SUPPLEMENTARY APPENDIX

Identification of a novel erythroid-specific enhancer for the *ALAS2* gene and its loss-of-function mutation which is associated with congenital sideroblastic anemia

Kiriko Kaneko,[1,2] Kazumichi Furuyama,[1,6] Tohru Fujiwara,[3] Ryoji Kobayashi,[4] Hiroyuki Ishida,[5] Hideo Harigae,[3] and Shigeki Shibahara[1]

[1]Department of Molecular Biology and Applied Physiology, [2]Department of Endocrinology and Applied Medical Science, [3]Department of Hematology and Rheumatology, Tohoku University Graduate School of Medicine, Sendai, Miyagi; [4]Department of Pediatrics, Sapporo Hokuyu Hospital, Sapporo; [5]Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto; and [6]Laboratory of Molecular Biochemistry, Iwate Medical University, Yahaba, Iwate, Japan

 doi:10.3324/haematol.2013.085449
Manuscript received on February 4, 2013. Manuscript accepted on August 1, 2013.
Correspondence: furuyama@iwate-med.ac.jp

Supplemental methods

Reagents

Chemical reagents were purchased from SIGMA-ALDRICH (St. Louis, MI), Nacalai Tesque (Kyoto, Japan) and Wako pure chemicals (Osaka, Japan).

Identification of the mutations of ALAS2 gene

Genetic analyses performed in this project had been approved by the ethical committee of Tohoku University School of Medicine. Blood samples were withdrawn from the probands and the family members after informed consent. Genome DNA was then extracted 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 (GeneBank genomic: NG_8983.1, GenBank mRNA: NM_000032.4, GenBank: protein; NP_000023.2) were amplified using ExTaq DNA polymerase. Sequences of primers and the conditions for PCR were reported previously.[1] In addition, the first intron of ALAS2 gene was amplified with PrimeStar DNA polymerase. Amplified DNA fragments were purified using QIAquick gel purification kit (QIAGEN GmbH), and the sequences of each amplified DNA were determined directly using BigDye terminator sequencing kit ver. 1.1 with ABI3100 genetic analyzer (Applied Biosystems, Foster city, CA). The sequences of primers for amplification of the first intron of ALAS2 gene are listed in supplemental table 1.

ChIP-quantitative PCR analysis

Real-time-PCR-based quantitative chromatin immunoprecipitation (ChIP) analysis was conducted essentially as described,[2] K562 cells were cross-linked with 1% formaldehyde (Sigma) for 10 min at room temperature. The nuclear lysate was sonicated to reduce DNA length, and the protein-DNA complexes were then immunoprecipitated with anti-GATA1 antibody (ab11963, Abcam, Cambridge, United Kingdom) or control rabbit IgG and Protein A Sepharose (Sigma). Immunoprecipitated DNA fragments were quantified by real-time PCR using the primer set listed in Fig. 2A and supplemental table 1. Product was measured by SYBR Green fluorescence (QIAGEN) in a reaction mixture of 20 μL, and the amount of product was determined relative to a standard curve generated from titration of input chromatin. Analysis of post-amplification dissociation curves showed that primer pairs generated single products.

Identification of the mutation of the mutation of SLC25A38, ABCB7, GLRX5, PUS1 and SLC19A2 gene

All exons encode each protein were amplified with their exon-intron boundaries using ExTaq DNA polymerase. Primer sequence for the amplification of SLC25A38, ABCB7, GLRX5 and SLC19A2 genes were listed in supplemental table 2, 3, 4 and 5, respectively. Primer sequence for PUS1 gene was reported previously.[3]

Cell culture

K562 erythroleukemia cells were maintained in RPMI-1640 medium (WAKO Pure

Chemical Industries Ltd.), and HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium with high glucose (WAKO). Both mediums were supplemented with 10% heat-inactivated FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin.

