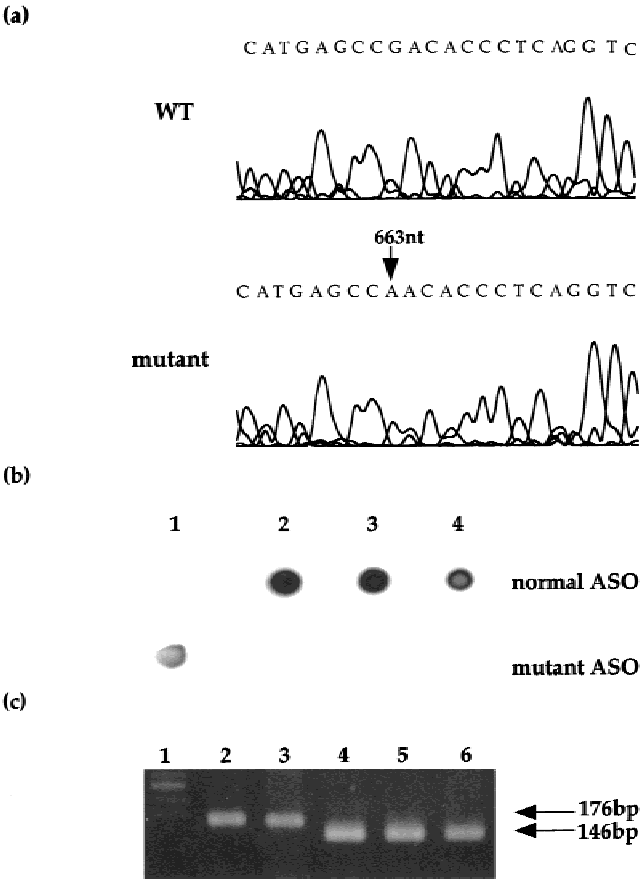

Fig. 1. Analysis of ALAS2 mutation in the patient. a: Nucleotide sequence analysis. WT, Subcloned exon 5 of normal ALAS2. Mutant, Subcloned exon 5 of the patient's ALAS2 in peripheral blood. Guanine at nt 663 is substituted with adenine. b: ASO analysis. Amplicons of exon 5 was blotted to nylon membranes, and hybridized with the end-labeled oligonucleotide carrying the normal sequence (normal ASO), or the mutant sequence (mutant ASO). lane 1, proband; lane 2, mother; lane 3, sister; lane 4, father. c: Mismatch PCR analysis. The mismatch PCR product from a mutant allele is resistant to *Hinf*I digestion, so that the size is not affected by digestion with *Hinf*I (176bp). In contrast, the mismatch PCR product from a normal allele is restricted by *Hinf*I (146bp), since it has an artificial *Hinf*I site. lane 1, marker DNA; lane 2, peripheral blood of the proband; lane 3, oral mucosal membrane of the proband; lane 4, sister; lane 5, mother; lane 6, father.

RESULTS AND DISCUSSION

Sequence analysis of the proband's DNA from his peripheral blood revealed a point mutation, G663A, in exon 5 (Fig. 1a). To identify the mutation in his family members, ASO analysis was performed. The results clearly showed that, although the proband carries the mutation, other family members of the proband do not (Fig. 1b). For further confirmation, mismatch PCR was carried out. Since mismatch PCR introduces a new *Hinf*I site only into the amplicon from exon 5 of the normal allele, amplicons from the normal, but not from the mutant allele should be shorter by 30 bp by digestion with *Hinf*I. In fact, amplicons from all family members of the proband were 30 bp shorter than that of the proband with *Hinf*I digestion (Fig. 1c). These results are consistent with the findings by ASO analysis. Furthermore, mis-

match PCR demonstrated that the amplicon from the oral mucosal membrane of the proband also carries the same mutation. The result of mismatch PCR was also confirmed by sequence analysis (data not shown). These findings indicate that G663A mutation identified in the proband may be an index (or a founder) mutation in this pedigree, or a somatic mutation.

We then examined the enzymatic activity of the mutant ALAS2 protein by bacterial expression that carries an amino acid substitution, R204Q, that is produced by the G663A mutation. Activities of the wild-type and the mutant ALAS2 protein expressed in *Escherichia coli* in the absence of PLP were 8181 ± 1118 nmol ALA/h/mg protein, and 1233 ± 231 nmol ALA/h/mg protein, respectively ($n = 3$, $p < 0.001$), while activities of the wild-type and the mutant ALAS2 in the presence of PLP (200 μM) were 10767 ± 2191 nmol ALA/h/mg protein and 3713 ± 319 nmol ALA/h/mg protein, respectively ($n = 3$, $p < 0.001$). Thus the specific activity of the mutant ALAS2 was 15.1% and 34.5% of the wild-type enzyme, in the absence and the presence of PLP, respectively. This finding is consistent with the fact that oral pyridoxine treatment *in vivo* in the patient remarkably improved anemia, and clearly establishes the fact that G663A mutation of the ALAS2 gene is responsible for the pyridoxine-responsive SA in the patient.

ACKNOWLEDGMENTS

We thank Takako Saito, Sakiko Kusunoki, and Luba Garbaczewski for their technical assistance.

REFERENCES

1. Cotter PD, Baumann M, Bishop DF. Enzymatic defect in "X-linked" siberoblastic anemia: molecular evidence for erythroid delta-aminolevulinate synthase deficiency. Proc Natl Acad Sci USA 1992; 89:4028–4032.
2. Edgar AJ, Vidyatilake HM, Wickramasinghe SN. X-linked siberoblastic anaemia due to a mutation in the erythroid 5-aminolaevulinate synthase gene leading to an arginine^{170} to leucine substitution. Eur J Haematol 1998;61:55–58.
3. Furuyama K, Uno R, Urabe A, Hayashi N, Fujita H, Kondo M, Sassa S, Yamamoto M. R411C mutation of the ALAS2 gene encodes a pyridoxine-responsive enzyme with low activity. Br J Haematol 1998; 103:839–841.
4. May A, Bishop DF. The molecular biology and pyridoxine responsiveness of X-linked sideroblastic anaemia. [Review]. Haematologica 1998;83:56–70.
5. Furuyama K, Fujita H, Nagai T, Yomogida K, Munakata H, Kondo M, Kimura A, Kuramoto A, Hayashi N, Yamamoto M. Pyridoxine refractory X-linked sideroblastic anemia caused by a point mutation of the erythroid-specific 5-aminolevulinate synthase gene. Blood 1997;90: 822–830.
6. Wood WI, Gitschier J, Lasky LA, Lawn RM. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc Natl Acad Sci USA 1985;82:1585–1588.