LIBYAN JOURNAL OF MEDICINE
2024, VOL. 19, 2406620
https://doi.org/10.1080/19932820.2024.2406620

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Interactions of electrophoretically silent hemoglobin Hekinan II [HBA1:c.84G>T] with various forms of α-thalassemias and other hemoglobinopathies: novel insights into the molecular and hematological characteristics and genetic origins

Sitthichai Panyasai [a], Prasert Chantanaskulwong[b], Nopphadol Permsripong[c] and Thippawal Mokmued[c]

[a]Department of Medical Technology, School of Allied Health Sciences, University of Phayao, Phayao, Thailand; [b]Department of Medical Technology, Phare Hospital, Phare, Thailand; [c]Department of Medical Technology, Uttaradit Hospital, Uttaradit, Thailand

ABSTRACT

To determine the molecular basis, genotype – phenotype relationship, and genetic origin of Hemoglobin (Hb) Hekinan associated with several forms of α-thalassemia and other hemoglobinopathies for a better understanding of its diverse clinical phenotypes. Seventeen participants with suspected abnormal Hb were studied. Hb analysis was performed using high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Mutational and α-haplotypic and structural analyses were conducted, and the effects of mutations on globin-chain stability were determined. All participants harbored Hb Hekinan II (HBA1:c.84 G>T) co-inherited with another α-globin gene anomaly. Three novel genotypes, $(\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha)$, $(\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha,\beta^{A}/\beta^{E})$, and $(\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha,\beta^{E}/\beta^{E})$, were characterized. Despite being co-inherited with both α- and β-Hb variants Hb Hekinan II led to minimal changes in erythrocyte parameters, suggesting a non-pathological nature. HPLC but not CE revealed a distinct small shoulder-like Hb pattern. Thai Hb Hekinan II was strongly associated with haplotype [+ - S + - - -] and the possibility of four different haplotypes, while two Burmese Hb Hekinan II were associated with haplotypes [± - S + - + -] and [± - S + - - -]. The novel genotypes identified provide a fresh perspective on Hb Hekinan II diversity. HPLC has superior identification capabilities for samples of Hb Hekinan II co-inherited with α-thalassemia. Thai and Burmese Hb Hekinan II have diverse origins.

ARTICLE HISTORY
Received 24 July 2024
Accepted 17 September 2024

KEYWORDS
Hemoglobin hekinan II; α-hemoglobin variant; α-globin haplotype; high-performance liquid chromatography; capillary electrophoresis

1. Introduction

Hemoglobinopathies are genetic disorders characterized by structural alterations and/or insufficient globin-chain production. These disorders have a significant impact worldwide [1]. More than 1,800 variants have recently been registered in the Globin gene server's HbVar database [2]. Although most hemoglobin (Hb) variants are non-pathological, some can cause severe clinical syndromes with hemolysis. Furthermore, the interaction between pathological Hb variants and various structural Hb variants results in a wide range of disorders of varying clinical severity. Therefore, it is important to identify and characterize Hb variants in populations with a high prevalence and heterogeneity of thalassemia. Hemoglobin Hekinan is a genetic variant that affects the structure of the α-globin protein. This variant is caused by a change from G to T in the third nucleotide base at codon 27 of the α-globin gene. This results in the replacement of glutamic acid with aspartic acid at position 8 of the B helix [3]. Hb Hekinan was initially reported in a Japanese male [3] and has since been identified in several populations, including Chinese-Black individuals from French Guyana [4], Chinese individuals from Macau [5], Thai individuals [6–8], a Burmese woman working in Thailand [8], and Taiwanese individuals [9]. Notably, all reported cases of the GA*G*>GA*T* mutation have occurred on the α_1-globin gene. However, a similar substitution of amino acids in the same position with a different nucleotide change (GA*G*>GA*C*) has been observed on the α_2-globin gene, resulting in Hb Hekinan. This substitution was reported to be co-inherited with a 3.7 kb deletion α^{+}-thalassemia [10]. Although different nucleotide substitutions (G>T and G>C) between the α_1 and α_2-globin genes cause Hb Hekinan II and Hb Hekinan, respectively, their hematological data are similar, but a relatively high percentage of Hb Hekinan was found due to a mutation on the α_2-globin gene. Hb Hekinan has an acid-to-acid amino acid substitution, leading to a lack of an electron charge compared to Hb A. However, its electrophoretic behavior is similar to that of Hb A, making it difficult to distinguish using high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Hence, it is frequently misdiagnosed using routine Hb analysis [3–10]. The challenge in distinguishing Hb Hekinan from Hb A is significant. The presence of α^{0}-thalassemia makes it easy to distinguish Hb Hekinan

CONTACT Sitthichai Panyasai sitthichai.pa@up.ac.th Department of Medical Technology, School of Allied Health Sciences, University of Phayao, 19, Phahonyothin Road, Muang Phayao, Phayao 56000, Thailand

from Hb A, allowing for estimation through HPLC but not CE [6,8,10]. Therefore, the co-inheritance of Hb Hekinan and α^0-thalassemia is frequently reported and is often detected accidentally during routine laboratory tests. However, isoelectric focusing (IEF) and weak cation-exchange materials coupled with polyaspartic acid techniques have demonstrated the capability to completely separate Hb Hekinan II from Hb A in the heterozygote state [7,9]. Studies employing these techniques have estimated Hb Hekinan in heterozygotes to be 13.0–14.7% [4,5,9,10]. The co-inheritance of Hb Hekinan II with α^0-thalassemia has been reported, and its interaction with Hb E (HBB:c.79 G>A) heterozygotes, with or without α^0-thalassemia, has been observed in Thai patients [6,8]. Interestingly, their hematological parameters showed minimal changes, similar to those of the equivalent genotype lacking $\alpha^{Hekinan}$. Furthermore, its combination with Hb E homozygote and compound heterozygosity of Hb E and Hb Hope (HBB:c.410 G>A), leading to a complex genotype ($\alpha\alpha^{Hekinan}/\alpha\alpha$, β^E/β^E and $\alpha\alpha^{Hekinan}/\alpha\alpha$, β^E/β^{Hope}), has also been reported [11]. Despite their complexity, these genotypes do not exhibit clear clinical symptoms. The challenge of distinguishing Hb Hekinan II from other Hb by IEF or HPLC highlights the potential diagnostic obstacles in Hb analysis.

In areas with a wide variety of thalassemic alleles, such as Thailand, the possibility of interactions with Hb Hekinan II resulting in complex syndromes is common. These syndromes have been increasingly reported since the introduction of nationwide prevention and control programs, highlighting the pressing need for further research. A better understanding of the hematological characteristics of Hb Hekinan II with diverse genotypes is essential for the accurate and timely diagnosis and genetic counseling of thalassemia and other hemoglobinopathies, especially in areas with a high prevalence and heterogeneity of thalassemia and hemoglobinopathies. Furthermore, the genetic origin and evolution of this variant remain to be established. Therefore, in this study, we conducted a comprehensive analysis of participants with Hb Hekinan II. Our investigation focused on characterizing its molecular background, genotype–phenotype relationship, and genetic origin, providing valuable insights into this variant.