Electrophoretic Mobility Sift Assay (EMSA)

EMSA was performed using "DIG Gel Shift Kit, 2nd Generation" (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. Sequences of oligonucleotides for probes are indicated by the horizontal bar in Fig. 2B and Fig 4A, and are listed in supplemental table 1. Nuclear extracts were prepared, as described previously,[4] from K562 cells or HEK293 cells that were transfected with GATA1 expression vector or its backbone vector. For construction of the GATA1 expression vector that expresses GATA1 as a FLAG-tagged protein, human GATA1 cDNA was amplified by PCR, and was then cloned into pFLAG-CMV5a plasmid (SIGMA). The resulting FLAG-fused GATA1 expression vector or pFLAG-CMV5a was introduced into HEK293 cells using Lipofectamine 2000 transfection reagent (Invitrogen) at 48 hours before harvest.

Promoter/enhancer activity assay.

The human ALAS2 proximal promoter region (g.4820_5115, between -267 and +29 from the transcription start site) was amplified by PCR, and cloned between BglII and HindIII site of pGL3basic plasmid (Promega Corporation, Madison, WI). The resulting plasmid was referred as pGL3-AEpro(-267), which was used as a parent plasmid. For examining the

enhancer activity of the first intron, a single DNA fragment (5.2 kbp), carrying ALAS2 proximal promoter, first exon, first intron and the untranslated region of second exon, was amplified by PCR, and was subcloned between KpnI and HindIII sites of pGL3basic plasmid (referred as pGL3-AEpro(-267)+intron1). A DNA fragment containing GATA1 binding region in the first intron of ALAS2 gene, which was defined by ChIP-seq analysis,[2] was amplified by PCR using genome DNA prepared from healthy volunteer, proband 1 or proband 3, and was cloned into pCRII-blunt-TOPO vector (Invitrogen). Each DNA fragment obtained after the digestion with appropriate enzymes, which corresponds to g.7488_7960 for wild-type, was referred as ChIP-peak(WT), ChIP-peak(GGTA) or ChIP-peak(delGATA), respectively. The length of ChIP-peak(WT) is 473 bp. In addition, a 130-bp fragment containing ALAS2int1GATA in ChIP-peak was amplified by PCR using genome DNA prepared from healthy volunteer, proband 1 or proband 3, and referred as ChIPmini(WT), ChIPmini(GGTA) or ChIPmini(delGATA), respectively, and cloned into pCRII-blunt-TOPO. Primer sets for PCR were listed in supplemental table 1. Each of these DNA fragments was inserted at the upstream of ALAS2 proximal promoter or the downstream of the luciferase gene of pGL3-AEpro(-267) vector using appropriate restriction enzymes. pGL3-TKpro plasmid was constructed by transferring herpes simplex virus thymidine kinase (HSV-TK) promoter from pRL-TK plasmid (Promega) to the upstream region of luciferase gene of pGL3basic plasmid. Then, ChIP-peak(wt) or ChIPmini(WT) was inserted at the downstream of the luciferase gene of pGL3-TKpro, resulting in pGL3-TKpro+ChIP-peak or pGL3-TKpro+ChIPmini, respectively. To examine the erythroid-specific or non-specific

enhancing activity of each DNA fragment on ALAS2 promoter or HSV-TK promoter, each reporter vector (0.075 pmol/well) and pEF-RL[5] (5 ng/well) were introduced into K562 human erythroleukemia cells or HEK293 human embryonic kidney cells using X-tremeGENE HP transfection reagent (Roche) or Fugene HD transfection reagent (Roche), respectively. Cells were incubated for 24 hours after transfection and were collected to determine luciferase activity using Dual-luciferase reporter system (Promega). Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase activity.

Measurement of ALAS2 mRNA in purified erythroblasts

Bone marrow aspirates were overlayed on Ficoll-Paque PLUS (GE Healthcare UK Ltd, Buckinghamshire, England) to obtain mononuclear cells, and glycophorine A-positive cells were then separated using MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Total RNA was extracted from these cells with TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized from total RNA using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). Reaction mixtures (20 μL) for real-time quantitative RT-PCR consisted of 2 μL of cDNA, 10 μL of SYBR green master mix (QIAGEN) and appropriate primers. Product accumulation was monitored by measuring SYBR Green fluorescence and normalized with GAPDH (MIM# 138400) mRNA.

