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SHORT COMMUNICATION

INTERACTION OF Hb ADANA (*HBA2*: c.179G>A) WITH DELETIONAL AND NONDELETIONAL α^+-THALASSEMIA MUTATIONS: DIVERSE HEMATOLOGICAL AND CLINICAL FEATURES

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□ *We describe 27 cases of mild-to-severe α-thalassemia (α-thal) syndrome caused by interaction of Hb Adana [α59(E8)Gly→Asp, GGC>GAC (α2)] with deletional and nondeletional α^+-thal mutations in Indonesian patients. Hematological profiles and clinical manifestations of all patients were assessed by routine procedures. The genotypes were generated by a multiplex-polymerase chain reaction (m-PCR), PCR-RFLP (restriction fragment length polymorphism)-based method, and DNA sequencing. The α-thal patients who had Hb Adana in combination with the 3.7 kb deletion mostly have mild-to-moderate anemia. In contrast, patients who were compound heterozygotes for Hb Adana and nondeletional mutations, generally showed a more severe anemia and it mostly presented in childhood. Thus, accurate diagnosis of α-thal disorders is not only important for future management of these patients but also for providing proper genetic counseling to the family.*

Keywords Hb Adana, Hematological features, α-Thalassemia (α-thal) mutations

Hb Adana is a highly unstable variant hemoglobin (Hb) resulting from a mutation at codon 59 on the α1- or α2-globin gene (*HBA1*: c.179G>A or *HBA2*: c.179G>A) (1,2). In Indonesia, we found Hb Adana on the α2-globin gene (3,4). The most severe phenotype due to homozygous Hb Adana, manifesting as

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hydrops fetalis, has been previously reported (3). Study of the frequency of Hb Adana trait in the Indonesian population has not yet been finished; however, our preliminary population study showed that this Hb variant was detected at a relatively low frequency (0.4%) in the Javanese population, although it is quite common (16.0%) in Indonesian patients (4). It contributes to many kinds of α-thalassemia (α-thal) syndromes, especially in compound heterozygosity with the 3.7 kb deletion, which is the most common α^+-thal deletion in Indonesia (4). Interestingly, we also found the combination of Hb Adana with other nondeletional mutations such as Hb Constant Spring (Hb CS, *HBA2*: c.427T>C (5), codon 22 (α2, *HBA2*: c.69C>T) (6), a regulatory single nucleotide polymorphism (rSNP) 149709T<C (GenBank DQ431198) in a non genic region between the ζ- and α-globin genes (7) that creates a new promoter causing α-thal [Online Mendelian Inheritance in Man (OMIM) 141800.0218] and IVS-II-142 (α2, *HBA2*: c.440+2G>A) (8). In this study, we report the hematological and clinical features of patients having Hb Adana in combination with either deletional or nondeletional α^+-thal mutations.

Selection of Patients and Features

Data used in this study was retrospectively evaluated including patients who were referred to the GenNeka Clinic (Yayasan GenNeka, Eijkman Institute for Molecular Biology, Jakarta, Indonesia) and were diagnosed with α-thal based on hematological parameters and molecular diagnosis. Clinical features including age of first presentation, clinical course and physical examination were recorded.

Hematology Analysis and Molecular Studies

Hematological analysis consisted of complete blood counts (CBC), blood smear and Hb analysis. The CBCs were measured using Cell Dyne 1700 (Abbot Diagnostics, Abbott Park, IL, USA), erythrocyte morphology was assesed by blood smear examination under light microscope and Hb analysis was performed using high performance liquid chromatography (HPLC) on a VARIANT™ Hemoglobin Testing System (β-Thalassemia Short Program; Bio-Rad Laboratories, Hercules, CA, USA). Genomic DNA was isolated from leukocytes using modified Gentra Puregene Blood Kit (Qiagen Inc., Valencia, CA, USA) and used as a template for the polymerase chain reaction (PCR)-based mutation detection.

Single α Gene Deletions [$-\alpha^{3.7}$ (rightward) and $-\alpha^{4.2}$ (leftward) types]

We performed multiplex-PCR (m-PCR) using primers as previously described (4). Each 25 μL reaction mixture contained 5× KAPA2G GC Buffer (with $MgCl_2$), 200 μM dNTPs (Invitrogen, Life Technologies Corp.,

TABLE 1 List of Primers Used to Detect Single α-Globin Gene Deletions (Ivy Ng, personal communication)

Primers	Sequences (GenBank: J00153.1) (5'>3')
2/3P	TGT TGG CAC ATT CCG GGA CAG
XY1	GCG CCG AGC CTG GCC AAA CCA TCA CTT TTC
3R1	TGC ATC CTC AAA GCA CTC TAG GGT CCA GCG T
SA3P	TAA GCT AGA GCA TTG GTG GTC ATG C
XYHA	GAA GTA GCT CCG ACC AGC TTA GCA A