2. Materials and methods

2.1. Subjects and hematological analysis

The study protocol was approved by the Ethics Committee, Institutional Review Board (IRB) of the University of Phayao, Phayao, Thailand (1.2/026/67). Informed consent was obtained from all participants before their participation. Seventeen blood specimens suspected of harboring Hb variants were collected from peripheral hospitals in northern Thailand. The School of Allied Health Sciences, University of Phayao, Phayao, Thailand, was responsible for the DNA analysis of these samples. During the transfer, the samples were kept at 4°C to maintain sample completeness. Hematological parameters were recorded using standard blood cell counters. Hemoglobin analysis was conducted using automated HPLC (VARIANT II, β-Thalassemia Short Program; Bio-Rad Laboratories, Hercules, Calif., USA) and CE (MINICAP Flex Piercing; Sebia, Lisses, France).

2.2. Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes using a standard method [12]. Complete HBA1 and HBA2 genes were analyzed as previously described [13]. Selective amplification of α_1- and α_2-globin genes were performed employing the specific primers C1 (5′-TGGAGGGTGGAGACGTCCTG-3″) and C2 (5″-CCATGCTGGCACGTTTCTGA-3″) and C1 and C3 (5″-CCATTGTTGGCACATTCCGG-3′), respectively. Direct DNA sequencing of the amplified α-globin genes was performed with an ABI PRISM^{TM} 3130 ×l analyzer (Applied Biosystems, Foster City, CA, USA). Mutation positions were analyzed and identified in the human abnormal Hb thalassemia library (http://globin.bx.psu.edu). Multiplex allele-specific polymerase-chain reaction (PCR) previously established in our laboratory was used to confirm Hb Hekinan II (HBA1:c.84 G>T) [14]. Identification of the two most common α^0-thalassemia deletions ($-^{SEA}$ and $-^{THAI}$), α^+-thalassemia [$-\alpha^{3.7}$ (rightward) and $-\alpha^{4.2}$ (leftward)] deletions as well as the common Hb Constant Spring (HbCS; HBA2: c.427T>C) and Hb Paksé (HBA2: c.429A>T), were routinely performed in our laboratory using Gap-PCR and allele-specific PCR [15–17]. Analysis of HbE (HBB:c.79 G>A) was performed using allele-specific PCR, as described previously [18].

2.3. Analysis of α-globin gene haplotypes

The α-globin gene haplotypes were assessed using seven of the most common polymorphisms, consisting of six restriction fragment length polymorphisms (RFLP); *Xba*I site of the 5′ ζ2-globin gene, *Bgl*I site of the 3′ ζ2-globin gene, *Acc*I site of the 3′ ψα2-globin gene, *Rsa*I site of the 5′ α2-globin gene, *Pst*I sites of the 5′ α1- and 5′ θ1-globin gene, and one broadly triallelic inter ζ-globin hypervariable region (HVR). Amplification of the RFLP and inter ζ-globin HVR regions was performed as described previously [19]. Haplotypes were created by determining the presence or absence of cleavage at each site and gathering the results into one pattern.

2.4. Bioinformatics analysis of hemoglobin Hekinan II

To improve our understanding of the impact of amino acid substitution on the α-globin chain, structural analysis of the positions altered on the α-globin chain was conducted using iCn3D: web-based 3D structure viewer [20], which is now available on NCBI web servers and GitHub (https://github.com/ncbi/icn3d). Additionally, the effect of a single amino acid substitution on the structure and function of the α-globin chain was predicted using network-based HumDiv-trained Polymorphic Phenotype V.2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/).

3. Results

We studied 17 healthy participants aged 3–76 years with no history of blood transfusion or hepatosplenomegaly. Of these, two participants were of Burmese male descent (23 and 24 years old) who attended an antenatal care clinic. A one-tube osmotic fragility test for thalassemia and hemoglobinopathies screening revealed positive results in all participants, with three participants also positive for the dichlorophenol indophenol precipitation (DCIP) test, indicating Hb E presence. Hb analysis using an automated cation exchange HPLC showed A_2A with an abnormal Hb partially distinguished from Hb A (Figure 1(a)) with relatively low Hb A_2 levels (1.96% ± 0.3) in eight participants, and A_2A with an unusual peak of Hb A (left shoulder-like Hb pattern) (Figure 1(b,c)) in six participants. Furthermore, an additional suspected HbCS fraction was observed in one participant (Figure 1(c)). This Hb analysis result indicated an unknown Hb variant co-eluting with Hb A. Additionally, an unusual Hb A peak, along with a small abnormal peak ahead of the Hb E fraction, was observed in the two participants that harbored Hb E. One of these two participants indicated a possibility of the HbCS fraction, indicating a complex interaction of triple hemoglobinopathies, as shown in Figure 2(a). Surprisingly, one participant with an Hb E pattern showed an unknown Hb variant that co-eluted with Hb E, resulting in an unexpected Hb E peak, as shown in Figure 2(b). However, no abnormal Hb peaks, besides those of Hb E and Hb CS, which were analyzed by automated CE, were observed in any patient (Figures 1(d–f) and 2(c,d)). HPLC-chromatogram analysis revealed that the abnormal Hb was similar to Hb Hekinan II or Hb Wiangpapao, previously described in the Thai population [6,7,14].

Both α_1- and α_2-globin genes were analyzed using PCR and direct DNA sequencing and revealed a substitution of G to T on the third base of codon 27 of the α_1-globin gene (Figure 3(b,c)). No anomalies were detected in the α_2-globin gene. A G to T mutation at a specific position results in the substitution of glutamic acid with aspartic acid in the α-globin chain responsible for Hb Hekinan II, which has been previously observed in the Asian population [3–9]. The mutation was confirmed in all participants by analysis using a previously developed allele-specific PCR assay (Figure 3(d)). Further DNA analysis of the α- and β-globin genes revealed additional anomalies in the α-globin gene in all participants. Allele-specific PCR revealed that three participants also harbored β^E-globin genes (data not shown); therefore, five different genotypes were identified. We identified the SEA deletion α^0-thalassemia in eight heterozygous Hb Hekinan II ($-^{SEA}/\alpha\alpha^{Hekinan}$) and interactions with 3.7 kb deletion α^+-thalassemia ($-\alpha^{3.7}/\alpha\alpha^{Hekinan}$) in five participants. Additionally, the HbCS gene was identified in four heterozygous Hb Hekinan II ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$); two of them co-inherited with Hb E heterozygote, generating a complex genotype ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$, β^E/β^A) and another one co-inherited with Hb E homozygote causing a more complex genotype ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$, β^E/β^E). The genotypes ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$), ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$, β^E/β^A), and ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$, β^E/β^E) are novel interactions that have not yet been observed in any population so far.

