



Analysis of $\alpha 1$ and $\alpha 2$ globin genes among patients with hemoglobin Adana in Malaysia

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ABSTRACT. Hemoglobin (Hb) Adana [HBA2: c179G>A (or HBA1); p.Gly60Asp] is a non-deletional α -thalassemia variant found in Malaysia. An improvement in the molecular techniques in recent years has made identification of Hb Adana much easier. For this study, a total of 26 Hb Adana α -thalassemia intermedia and 10 Hb Adana trait blood samples were collected from patients. Common deletional and non-deletional α -thalassemia genotypes were determined using multiplex gap polymerase chain reaction (PCR) and multiplex ARMS PCR techniques. Identification of the Hb Adana location on the α -globin gene was carried out using genomic sequencing and the location of the mutation was confirmed via restriction fragment length polymorphism-PCR. Among the 36 samples, 24 (66.7%) had the $-\alpha^{3.7}/\alpha^{Cd59}\alpha$ mutation, while the $-\alpha^{3.7}/\alpha^{Cd59}\alpha$ mutation accounted for 2 samples (5.6%) and the remaining

10 (27.8%) samples were $\alpha/\alpha^{Cd59}\alpha$. All 36 samples were found to have the Hb Adana mutation on the $\alpha 2$ -globin gene. The position of the α -globin gene mutation found in our cases was similar to that reported in Indonesia (16%) but not to that in Turkey (0.6%). Our results showed that the Hb Adana mutation was preferentially present in the $\alpha 2$ -globin genes in Malays compared to the other ethnicities in Malaysia. Thus, the Malays might have similar ancestry based on the similarities in the Hb Adana position.

Key words: Hb Adana; α -thalassemia; α -globin genes; RFLP-PCR; Genotyping

INTRODUCTION

α -thalassemia is a highly prevalent genetic disease that is characterized by various degrees of α -globin chain deficiency caused either by deletional or non-deletional mutations. Non-deletional α -mutations are denoted as $\alpha^T\alpha$ or $\alpha\alpha^T$ depending on the location of the mutation either on the $\alpha 2$ or $\alpha 1$ gene (Bain, 2006). Non-deletional mutations, which involve the $\alpha 2$ gene are often associated with more severe clinical phenotypes than the $\alpha 1$ gene, as the former encodes for 2-3 fold more α -globin mRNA than the latter (Liebhaber et al., 1986). Hb Adana [HBA2: c179G>A (or HBA1); p.Gly60Asp] is a common non-deletional α -thalassemia variant found among Malays (Rahimah et al., 2012). This highly unstable variant is located at position Cd 59 either on $\alpha 1$ - or $\alpha 2$ -globin gene. This point mutation disrupts protein stability since it involves the substitution of the amino acid glycine with a charged aspartic acid (Cürük et al., 1993).

Thalassemia is a public health issue in Malaysia, because it has been estimated that a total of 3-4.5% of Malays and Chinese are thalassemia carriers (George and Ann, 2010). The frequency of α -thalassemia was found to be 4.1% in certain regions in Malaysia (Rahimah et al., 2012). The intermediate forms of α -thalassemia are typically selected since the phenotypic changes are commonly observed in this group than that of the α -thalassemia trait and Hb Barts hydrops fetalis patients. For α -thalassemia trait, the phenotypes are normally too mild and it is difficult to observe any severe changes. Most of the Hb Barts hydrops fetalis patients die at a young age due to the severity of the disease (Weatherall, 1995). This study will be informative regarding the location of Hb Adana on the α -globin gene in Malaysia and its origin compared to other Hb Adana studies.

MATERIAL AND METHODS

Study subjects

Hb Adana was identified in the samples sent to Hospital Kuala Lumpur for α -thalassemia molecular studies. A total of 26 α -thalassemia intermedia with Hb Adana and 10 Hb Adana trait blood samples were collected from patients at the Hospital Kuala Lumpur. This study was approved by the National Medical Research Register Ethics Committee (NMRR-12-1387-13958) and was performed in accordance with the Declaration of Helsinki. Verbal and written consent was obtained from all study subjects.