Carlsbad, CA, USA), 0.2 μM of primers SA3P, XYHA and XY1, 0.1 μM of primers 3R1 and 2/3P (Table 1), 4.0% DMSO, 0.5 units of KAPA2G Robust HotStart DNA polymerase (5 U/μL) (KAPA Biosystems Inc., Woburn, MA, USA), and 100 ng of genomic DNA as template. The PCR was carried out on a Veriti™ 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following conditions: initial denaturation at 95°C for 3 min.; 35 cycles at 95°C for 30 seconds, 62°C for 45 seconds, 72°C for 2 min.; then final extension at 72°C for 5 min. Product sizes of PCR were analyzed by electrophoresis on 1.0% agarose gel (LE Agarose, Roche Applied Science, Mannheim, Germany). The gel was photographed using Molecular Imager Gel Doc™ XR System (Bio-Rad Laboratories). Product sizes of PCR were 1940 bp for the normal band, 2220 bp for the –3.7 kb mutant band, and 1673 bp for the –4.2 kb mutant band. For nondeletional mutation detection, we first performed DNA sequencing and then developed and applied direct mutation detection [PCR-RFLP (restriction fragment length polymorphism)].

We carried out PCR-RFLP to detect the codon 59 mutation as previously described (3). We developed a PCR-RFLP-based method to detect Hb CS, codon 22 and the IVS-II-142 mutations. For the Hb CS mutation detection, 25 μL reaction mixture contained 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8, 200 μM dNTPs, 0.4 μM of each primer (Table 2), 0.625 units of Taq DNA polymerase (New England BioLabs Inc, Ipswich, MA, USA) and 100 ng of genomic DNA. Polymerase chain reactions were carried out on a 9700 thermal cycler (Applied Biosystems) using the following conditions: initial denaturation at 95°C for 5 min.; 35 cycles at 95°C for 30 seconds, 68°C for 30 seconds, 72°C for 1 min.; then a final extension at 72°C for 5 min.

For the detection of the codon 22 and IVS-II-142 mutations, each 25 μL reaction contained 5× Phusion GC Buffer containing 7.5 mM $MgCl_2$ as 1× final concentration, 200 μM dNTPs, 0.4 μM of each primer (Table 2), 6.0% DMSO, 0.5 Units of Phusion DNA Polymerase (New England BioLabs Inc.) and 100 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 95°C for 5 min.; 35 cycles at 95°C for 1 min., 62°C for 30 seconds, 72°C for 1 min.; then a final extension at 72°C for 5 min.

TABLE 2 List of Primers, Polymerase Chain Reaction Product Sizes, Restriction Enzymes and Restriction Fragment Length Polymorphism Product Sizes to Detect Nondeletional α-Thalassemia Mutations

Mutation	Primer Names and Sequences (GenBank: J00153.1) (5'>3')	PCR Product Size (bp)	Restriction Enzymes	RFLP Product Size (bp)		
				Normal	Heterozygote	Homozygote
Hb CS	E3a2: GCG GGT TGC GGG AGG T HbCS: GAA CGG CTA CCG AGG CTC CAG CT*C*	222	*Taq*$^{\alpha}$-I	222	222; 200; 22	200; 22
Codon 22	α2α1PromF: GTG GAG GGT GGA GAC GTC α2R: GGA GGC CCA GCG GGC AGG AGG AAC	971	*Msl*I, *Alu*I	559; 241; 130; 41	559; 495; 241; 130; 41; 64	495; 241; 130; 41; 64
rSNP[a] 149709T>C	0745F: GGG AGC ACC AGG ACA CAG ATG 149776R: CTT GCA CCA ACA GCT TTT CA	164	*Hpy*188I	164	164; 85; 79	85; 79
IVS-II-142	E2a2: CCC GCC CGG ACC CAC A α2R: GGA GGC CCA GCG GGC AGG AGG AAC	599	*Alu*I	258; 169; 130; 42	299; 258; 169; 130; 42	299; 258; 42

Hb CS: Hb Constant Spring.

[a] Primers and PCR-RFLP information provided by Dr. D.R. Higgs (MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, UK.)

For the detection of the rSNP 149709T<C mutation, each 25 μL reaction contained 1× PCR buffer (Qiagen), 1× Q solution (Qiagen), 200 μM dNTPs, 0.2 μM of each primer (Table 2), 1 unit of HotStar Taq Polymerase (Qiagen) and 100 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 95°C for 15 min.; 35 cycles at 95°C for 1 min., 54°C for 1 min., 72°C for 1 min.; then a final extension at 72°C for 5 min.