The hematological data for each genotype are summarized in Table 1. None of the genotypes were associated with anemia. Remarkedly, all participants with the ($-^{SEA}/\alpha\alpha^{Hekinan}$) genotype exhibited an evident decrease in MCV and MCH. Furthermore, HPLC allowed for the partial separation of Hb Hekinan II from Hb A, making it possible to determine the Hb Hekinan II ($\alpha_2{}^{Hekinan}\beta_2{}^A$) level as 24.6 ± 1.6% of total Hb. The participants with genotype $-\alpha^{3.7}/\alpha\alpha^{Hekinan}$ showed no anemia and red blood cell abnormality. However, the Hb Hekinan II peak was not observed on the HPLC chromatogram. A participant with compound heterozygous Hb Hekinan II and HbCS ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$) exhibited no anemia and slightly decreased mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). The HPLC chromatogram and CE electropherogram showed the HbCS peak but no Hb Hekinan II peak (Figure 1(c–f)). Hematological parameters of two participants carrying triple different Hb variants with genotype ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$, β^E/β^A) showed normal red blood cell parameters. HbCS was detected in one patient, allowing an estimation of 0.5%; however, Hb E separated in both cases showed relatively low levels (15.8% and 22.2%). The combination of Hb Hekinan II with heterozygous HbCS and homozygous Hb E, causing a more complex genotype ($\alpha^{CS}\alpha/\alpha\alpha^{Hekinan}$, β^E/β^E), led to a distinct decrease in MCV and MCH and an Hb E level of 86% of total Hb accepted range in the homozygous Hb E. The suspected HbCS and Hb Hekinan II peaks were not visible on the HPLC-chromatogram or CE-electropherogram (Figure 2(b–d)).

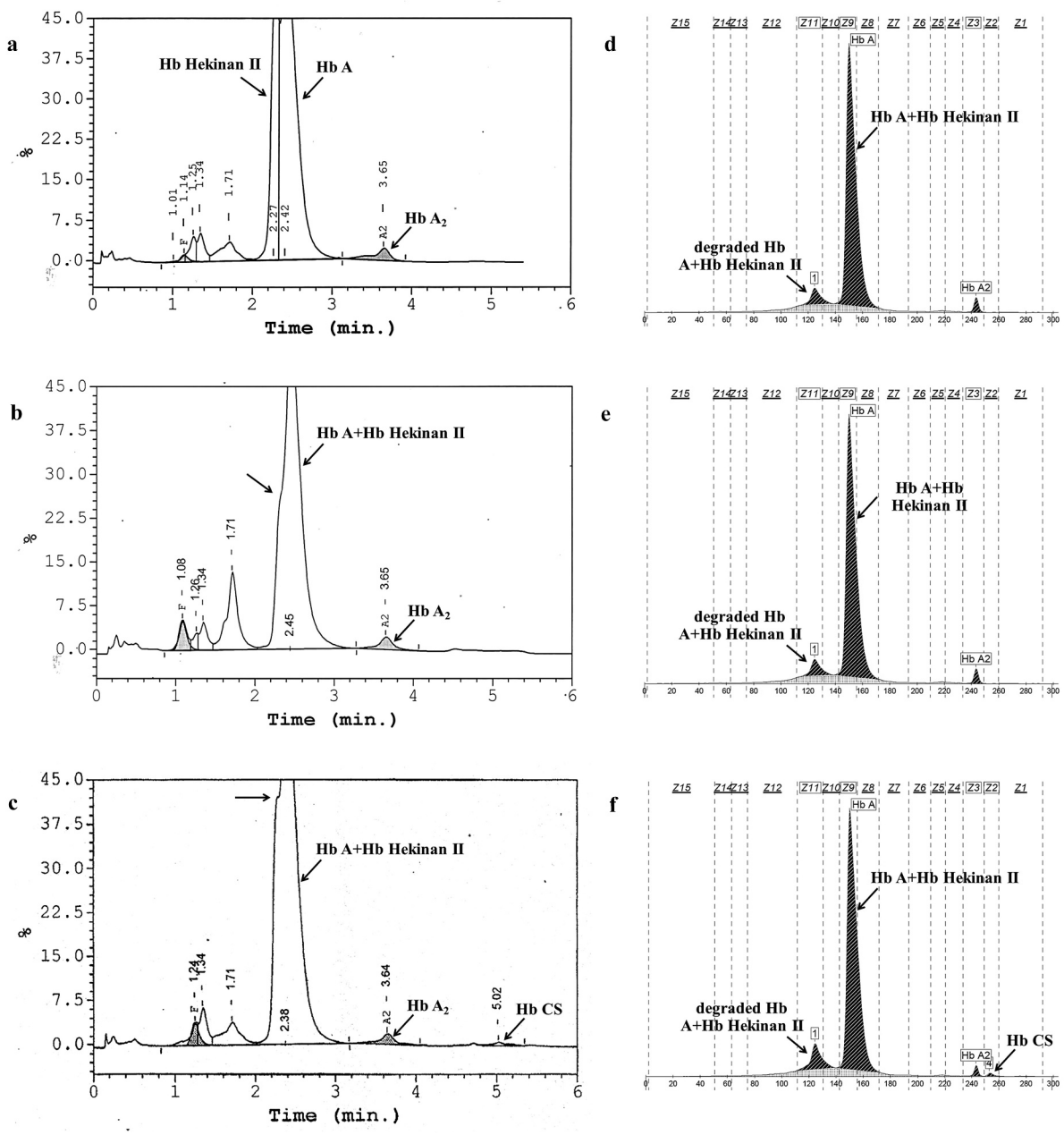


Figure 1. Hb separation profiles of Hb Hekinan II using automated HPLC. HPLC-chromatogram pattern of a compound heterozygous Hb Hekinan II and SEA deletional α^0-thalassemia (a) showing slightly faster migration of Hb Hekinan II than Hb A and incomplete separation from Hb A. (b) HPLC-chromatogram pattern of a compound heterozygous of Hb Hekinan II and 3.7 kb deletion α^+-thalassemia. (c) HPLC-chromatogram pattern of a compound heterozygous of Hb Hekinan II and Hb CS. D-F: Representative Hb separation profiles obtained by automated capillary electrophoresis (CE). d: compound heterozygous Hb Hekinan II and SEA deletional α^0-thalassemia, e: compound heterozygous of Hb Hekinan and 3.7 kb deletion α^+-thalassemia, and f: compound heterozygous of Hb Hekinan and Hb CS. Arrows indicate shoulder-like Hb fractions.

The analysis of the structural alterations using the 3D ribbon structure, conducted using iCn3D, revealed the replacement of glutamic acid at codon 27 with aspartic acid in the alpha chain and that this is specifically located on the α-helix B segment, which is outside of the crucial functional position related to the binding sites for oxygen, proteins, and heme (Figure 4). Thus, the substitution of aspartic acid did not affect the function of the alpha globin chain. The pathogenic prediction of replacing glutamic acid 27 with aspartic acid in the alpha chain using PolyPhen-2 indicated that it was unlikely to affect protein function, with a score of 0.371 (sensitivity, 0.85; specificity, 0.78).