References

1. Kadirvel S, Furuyama K, Harigae H, Kaneko K, Tamai Y, Ishida Y, et al. The carboxyl-terminal region of erythroid-specific 5-aminolevulinate synthase acts as an intrinsic modifier for its catalytic activity and protein stability. Exp Hematol. 2012;40(6):477-86 e1.

2. Fujiwara T, O'Geen H, Keles S, Blahnik K, Linnemann AK, Kang YA, et al. Discovering hematopoietic mechanisms through genome-wide analysis of GATA factor chromatin occupancy. Mol Cell. 2009;36(4):667-81.

3. Bergmann AK, Campagna DR, McLoughlin EM, Agarwal S, Fleming MD, Bottomley SS, et al. Systematic molecular genetic analysis of congenital sideroblastic anemia: evidence for genetic heterogeneity and identification of novel mutations. Pediatr Blood Cancer. 2010;54(2):273-8.

4. Vargas PD, Furuyama K, Sassa S, Shibahara S. Hypoxia decreases the expression of the two enzymes responsible for producing linear and cyclic tetrapyrroles in the heme biosynthetic pathway. FEBS J. 2008;275(23):5947-59.

5. Muto A, Hoshino H, Madisen L, Yanai N, Obinata M, Karasuyama H, et al. Identification of Bach2 as a B-cell-specific partner for small maf proteins that negatively regulate the immunoglobulin heavy chain gene 3' enhancer. EMBO J. 1998;17(19):5734-43.

Supplemental table 1

		forward (sense) primer	reverse (antisense) primer
Amplification of proximal promoter and intron 1 of ALAS2 gene		GGTACCTCAAGGTATCCCAGCTCTATG	AAGCTTGAACCTAAAGTCCTGCAGAAGAC
Amplification of ALAS2 GATA1 ChIP-peak		GCCAAAAGCCCCCTAGTAATGATGT	CAGAAGGGTTGTTGGAGGGACTAAAT
Primer set used in Figure 2A	1	GGGGTACACTAGAGGGAGGGGC	GGACGAACGAATGACAGGTGGGT
	2	GGCAACAGGTAAGAGCTGCTTTCAG	TCAGATCTACTCAGACCAGAGAGTCA
	3	ACTGACTCTCTGGTCTGAGTAGAT	TCAGAGAATGATGAAATGAAATGAATGATC
	4	TTTCATTTCATCATTCTCTGACACTCTTGC	GGAGAGAGGAATGGGGAATTGCAG
	5	GGCTTGGCCTGTAATTTCCACCACC	TCCTAGTACCCCTTTCCCACCATGA
	6	TGGGTGCTGACACAAGAGAGGATT	TGTCACCAATGCCTTACCAAAGGAACA
	7	AAGAGGCCTATGTTAGTGCAGCAGA	TGCCAGGCCGTGTTCTCATGT
	8	TGAGAACACGGCCTGGCACA	GAACGTACAGCCAAGGGAAGTCACA
	9	TGTGACTTCCCTTGGCTGTACGTT	AACAAGGACAATCTGCATCACAGGAA
	10 (ChIPmini)	TCTCCCACGCCCTGGTCTCA	TTCCCTTCCCCTGCCTGCTTGT
	11	CCTCAAAACATAAGGCTGAGGGTGC	GCAGGGGGATGAGGGTTAAGAGGGGTG
	12	TGTCCCTTTCGTGCCCCTTGG	GGTAGAGGGAAAGGGAGCAGGGT
	13	ACCCTGCTCCCTTTCCCTCTACCT	AGCAAATGACACACAGGCACTCAA
probe for EMSA	wild type	TAAACTCTGGCAACTTTATCTGTGGTCTGCAGGCTC	GAGCCTGCAGACCACAGATAAAGTTGCCAGAGTTTA
	GGTA mutant	TAAACTCTGGCAACTTTACCTGTGGTCTGCAGGCTC	GAGCCTGCAGACCACAGGTAAAGTTGCCAGAGTTTA
	delGATA mutant	TGGGGAGTGGTCAGACCCCTGCAGGCTCAGCCCCAA	TTGGGGCTGAGCCTGCAGGGGTCTGACCACTCCCCA