Polymerase chain reactions to detect the nondeletional mutations were carried out on a 9700 thermal cycler (Applied Biosystems). Digestion, using appropriate restriction enzymes, was according to the manufacturer's instructions. All digested products were electrophoresed in 2.0% agarose. The gels were photograped using Gel Doc™ XR (Bio-Rad Laboratories). The PCR product sizes, restriction enzymes and RFLP product sizes are given in Table 2.

Twenty-seven cases were studied, 25 were of Indonesian ethnic background and two were Chinese. Eighteen cases (67.0%) were compound heterozygotes for Hb Adana and either the $-\alpha^{3.7}$ (17 cases) or $-\alpha^{4.2}$ (one case) mutations (Table 3), and nine cases (33.0%) were compound heterozygotes for Hb Adana and other nondeletional mutations (Table 4).

Although the clinical manifestations in patients with Hb Adana in combination with single gene deletions were mostly mild, the onset varied widely from infant to late adult (1 month to 49 years old) with the steady state Hb levels ranging from 6.1 to 11.9 g/dL (9.4 ± 1.2) (Table 3). Only two patients required regular blood transfusions, one of whom started at 3 months of age and one at 3 years old. Two patients first presented at quite a late age (42 and 49 years). Several patients had a slight increase in Hb F levels (patients 3, 4, 6 and 7 in Table 3) as previously described (1,9). This phenomenon might be related with the increase of γ-globin gene expression, which should be further elucidated.

The nondeletional mutations in *trans* to Hb Adana were Hb CS (four cases), codon 22 (three cases), rSNP 149709T>C mutation (one case) and IVS-II-142 (one case) (Table 4). The first presentation varies from 7 days to 28 years old, with the steady state Hb level ranging from 4.9 to 11.1 g/dL. Most patients (five out of nine) required regular blood transfusions, two patients required irregular blood transfusions, and only one patient did not require any blood transfusions. One patient, a compound heterozygote for Hb Adana and rSNP 149709T>C required regular blood transfusions since 2 months of age, but did not require them after a splenectomy at age 9 years. Two related patients (case 6 and 7) with the same genotype (Hb Adana and codon 22) showed very different clinical severity as thalassemia major and mild thalassemia intermedia. This situation was also found in patients with a compound heterozygosity for Hb Adana and Hb CS (Table 4).

Although Indonesian α-thal patients who had Hb Adana in combination with the 3.7 kb deletion generally have mild-to-moderate anemia, a few of them could manifest as severe anemia. This phenotype variation has been

TABLE 3 Hematological and Clinical Data of Hb Adana Patients in Combination With One Gene Deletion at Diagnosis

n	Sex-Age	Hb (g/dL)	MCV (fL)	MCH (pg)	RDW (%)	Hb A_2 (%)	Hb F (%)	Hb X	Clinical Manifestation
1	M-0.25	6.1	71.0	19.0	20.0	1.4	22.0	Bart's[a]; H	has been transfused since 3 months old[b]
2	M-0.25	9.5	65.2	21.4	19.6	1.1	8.5	–	one transfusion at 1 month old[c]
3	M-2	9.4	65.0	23.7	23.1	2.5	7.4	Bart's[a]	splenomegaly; no transfusions
4	F-4	8.3	73.4	23.6	31.9	2.6	4.0	Bart's[a]	has been transfused since at 3 years old
5	F-5	9.2	71.0	23.0	21.6	2.0	1.4	Bart's[a]	anemic since 4 years old
6	F-7	10.3	69.7	23.8	17.5	2.1	6.6	Bart's[a]	transfused once at 6 years old (fever)
7	F-12	7.8	72.3	22.2	23.9	1.9	4.8	Bart's[a]	not reported
8	M-14	10.2	65.9	22.5	17.9	2.4	1.3	–	not reported
9	F-17	9.5	70.7	23.1	21.1	2.4	1.8	–	high ferritin levels; no transfusions
10	M-22	8.4	70.4	21.5	22.7	3.1	1.5	Bart's[a]	splenomegaly; transfused once at age 18
11	M-24	9.7	83.0	25.0	N.D.	1.8	1.9	–	no transfusions[c]
12	F-26.5	9.7	69.4	24.1	21.9	2.5	1.6	Bart's[a]	transfused at age 25 (pregnancy) and again at age 25.5 old (infection); splenomegaly
13	F-31	7.5	71.7	24.4	42.1	2.6	1.7	Bart's[a]	transfused twice at age 29 (Hb 6.0 g/dL)
14	M-32	11.9	75.6	24.0	17.7	2.6	1.7	–	splenomegaly; high ferritin levels
15	M-34	9.5	83.8	25.5	19.4	2.1	1.8	Bart's[a]	no transfusions
16	F-34	9.0	77.2	23.0	23.3	2.4	1.5	N.D.	transfused during every pregnancy (P_4A_3), for a total of six times[c]
17	F-42	8.4	72.6	23.4	20.2	2.2	1.8	–	transfused twice at age 33 (pregnancy); twice at 41–42 years old (infection); hepatosplenomegaly
18	M-53	11.7	76.2	25.9	16.3	2.3	0.6	–	transfused once at 49 years old
		9.4±1.2	73.0±5.2	23.7±1.2	22.7±6.5	2.3±0.3	2.5±0.3		