Table 2 summarizes the α-globin gene haplotype and segregation. Given that heterozygosity of multiple polymorphic sites was observed, accurate segregation of haplotypes could not be performed. However, complete segregation of Thai Hb Hekinan II was observed with one allele, which appeared to be associated with [+ - S + - - -]. Additionally, Thai Hb Hekinan II may be associated with four different haplotypes: [± - S + - + -], [- - M ± - + -], [± - M + ± + -], and [± - M + ± - -]. This is suggested by the segregation of

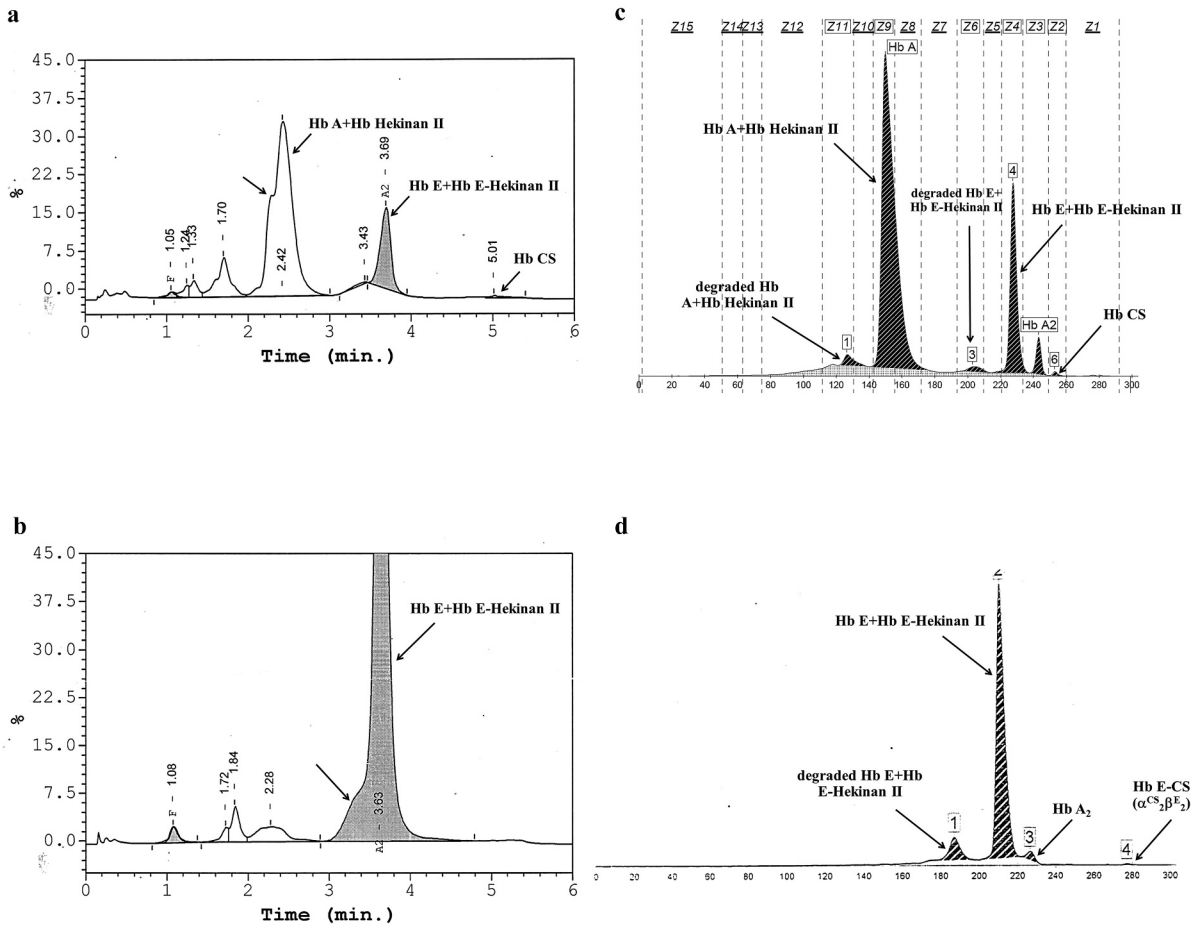


Figure 2. Representative Hb separation profiles of Hb Hekinan II using automated HPLC and capillary electrophoresis (CE). HPLC-chromatogram pattern of (a) a triple heterozygous Hb Hekinan II with Hb CS and Hb E, (b) a compound heterozygous of Hb Hekinan II with Hb CS and homozygous Hb E. Hb separation profiles using automated capillary electrophoresis of (c) a triple heterozygous Hb Hekinan II with Hb CS and Hb E and (d) a compound heterozygous Hb Hekinan II with Hb CS and homozygous Hb E, in which the small peak occupying zone 1 is visualized in addition to that of Hb E.

these haplotypes based on the polymorphic sites exhibiting homozygous patterns. These findings suggest that Hb Hekinan II has multiple origins in the Thai population. Furthermore, two Burmese Hb Hekinan II are likely associated with haplotypes [± - S + - + -] and [± - S + - - -].

4. Discussion

Hb analysis is an effective screening strategy worldwide for the detection of thalassemia and hemoglobinopathies. Intensive prevention and control programs for this disease are currently being implemented in Thailand, where hemoglobinopathies are prevalent. Consequently, there has been an increase in the number of new Hb variants whose differential electrophoretic mobility can be visualized on electropherograms and chromatograms. In this study, we characterized the molecular and hematological characteristics of participants whose Hb-chromatogram displays either an unknown Hb fraction overlapped with Hb A or an unusual Hb A fraction that differs from typical Hb A to identify individuals harboring Hb Hekinan II and investigate its molecular background, genotype – phenotype relationship, and genetic origin. Molecular analysis revealed a G to T substitution at position 27 of the α_1-globin gene responsible for Hb Hekinan II, previously documented in the Asian population [3–9]. The analysis of α- and β-globin genes enabled the identification of five genotypes, including three novels and two previously documented genotypes. Interestingly, all genotypes exhibit an additional α-globin gene anomaly, but no simple heterozygosity of Hb Hekinan II was observed. Furthermore, both heterozygous and homozygous Hb E genes were found in the compound heterozygous Hb Hekinan II and Hb CS, leading to complex genotypes, previously unidentified in any population. This finding highlights the heterogeneity of thalassemia and hemoglobinopathies and emphasizes the importance of further research in this area.

Different genetic variations of the Hb Hekinan II result in various hematological characteristics. The genotype with $\alpha\alpha^{Hekinan}/-\alpha^{3.7}$ exhibited no altered erythrocyte parameters and a decrease in Hb A_2 levels, consistent with a case previously reported [7].