Supplemental table 2

Primers for amplification of each exon of human SLC25A38 gene (coding region)

	forward primer	reverse primer
exon 1	GTCGTCCACGCTGGTCTCCA	CCCCGGCAATTCCGCCCTTT
exon 2	TGAGGCACCACCAGGTAAGTGT	GCTGCTCAGGAACGGACCCC
exon 3	AGGAAGTGTTTGAGTGGGGAATTGTTT	AGACCACATAGGTACTCCCACCACT
exon 4	TGGGGTCTTTTGGGAAAACCCAGC	GTGACTCGCATGGAGGCGCT
exon 5	GCCCCATAACCTGCAGTCTGCTT	CACCCTATCCTCACCCCGCCA
exon 6	GGTGGGCAACTTGCACTGACCT	GCCTAGATTTTAACCTGGGCATGGGG
exon 7	ACCCTCACTGTGGTACCAAG	CCTGGTTTTCCAGGTAGGAC

Supplemental table 3

Primers for amplification of each exon of human ABCB7 gene

	Forward primer	Reverse primer
exon 1	ACAGCTGAAGCCTCCTCCCAGG	CCCCGAGGTCAGGAGGGCAA
exon 2	TGATCCGCCCGCCTTAGCCT	TCTCTGCATTTCCAGAAGCAGAAACAT
exon 3	AGTGAATGACACTGGGAAAGCCAG	ACCTTGAAGCACACGCACACACA
exon 4	ACCAAGCCCTCTGCTTTCCTAAAAGG	AGTGATTTACACCAGGCCCAGGA
exon 5	AGCCTGAAATGACAGCTCTCCCA	AACCTCCTTGAAGAAAGTCAACACCTG
exon 6	TCCACAGTAATGCCATGTGGGCT	CCCATGGGCATGCAACAGTACA
exon 7 & 8	CACGTACATAACTTCACGCCACCA	GGGACCAACATCATAGATGCCAAAACA
exon 9	TCAGGGGAAGGCTTTGTGAAGGA	CCAATCAGTGAGTGAGGCAGTGCT
exon 10	GGTGGGTCTTTCCCATTCCTAACG	AGCACCCCCACCCCTGACAA
exon 11 & 12	CCCTCCCCAACCCCACCTCA	GAGGCCCCAGGCCACACAAC
exon 13	ACCCCTGGGAAGGGAATGGGA	ACCCAATCAAATGTGACTCAACGAGCA
exon 14	GCCTCATTCTCATTCTTCCCACCTGC	TGGAAAAAGGGGGATAGGCATTTTGCT
exon 15	AGTTGCCTTCTCTTTTTGCTTTCTCCT	AGGGGCTAAAAACAGAATCGTAACAGG
exon 16	GGCACTGGGTAGCTCAACAGGGA	TGAGCACAACCAGGACAGTGACA

Supplemental table 4

Primers for amplification of each exon of human GLRX5 gene

	forward primer	reverse primer
exon 1	CCGCGCCTCTCCCAGTTGTCT	CCGGCTCGAACCTTCAGACAGAC
exon 2	GGGAAGCCAGGGAGGGACAGTG	CAGGGCTCCAGAGATAGGCAGGTG

Supplemental table 5

Primers for amplification of each exon of human SLC19A2 gene

	forward primer	reverse primer
exon 1	CAATGGAAGAGCAGGCAAGT	CGCTTTTCTCGGTCCTCTCT
exon 2	CCAGGTCCTTTCATCACTAATGT	GCCCCCATAGTAGCAATTACA
exon 3	TGGGCCTGTAAATTGCTTTC	CAAATTTGGGAGGGGTGAAT
exon 4	GCAACAGCATTTGTGTAGCA	ACAATGCTTCCTCCCATTTG
exon 5	CATTTGGTTGGAAAGGCAAT	TCACCCTGATCAAGTCACACA
exon 6	GGCACGTGGTGTAAGTATGC	TGCTGTGCAGAGTTCTTGCT