DNA isolation

A total of 2.5 mL venous blood was collected in EDTA-vacutainers from α -thalassemia

patients. Genomic DNA was extracted from leukocytes (in peripheral blood samples) using QIAamp DNA Midi kit (Qiagen GmbH, Hilden, Germany). Extracted genomic DNA was tested for its quality and quantity using Nanodrop 1000 Spectrophotometer (Thermo Scientific, Thermo Fisher Scientific Inc., Wilmington, DC, USA) and agarose gel electrophoresis.

α -globin genotyping

Seven deletional α -thalassemia [$-\alpha^{3.7}$ (NG_000006.1:g.34164_37967del3804), $-\alpha^{4.2}$ (not defined), $--^{SEA}$ (NG_000006.1:g.26264_45564del19301), $-\alpha^{20.5}$ (NG_000006.1:g.15164_37864del22701), $--^{THAI}$ (NG_000006.1:g.10664_44164del33501), $--^{FIL}$ (NG_000006.1:g.11684_43534del31851), $--^{MED}$ (NG_000006.1:g.24664_41064del16401)] were characterized using multiplex gap polymerase chain reaction (PCR) (Chong et al., 2000).

Six point mutations [initiation Cd (c.1A>G), Cd30 (c.91_93delGAG), Hb Evora (c.106T>C), Hb Adana (c.179G>A), Hb Quong Sze (c.377T>C), Hb Constant Spring (c.427T>C)] were characterized using multiplex ARMS PCR (Eng et al., 2001).

Detection of the location of Hb Adana in α 1- and α 2-globin genes

PCR products were purified, sent for bidirectional sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) and analyzed on the ABI 3100 Genetic Analyzer (Applied Biosystems). The sequence alignment and assembly was done using the DNA Baser sequence assembly software (Heracle Biosoft SRL, Arges, Germany).

Confirmation of Hb Adana on α 2-globin gene

Restriction fragment length polymorphism (RFLP)-PCR technique was performed to confirm the location of Hb Adana on the α 2-globin gene as described by Nainggolan et al. (2010). The forward and reverse primers 59modF (5'-GCT CTG CCC AGG TTA AGG GCC TCG-3') and α 2R (5'-GGG AGG CCC ATC GGG CAG GAG GAA C-3'), respectively, were used to amplify the 460-bp product using conventional PCR. PCR was performed in a 25- μ L reaction containing 2X Phusion Flash PCR Master Mix and 100 ng DNA template (Phusion Flash High-Fidelity PCR Master Mix, Thermo Scientific, Affibody AB, Sweden). PCR was carried out with an initial heat activation for 10 s at 98°C, followed by 35 cycles of 98°C for 1 s, 58°C for 5 s, and 72°C for 15 s, and then an extension step of 72°C for 1 min using a PCR thermal cycler (Takara, Shiga, Japan). About 20 μ L PCR products was used for restriction enzyme digestion with *Taq*- α I enzyme (New England Biolabs, Beverly, MA, USA). A total of 5 μ L PCR product and the restricted fragments were examined on a 1.5% agarose gel, stained in ethidium bromide solution, and viewed under a UV illuminator.

RESULTS

This study recruited 26 α -thalassemia intermedia patients with Hb Adana and 10 patients with Hb Adana trait. Representative results are shown in Table 1 in which Malays had the highest frequency of the genotype $-\alpha^{3.7}/\alpha^{Cd59}\alpha$ (66.7%), followed by $-\alpha^{4.2}/\alpha^{Cd59}\alpha$ (5.6%), and $\alpha\alpha/\alpha^{Cd59}\alpha$ (27.8%). Figure 1 shows the chromatograms of direct genomic sequencing for α -thalassemia Hb Adana and Hb Adana trait. All 36 (26 α -thalassemia intermedia with Hb Adana and 10 Hb Adana

trait) samples sequenced showed the Hb Adana mutation on the $\alpha 2$ -globin gene instead of $\alpha 1$. To confirm the position of Hb Adana on the $\alpha 2$ -globin gene, RFLP-PCR was done and the findings concurred with the DNA sequencing results obtained (Figure 2).