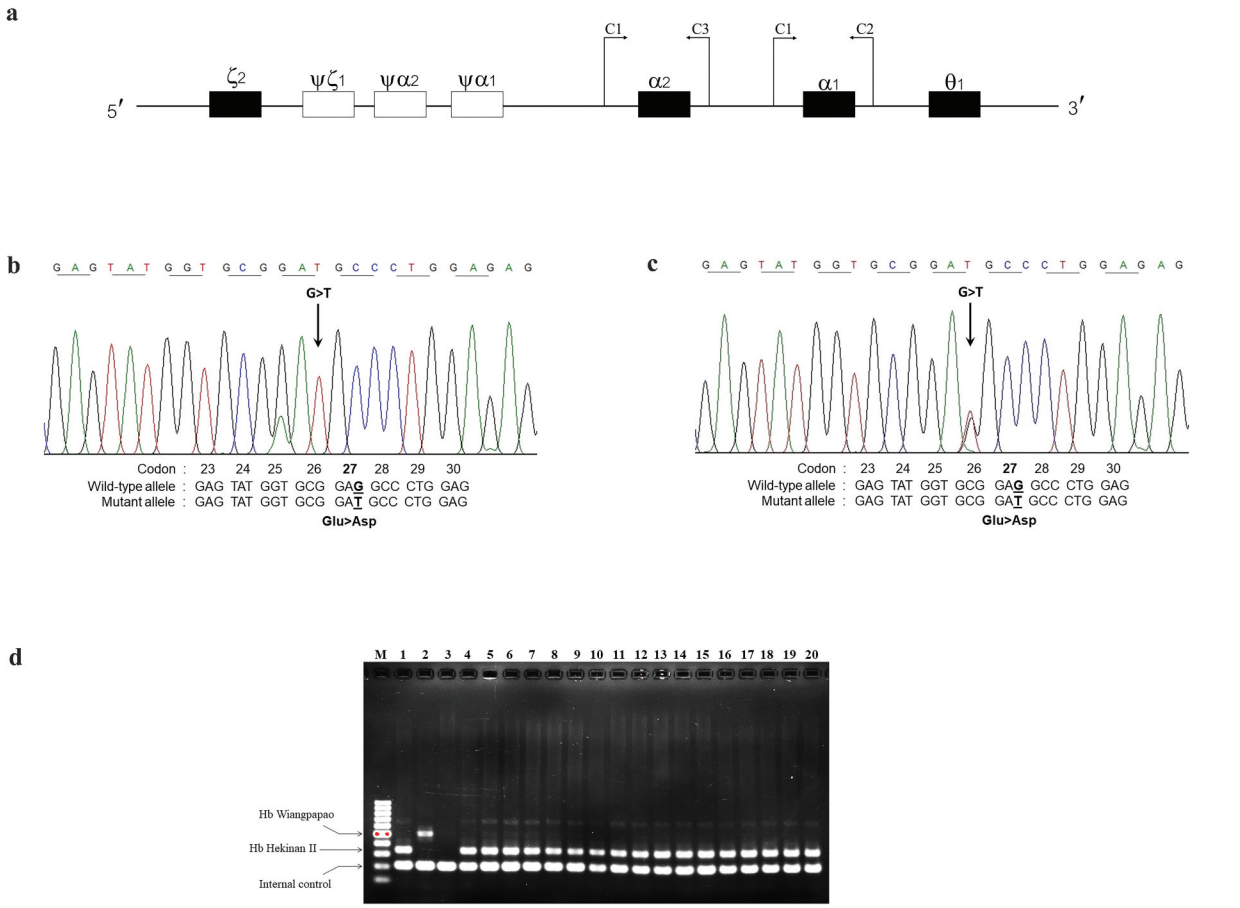


Figure 3. Identification of mutations by DNA sequencing of the selective amplification of *HBA1* and *HBA2* genes and direct sequencing of the amplicons. (a) horizontal arrows represent the locations and orientations of the primers used for α_1 and α_2-globin genes. The C1 and C2 primer pair directs α_1-globin specific amplification of a 1091 nucleotide sequence and the C1 and C3 pair directs α_2-globin synthesis of a fragment of 1085 bp. (b) direct DNA sequencing of the α_1-globin gene. The sequencing profile demonstrates the antisense strand sequences in codon 27 where GAG – GAT mutation causes Hb Hekinan II. The electropherogram denotes the presence of only T nucleotide at that position representing a compound heterozygous Hb Hekinan II and SEA deletion α^0-thalassemia. (c) electropherogram showing G and T substitution at codon 27, indicating the heterozygous Hb Hekinan II. (d) electrophoresis gel illustration. Lane M contains 100-bp leader DNA markers. Lane 1: positive DNA for Hhb Hekinan II, lane 2: positive DNA for Hb wiangpapao, lanes 3: normal DNA, and lanes 4-20: DNA from participants harboring Hb hekinan II.

The novel genotype with double heterozygous Hb Hekinan II with HbCS ($\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha$) exhibited a minimal change in red blood cell parameters, as seen in heterozygous HbCS carriers [21,22]. Remarkedly, they showed a remarkable decrease in Hb A_2 ($\alpha_2\delta_2$) (1.8%) compared with those with heterozygous Hb Hekinan II. The levels of Hb A_2 in simple heterozygous Hb Hekinan II have been reported to be 2.3 ± 0.3 of total Hb [23]. Hb CS may lead to a significant decrease in normal α-globin protein synthesis. Similarly, a new combination of Hb Hekinan II and Hb CS with heterozygous Hb E causing triple globin gene anomalies ($\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha$, β^A/β^E) exhibited remarkably decreased Hb E levels (15.8% and 22.2%). While the equivalent genotype lacking the $\alpha^{Hekinan}$ gene showed no significant change in Hb E levels compared with pure Hb E carriers (27.0 ± 1.6) [24]. Therefore, the decreased Hb E levels observed in $\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha$, β^A/β^E were similar to the effect seen in individuals with Hb E and α^0-thalassemia, where both α_1 and α_2 are deleted [24,25]. Generally, the Hb E protein level in Hb E carriers with α^0-thalassemia is 19.6 ± 1.3 [24]. Nevertheless, no changes were observed in red blood cell parameters in the $\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha$, β^A/β^E genotype, unlike the evident decrease in MCV and MCH observed in individuals with heterozygous Hb E with α^0-thalassemia [24] Interesting, this study identified a new complex genotype ($\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha$, β^E/β^E) that does not produce any Hb A and Hb Hekinan II. This genotype produced significant amounts of Hb E. Additionally, a new type of hemoglobin called Hb Hekinan II-E, composed of two $\alpha^{Hekinan}$ chains and two β^E chains, may also be synthesized. However, the challenge lies in the difficulty of distinguishing Hb Hekinan II-E from Hb E using HPLC and CE. In our analysis, hematological data and Hb E levels were consistent with those observed in cases of simple homozygous Hb E [26]. The most common genotype found in this study was Hb Hekinan II with α^0-

Table 1. Hematological parameters and genotypes of 17 carriers of Hb Hekinan II with α-thalassemia and hemoglobinopathies (values are presented as mean ± SD or as raw data where appropriate).