N.D.: not defined; RDW: red cell distribution width; patients 1 and 4 are transfusion-dependent; mean and standard deviations were calculated for patients more than 1-year-old.

[a] Was seen on the HPLC system (VARIANT™; Bio-Rad Laboratories) (10).

[b] In transfused patients, hematology analysis was carried out after one (or several) blood transfusions had been received (Hb steady state) except in patient 1 where it was carried out before he had received his first blood transfusion.

[c] The Hb analyses were not done on the HPLC system (VARIANT™; Bio-Rad Laboratories).

NB: All patients carry the Hb Adana mutation in combination with the $-\alpha^{3.7}$ kb type deletion except patient 16 who has the $-\alpha^{4.2}$ kb type instead.

TABLE 4 Hematological, Molecular and Clinical Data of Patients Carrying Hb Adana in Combination With Nondeletional α^{+}-Thalassemia at Diagnosis

n	Sex-Age	Hb (g/dL)	MCV (fL)	MCH (pg)	RDW (%)	Hb A_2 (%)	Hb F (%)	Hb X	Genotype	Notes
1	M-7 days	8.9	93.5	30.9	21.7	0.0	71.7	Bart's[a]; H	codon 59/ Hb CS	not reported
2	M-4	9.8	80.2	26.5	16.6	2.7	1.7	–	codon 59/ Hb CS	transfused since <1 year old
3	F-14	9.0	76.4	23.9	29.1	2.8	2.1	Bart's[a]	codon 59/ Hb CS	regularly transfused since 7 years old (3–4/year)
4	F-28	8.1	85.5	23.5	24.1	2.3	1.6	Bart's[a]; CS[a]	codon 59/ Hb CS	first presented as a young adult
5	M-5	8.2	67.3	24.3	21.8	9.0	1.2	–	codon 59/ codon 22	irregularly transfused (+), high Hb A_2 is not due to coinheritance of Hb E[b]
6	M-5	11.1	73.5	25.4	20.4	2.9	0.0	–	codon 59/ codon 22	regularly transfused since 1.5 years old (10–12/year); has not been transfused since age 14
7	F-24	9.4	73.2	24.5	16.7	1.9	3.0	Bart's[a]	codon 59/ codon 22	only transfused while pregnant
8	F-12	8.7	81.1	27.1	N.D.	2.1	0.1	Bart's[a]	codon 59/ rSNP 149709T>C	regularly transfused since 2-months-old but not since age 9 after being splenectomized
9	F-3	4.9	71.7	23.6	29.0	2.6	2.4	Bart's[a]	codon 59/ IVS-II-142	regularly transfused since 3.5 months old

RDW: red cell distribution width; N.D.: not defined; codon 59: Hb Adana; CS: Hb Constant Spring.

[a] Was seen on the HPLC system (VARIANT™; Bio-Rad Laboratories) (10).

[b] On HPLC, Hb E elutes at a similar time as Hb A_2; the parents do not carry Hb E trait, maybe the blood donor carried Hb E trait.

reported previously (2,9). Patients who had Hb Adana in combination with nondeletional mutations mostly showed more severe symptoms than those who had it with single gene deletion as we can see from their blood transfusion status, and who presented at an earlier age. The clinical severities varied widely between patients, even for those with the same genotype. The diversity of the hematology profiles and clinical manifestations might be due to genetic or non genetic factors, further research is required to explain this phenotype variation. Moreover, traits of the Hb Adana (3) and the other nondeletional α-thal mutations in this study exhibit a normal range of Hb level with mild reduction of red cell indices, such as codon 22 (MCV >70.0 fL, MCH >25.0 pg), rSNP 149709T>C (MCV >70.0 fL, MCH >22.0 pg) and IVS-II-142 (MCV >79.0 fL, MCH >26.0 pg). Therefore, routine DNA analysis, regardless of the hematology feature, is strongly suggested for diagnosis of α-thal in Indonesia, especially during antenatal screening.

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