Table 1. Genotype frequencies, ethnicity, and the position of Cd 59 on the α -globin gene in Hb Adana cases.

Genotypes	Ethnicity (No. of patients)	Frequency*	Position of Cd59 on α -globin gene
$-\alpha^{2.7}/\alpha^{Cd59}\alpha$	Malay (21) Chinese (1) Iban (2)	24 (66.7%)	$\alpha 2$
$-\alpha^{4.2}/\alpha^{Cd59}\alpha$	Malay (2)	2 (5.6%)	$\alpha 2$
$\alpha\alpha/\alpha^{Cd59}\alpha$	Malay (9) Chinese (1)	10 (27.8%)	$\alpha 2$
Total		36	

*Frequency data are reported as the number of subjects with the frequency percentage in parentheses.

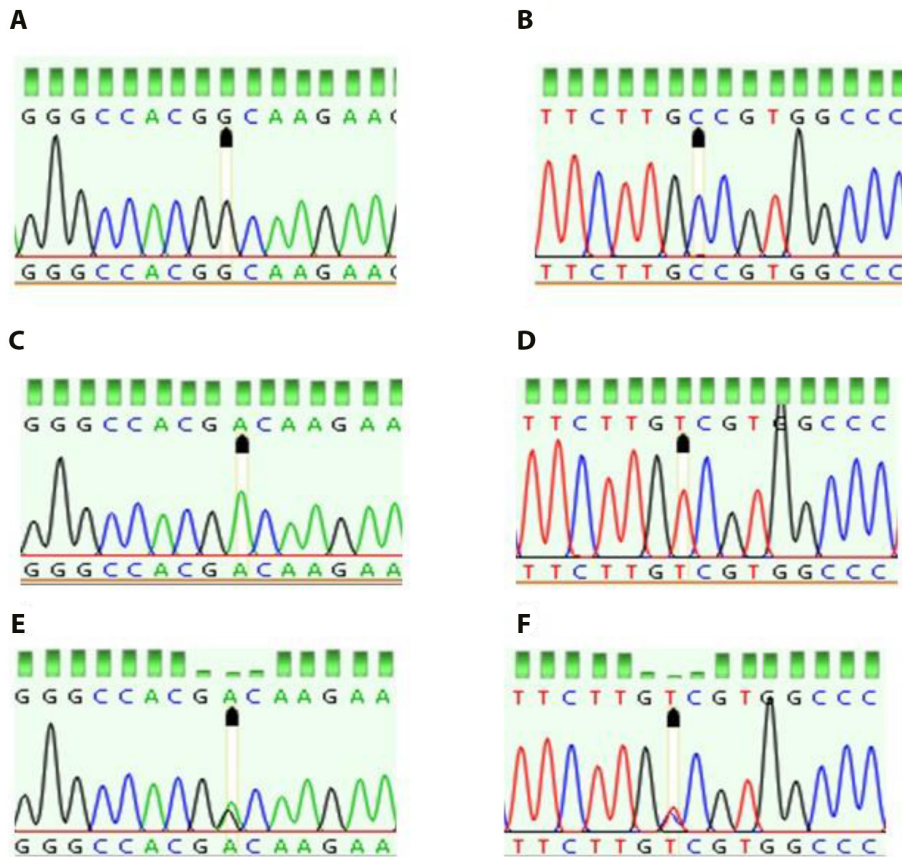


Figure 1. Chromatograms of direct genomic sequencing. **A.** Forward wild-type sequence for nucleotide G of the $\alpha 2$ -globin gene. **B.** Reverse wild-type sequence for nucleotide C of the $\alpha 2$ -globin gene. **C.** Forward mutant (α -thalassemia Hb Adana) sequence for nucleotide A on the $\alpha 2$ -globin gene. **D.** Reverse mutant (α -thalassemia Hb Adana) sequence for nucleotide T on the $\alpha 2$ -globin gene. **E.** Forward mutant (Hb Adana trait) sequence on the $\alpha 2$ -globin gene. Two peaks were detected at the same position for nucleotide G (wild-type allele) and nucleotide A (mutant allele) indicating a heterozygous state. **F.** Reverse mutant (Hb Adana trait) sequence on the $\alpha 2$ -globin gene. Two peaks were detected at the same position for nucleotide C (wild type-allele) and nucleotide T (mutant allele) indicating a heterozygous state.