Parameters	Hb Hekinan II/α^0-thalassemia	Hb Hekinan II/α^+-thalassemia	Hb Hekinan II/Hb CS	Hb Hekinan II/Hb CS/Hb E		Hb Hekinan II/Hb CS/Hb EE
Number	8	5	1	1	1	1
Age (year)	23–72 (37.5 ± 16.0)	3–41 (24.4 ± 14.6)	25	76	32	21
RBC (x10^{12}/L)	5.99 ± 0.7	5.43 ± 0.58	6.16	4.61	5.05	5.84
Hb (g/dL)	13.2 ± 2.0	13.9 ± 1.60	15.3	12.5	13.1	12.1
HCT (%)	41.3 ± 5.2	43.5 ± 4.52	48.3	38.4	40.6	38
MCV (fL)	67.9 ± 2.5	80.3 ± 7.50	78.0	83.5	80.3	64.2
MCH (pg)	21.4 ± 1.1	25.6 ± 2.43	24.8	27.3	26.0	20.7
MCHC (g/dL)	31.9 ± 1.6	31.8 ± 0.57	31.6	32.6	32.4	32.3
RDW-CV (%)	15.1 ± 1.2	13.9 ± 0.40	14.9	16.1	17.0	16
Hb profile[a]	A_2A with Hb Hekinan II	A_2A	CSA_2A	CSEA	EA	EE
Hb A (%)[a]	62.4 ± 1.7	86.1 ± 1.27	87.7	68.1	67.3	0.0
Hb A_2 (%)[a]	1.96 ± 0.3	2.30 ± 0.23	1.8	na	na	na
Hb A_2+E (%)[a]	0	0	0	15.8	22.2	86.0
Hb F (%)[a]	1.0 ± 1.5	1.10 ± 1.56	2.4	0.9	1.0	1.5
Hb Hekinan II (%)[a]	24.6 ± 1.6	0.0	0.0	0.0	0.0	0.0
Hb CS (%)[a]	0.0	0.0	0.5	0.5	0.0	0.0
α-globin genotype	$\alpha\alpha^{Hekinan}/-^{SEA}$	$\alpha\alpha^{Hekinan}/-\alpha^{3.7}$	$\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha$		$\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha$	$\alpha\alpha^{Hekinan}/\alpha^{CS}\alpha$
β-globin genotype	β^A/β^A	β^A/β^A	β^A/β^A		β^A/β^E	β^E/β^E

RBC, Red Blood Cell; Hb, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW-CV, Coefficient of Variation of the Red Cell Distribution Width; CS, Constant Spring; na, not available.
[a]Determined using high performance liquid chromatography (HPLC) (Variant II, Bio-Rad Laboratories).

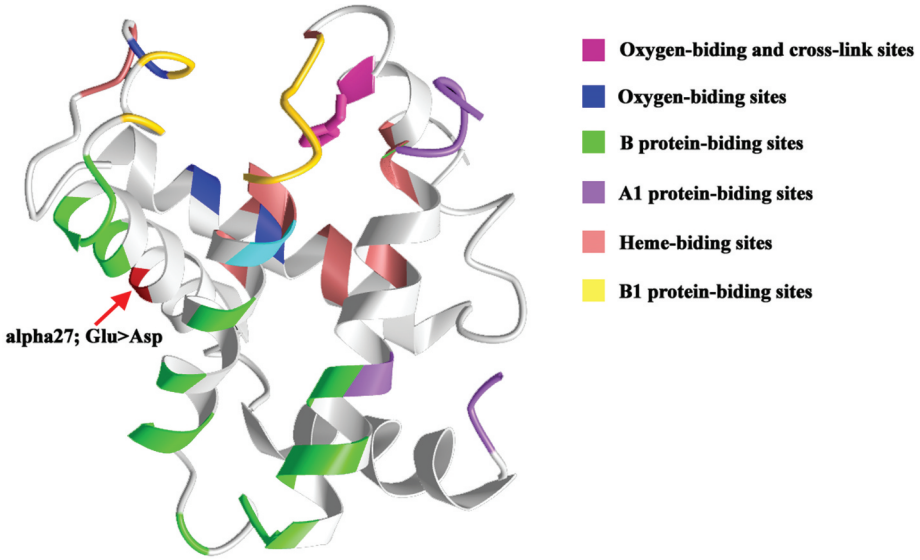
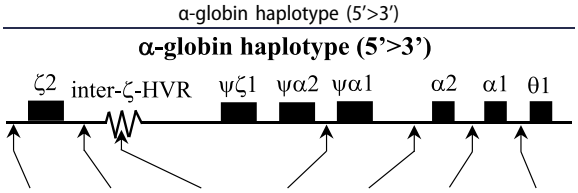


Figure 4. The structure of a monomer $\alpha^{Hekinan}$ visualized using iCn3d. Affected residues in Hb Hekinan II are indicated in red.

thalassemia ($\alpha\alpha^{Hekinan}/-^{SEA}$). The percentage of Hb Hekinan II quantified by HPLC was similar to that previously observed for the same genotype. The hematological parameters, mirroring those of heterozygous α^0-thalassemia, showed no anemia and microcytic red blood cells [27]. These findings are in line with those of many previous reports [8,10,11]. Remarkably, a distinct decrease in Hb A_2 was observed (1.96 ± 0.3), which is significantly different from the precisely quantified value in the heterozygous α^0-thalassemia (2.3 ± 0.3) [28]. The same findings in equivalent genotypes have been documented previously (1.7 ± 0.5) [23]. The distinct decrease in Hb A_2 levels observed in this genotype was in a range comparable with that observed in deletional Hb H disease with only one active α-globin gene or Hb H-CS with the deletion of two α-globin genes plus inactivation of the third α-globin gene, showing Hb A_2 levels of 1.84 ± 0.63 and 1.34 ± 0.89, respectively [29]. This implies that Hb Hekinan II may be slightly unstable. However, Hb analysis revealed no Hb H (β_4) or Hb Bart's (γ_4) fractions on both the chromatogram and electropherogram despite the similarity of the genotype of this combination to that of the non-deletional Hb H disease, consistently detected Hb H fraction by Hb analysis, indicating a need for globin genotyping for accurate diagnosis. The data observed in our cases, with several genotypes exhibiting minimal hematological phenotypic changes, reinforce that Hb Hekinan II is a non-pathological Hb variant, as previously known.

Further analysis of the impact of amino acid substitution on the α-globin chain revealed that the glutamic acid residue at position 27 lies in the α-helix B segment and is not involved in the heme pocket but

Table 2. α-Globin gene haplotypes associated with Hb Hekinan II in Thai and Burmese participants. + and − represent the presence and absence of the restriction enzyme sites, respectively. S and M indicate inter ζ hyper variable region (HVR). 0 indicates deletion.