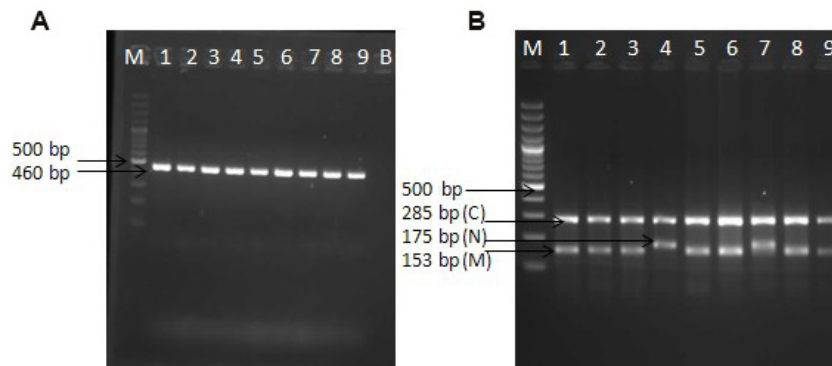


Figure 2. PCR-RFLP analysis of Hb Adana location on α 2 globin gene. **A.** Undigested PCR products. **B.** Digested PCR products. Lanes 1-3, 5-6, and 8-9 = α -thalassemia Hb Adana on α 2. Lane 4 acts as positive control for wild type. Lane 7 = Hb Adana trait on α 2. Lane M = marker for ladder, B = Blank, C = constant band, N = normal band, and M = mutant band.

DISCUSSION

Malaysia has a multiracial population, which consists of 3 major ethnic groups: Malays, Chinese, and Indian. As of 2010, Malay and Chinese populations constituted 65 and 26% of the 28.6 million people in Malaysia, respectively, based on data from the Department of Statistics Malaysia (Department of Statistics Malaysia, 2010).

Adana is a large city in southern Turkey situated near the Seyhan River at the north-eastern edge of the Mediterranean Sea. Major ethnic groups in Adana are Turks, Arabs, and Kurds. The first reported case of Hb Adana was found in a Turkish family and the Cd 59 mutation was located in α 1 of the α -globin gene complex. It was named Hb Adana since the first case was found in the Adana Province, Turkey (Cürük et al., 1993). Although the first case originated from Adana Province in Turkey, its frequency in that province itself is low (only 0.6%) as reported by Bozdogan et al. (2015). Based on Table 2, all Hb Adana cases reported in Turkey so far are in the α 1 globin gene whereas reports from other regions are all in the α 2 globin gene (Cürük et al., 1993; Durmaz et al., 2009; Aksu et al., 2014; Bozdogan et al., 2015). In the past, the prevalence of Hb Adana carriers was not known in Malaysia as the identification of this mutation required DNA analysis, which is not widely available in Malaysia (Alauddin et al., 2014). In addition, Hb Adana trait is asymptomatic and is often overlooked in thalassemia screening programs when the full blood counts and red blood cell indices are normal (Setianingsih et al., 2003). Nainggolan et al. (2010) reported that the frequency of Hb Adana in Indonesia is relatively high, accounting for as much as 16% of thalassemia intermedia and thalassemia major patients. Hb Adana is not a rare mutation as previously reported since the frequency in both Indonesia and Malaysia located in South East Asia has been found to be quite high as determined using the available DNA diagnostic technology (Rahimah et al., 2012; Azma et al., 2014; Yatim et al., 2014; Zainal et al., 2014).

All the Hb Adana cases reported here share the same Cd 59 mutation located in the α 2 globin gene, a feature similar to the cases seen in Indonesia. Our data reported herein is also in agreement with previous reports regarding the Hb Adana cases in Indonesia (Nainggolan et al., 2010; Nainggolan et al., 2013; Megawati et al., 2014). Both Malaysia and Indonesia are culturally

related neighbors located in the same region. The migration between Indonesia and Malaysia resulted in a similar language (Bahasa Malaysia and Bahasa Indonesia), culture, and religion. Constant travel since antiquity, particularly between the coastal port cities of Sumatra in Indonesia and Peninsular Malaysia, for trade also explains the current Hb Adana mutation pool in Malaysia (Rahimah et al., 2012).