										Hb Hekinan II ($\alpha\alpha^{HK}$) linked α-haplotype						
Patients	Ethnicity	α-genotype	*Xba* I	*Bgl* I	[S/M/L]	*Acc* I	*Rsa* I	α*Pst* I	θ*Pst* I							
1	Thai	$\alpha\alpha^{HK}/-\alpha^{3.7}$	[+/+]	[-/-]	[S]	[+/+]	[-/-]	[-/0]	[-/-]	+	-	S	+	-	-	-
2	Burmese	$\alpha\alpha^{HK}/-\alpha^{3.7}$	[±]	[-/-]	[S]	[+/+]	[-/-]	[+/0]	[-/-]	±	-	S	+	-	+	-
3	Thai	$\alpha\alpha^{HK}/-\alpha^{3.7}$	[±]	[-/-]	[M]	[±]	[-/-]	[+/0]	[-/-]	±	-	M	±	-	+	-
4	Thai	$\alpha\alpha^{HK}/-\alpha^{3.7}$	[-/-]	[-/-]	[M]	[+/+]	[±]	[+/0]	[-/-]	-	-	M	+	±	+	-
5	Thai	$\alpha\alpha^{HK}/-\alpha^{3.7}$	[±]	[-/-]	[S]	[+/+]	[-/-]	[+/0]	[-/-]	±	-	S	+	-	+	-
6	Thai	$\alpha\alpha^{HK}/-^{SEA}$	[±]	[-/-]	[S]	[+/0]	[-/0]	[-/0]	[-/0]	±	-	S	+	-	-	-
7	Thai	$\alpha\alpha^{HK}/-^{SEA}$	[±]	[-/-]	[S]	[+/0]	[-/0]	[-/0]	[-/0]	±	-	S	+	-	-	-
8	Thai	$\alpha\alpha^{HK}/-^{SEA}$	[±]	[-/-]	[S]	[+/0]	[-/0]	[-/0]	[-/0]	±	-	S	+	-	-	-
9	Burmese	$\alpha\alpha^{HK}/-^{SEA}$	[±]	[-/-]	[S]	[+/0]	[-/0]	[-/0]	[-/0]	±	-	S	+	-	-	-
10	Thai	$\alpha\alpha^{HK}/-^{SEA}$	[±]	[-/-]	[S]	[+/0]	[-/0]	[-/0]	[-/0]	±	-	S	+	-	-	-
11	Thai	$\alpha\alpha^{HK}/-^{SEA}$	[±]	[-/-]	[S]	[+/0]	[-/0]	[+/0]	[-/0]	±	-	S	+	-	+	-
12	Thai	$\alpha\alpha^{HK}/-^{SEA}$	[±]	[-/-]	[S]	[+/0]	[-/0]	[+/0]	[-/0]	±	-	S	+	-	+	-
13	Thai	$\alpha\alpha^{HK}/-^{SEA}$	[±]	[-/-]	[S]	[+/0]	[-/0]	[-/0]	[-/0]	±	-	S	+	-	-	-
14	Thai	$\alpha\alpha^{HK}/\alpha\alpha^{CS}$	[±]	[-/-]	[M]	[+/+]	[±]	[-/-]	[-/-]	±	-	M	+	±	-	-
15	Thai	$\alpha\alpha^{HK}/\alpha\alpha^{CS}$	[±]	[-/-]	[M]	[+/+]	[±]	[-/-]	[-/-]	±	-	M	+	±	-	-
16	Thai	$\alpha\alpha^{HK}/\alpha\alpha^{CS}$	[±]	[-/-]	[M]	[+/+]	[±]	[+/+]	[-/-]	±	-	M	+	±	+	-
17	Thai	$\alpha\alpha^{HK}/\alpha\alpha^{CS}$	[±]	[-/-]	[M]	[+/+]	[±]	[+/+]	[-/-]	±	-	M	+	±	+	-

in the α1β1 contact [30]. Notably, the replacement of glutamic acid with aspartic acid at that position in the α-globin chain does not disrupt its charge and acid-base properties. This is because both glutamic and aspartic acids are negatively charged. Additionally, the mutated residue occupies the α1β1 contact, but the shortened side chain due to the substitution may not lead to an attracting force between the α- and β-chains [3,23]. This substitution did not affect protein function or stability. In Hb Hekinan II, this substitution maintained its function, rendering it similar to that of Hb A. Additionally, the prediction software analysis substantiated that this variant is a benign mutation, aligning with the clinical symptoms.

Due to an amino acid substitution from acid to acid at α27(B8), Hb Hekinan II has similar electrophoretic behavior to Hb A, making it difficult to distinguish them using conventional cation exchange HPLC and CE [3]. Previous studies have demonstrated that Hb Hekinan II is poorly separated from Hb A using both conventional cation exchange HPLC and CE [7,9]. However, it can be distinguished from Hb A using reverse-phase HPLC [3], isoelectric focusing (IEF) [8,11], and weak cation exchange materials coupled with polyaspartic acid techniques [7,9]. We also found that conventional cation-exchange HPLC and CE could not distinguish Hb Hekinan II from Hb A despite its concomitant presence with α^{+}-thalassemia or the Hb CS gene. However, the Hb Hekinan II peak was observed as a small shoulder-like peak on the left side of Hb A; this unusual Hb A peak can be observed on the chromatogram, but not on the electropherogram. In addition, Hb Hekinan II-E was observed as a small notched peak on the left side of the Hb E fraction. Hb Hekinan II was better separated by HPLC when co-inherited with α^{0}-thalassemia, allowing for quantification of the Hb variant. Although well separated, Hb Hekinan II was not completely resolved from Hb A, potentially impacting the accuracy of its quantification. Our observations indicate that the Hb Hekinan II peak becomes progressively more visible on the chromatogram as the number of α-globin gene defects increases. This could explain why α-thalassemia, causing reducing normal α-globin chain production, allows α-Hb Hekinan II to increasingly bind with the β-globin chain. Co-inheritance of α-thalassemia could, therefore, greatly aid in its detection by HPLC. We assessed the chromatographic mobility of two variants, namely, Hb Wiangpapao and Hb Nakhon Ratchasima, which have been documented in Thai patients [14,31]. We found that they eluted with the same retention time as Hb Hekinan II, leading to chromatograms similar to those of Hb Hekinan II. Therefore, accurate diagnosis through DNA analysis is necessary in cases where such a chromatogram is observed.