Table 2. Recently reported cases of Hb Adana from 1993-2014.

Year (No. of cases)	Genotypes	Affected gene	Ethnicity	Reference
1993 (2)	-- _{20.5} /αα ^{CD59}	α1, exon 2	Turkish	Cürük et al., 1993
1997	-- _{SEA} /α ^{CD59} α	α2, exon 2	Chinese	Chan et al., 1997
2008	-- _{FIL} /α ^{CD59} α	α2, exon 2	Filipino	Henderson et al., 2008
2008 (2)	-α ^{3.7} /α ^{CD59} α	α2, exon 2	Albanian	Douna et al., 2008
2009	-- _{20.5} /αα ^{CD59}	α1, exon 2	Turkish	Durmaz et al., 2009
2010 (2)	α ^{CD59} α/α ^{CD59} α	α2, exon 2	Javanese	Nainggolan et al., 2010
2013 (17)	-α ^{3.7} /α ^{CD59} α	α2, exon 2	Indonesian	Nainggolan et al., 2013
2013	-α ^{4.2} /α ^{CD59} α	α2, exon 2	Indonesian	Nainggolan et al., 2013
2013 (4)	α ^{CD59} α/αα ^{CS}	α2, exon 2	Indonesian	Nainggolan et al., 2013
2013 (3)	α ^{CD59} α/αα ^{CD22}	α2, exon 2	Indonesian	Nainggolan et al., 2013
2013	α ^{CD59} α/αα ^{SNP}	α2, exon 2	Indonesian	Nainggolan et al., 2013
2013	α ^{CD59} α/αα ^{IL-142}	α2, exon 2	Indonesian	Nainggolan et al., 2013
2013	α ^{CD59} α/αα ^{CD24}	α2, exon 2	Sudanese	Megawati et al., 2014
2014 (2)	α ^{CD59} α/αα ^{CS}	α2, exon 2	Malay	Alauddin et al., 2014
2014 (2)	-α ^{3.7} /α ^{CD59} α	α2, exon 2	Malay	Alauddin et al., 2014
2014	αα ^{CD59} /αα ^{CD59}	α1, exon 2	Turkish	Aksu et al., 2014
2015	-α ^{3.7} /α ^{CD59} α	α1, exon 2	Turkish	Bozdogan et al., 2015
2015 (3)	α ^{CD59} α/αα	α1, exon 2	Turkish	Bozdogan et al., 2015

Hb Adana reported in Turkey is different from that described in Indonesia and Malaysia in terms of the position of Cd 59 in the α-globin gene complex. The cases reported in Turkey involved α1 and not α2 of the α-globin gene complex. α-thalassemia is diverse as the mutations are varied among populations due to natural selection along with some degree of founder effect and genetic drift (Higgs and Weatherall, 2009). Hb S is a β-globin chain variant with multicentric origins. The majority of the people who inherited hemoglobinopathy are African, as well as those from the Middle East and India. Similarly, Hb Adana may have more than one origin (Europe and Southeast Asia), which may explain its position in either α1 or α2 globin genes.

The results of this study should be interpreted in the context of the study's limitations. Haplotyping studies on the α-globin gene should be done on the Hb Adana patients to compare and trace the origins of different populations from different regions. This will facilitate the study of the α-globin gene flow among various populations using phylogenetics, among both the Turkish and non-Turkish population. Hb Adana among Malays in Malaysia might have the same ancestry based on the similarities of the Cd 59 position. Definitive diagnosis is dependent on the DNA analysis and we strongly recommend that DNA analysis is incorporated as part of the routine α-thalassemia screening programs carried out by the National Prevention and Control Programme for thalassemia patients in Malaysia. By doing so, appropriate genetic counselling can be given to couples or families with a risk of α-thalassemia Hb Adana.

Conflicts of interest

The authors declare no conflict of interest.

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