Notably, the concentration of Hb Hekinan II quantified from the eight participants with compound heterozygosity of Hb Hekinan II with α^{0}-thalassemia was lower than those quantified in patients with Hb Hekinan mutated on the α_2-globin gene [10]. Individuals with compound heterozygosity of Hb Hekinan II with α^{0}-thalassemia have only two active α-globin genes on the same chromosome, resulting in an equal number of remaining normal and abnormal

α-globin genes; one is a normal α_2 gene producing α^A globin chain, and another is a α1 gene mutated as $\alpha^{Hekinan}$ producing $\alpha^{Hekinan}$ globin chain (arranged as 5 '-α2-α1-3' in a haploid). Hb Hekinan II, and Hb A concentrations were expected to be equal. We found that the average percentage of Hb Hekinan II was 24.6 ± 1.6, while those of Hb A were 62.4 ± 1.7. These percentages correspond to a ratio of α2:α1 of 2.5. According to Molchanova et al. [10], the average protein production rate of the α_2-globin is 2–3-fold higher than that of the α_1-globin observed in normal individuals. Our observation indicates that Hb Hekinan II on the α_1-globin gene does not affect the proportion of abnormal protein production, unlike Hb Shuangfeng (HBA2:c.82 G>A (or HBA1)), Hb Fort Worth (HBA2:c.83A>G (or HBA1)), and Hb Spanish Town (HBA2:c.83A>T (or HBA1)) with the same position mutation but different amino acid substitution on the α_1-globin gene. These three variants have fewer variants due to abnormal mRNA processing [32–34].

Although Hb Hekinan II has been observed in various Asian populations, its origin and spread remain unclear. Therefore, we first characterized the α-globin gene haplotype of Hb Hekinan II in the Thai and Burmese participants. We found the Thai Hb Hekinan II to be associated with haplotypes [+ - S + - - -] and potentially with four different haplotypes, while Burmese Hb Hekinan to be potentially associated with haplotype [± - S + - + -] and [± - S + - - -]. These haplotypes were similarly observed in Thai individuals. Therefore, the $\alpha^{Hekinan}$ gene in Burmese may share the same origin as two of the Thai $\alpha^{Hekinan}$ genes with the haplotype [± - S + - + -] and [± - S + - - -]. The discovery of several Hb Hekinan II genotypes in the Thai population may be linked to the observation of multiple haplotypes of this variant. Various gene conversion and recombination events explain the different haplotype backgrounds. The multiple origins of Hb Hekinan II in Thailand suggest its prevalence in this region. Despite routine techniques for Hb analysis, especially in the heterozygote state, the challenge of distinguishing Hb Hekinan II from Hb A prevents the precise prevalence of Hb Hekinan II in the Thai population.

One limitation of this study is the absence of family members among the participants. When family members are unavailable, conducting a complete segregation analysis of haplotypes associated with Hb Hekinan II becomes challenging. Segregation analysis typically involves tracking the inheritance of specific genetic markers across generations to determine which alleles are passed from parents to offspring. Without family members, this type of segregation cannot be performed directly. However, further analysis using alternative methods such as computational phasing, population-based linkage disequilibrium (LD) analysis, and reference panels can still provide valuable insights.

5. Conclusions

We investigated Thai and Burmese cases with various combinations of Hb Hekinan II with other thalassemia and confirmed that Hb Hekinan II is a nonpathological hemoglobinopathy. Thai Hb Hekinan II has multiple origins, and some share the same origins as the Burmese Hb Hekinan II. We highlighted that various complex genotypes found in areas where thalassemia and hemoglobinopathies are particularly prevalent and the challenges in accurately quantifying Hb Hekinan II and distinguishing it from Hb A during routine thalassemia screening, underscoring the importance of DNA analysis for accurate diagnosis. Furthermore, co-inheriting this variant with α-thalassemia or another α-Hb variant leads to an unusual Hb A or Hb E peak by HPLC, indicating its utility in screening this Hb variant. This study provides insights for accurate genetic diagnoses, timely genetic counseling, and prenatal diagnostic assistance.

Acknowledgments

We wish to acknowledge with sincere appreciation the contribution of all patients who participated in this study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This study was supported by grants provided by the Thailand Science Research and Innovation Fund and the University of Phayao, Thailand under Grant [FF66-RIM091].

Author contributions

Panyasai S was involved in the conception and design; analysis and interpretation of the data; the drafting of the paper and revising it critically for intellectual content; Chantanaskulwong P was involved in the analysis and interpretation of the data; the drafting of the paper; Permsripong N, Mokmued T were involved in the analysis and interpretation of the data.

All authors agreed to be accountable for all aspects of the work and have reviewed and approved the final manuscript.

Data availability statement

All data produced in this study have been included in the corresponding published article. The datasets utilized in this research are accessible upon request from the corresponding author.

ORCID

Sitthichai Panyasai http://orcid.org/0000-0002-7589-3849

References

[1] Harteveld CL, Achour A, Arkesteijn SJG, et al. The hemoglobinopathies, molecular disease mechanisms and diagnostics. Int J Lab Hematol. 2022 Sep;1(Suppl 1):28–36. doi: 10.1111/ijlh.13885

[2] Giardine BM, Joly P, Pissard S, et al. Clinically relevant updates of the HbVar database of human hemoglobin variants and thalassemia mutations. Nucleic Acids Res. 2021 8;49(D1):D1192–D1196. doi: 10.1093/nar/gkaa959

[3] Harano T, Harano K, Imai N, et al. An electrophoretically silent hemoglobin variant, hb hekinan [alpha 27(B8)Glu —-asp] found in a Japanese. Hemoglobin. 1988;12 (1):61–65. doi: 10.3109/03630268808996883

[4] Merault G, Keclard L, Desfontaines L, et al. Hemoglobin Hekinan [Alpha (2)27(B8)Glu—asp beta 2] detected in Guyana. Hemoglobin. 1989;13(4):397–402. doi: 10.3109/03630268909003402

[5] Zhao W, Wilson JB, Webber BB, et al. Hb Hekinan observed in three Chinese from Macau; identification of the GAG—-GAT mutation in the alpha 1-globin gene. Hemoglobin. 1990;14(6):627–635. doi: 10.3109/03630269009046971

[6] Fucharoen S, Changtrakun Y, Ratanasiri T, et al. Complex interaction of hb hekinan [alpha27(B8) glu-asp] and hb E [beta26(B8) glu-lys] with a deletional alpha-thalassemia 1 in a Thai family. Eur J Haematol. 2003;70(5):304–309. doi: 10.1034/j.1600-0609.2003.00049.x

[7] Chunpanich S, Ayukarn K, Sanchaisuriya K, et al. Laboratory diagnosis of a compound heterozygosity for hb hekinan [alpha27(B8) glu-asp] and a deletional alpha-thalassaemia 2 in Thailand. Clin Lab Haematol. 2004;26(5):355–358. doi: 10.1111/j.1365-2257.2004.00627.x

[8] Ngiwsara L, Srisomsap C, Winichagoon P, et al. Two cases of compound heterozygosity for hb hekinan [alpha27(B8)Glu–>asp (alpha1)] and alpha-thalassemia in Thailand. Hemoglobin. 2004;28(2):145–150. doi: 10.1081/hem-120035913

[9] Shih HC, Shih MC, Chang YC, et al. Hb hekinan in a Taiwanese subject: a G–>t substitution at codon 27 of the alpha1-globin gene abolishes an HaeIII site. Hemoglobin. 2007;31(4):495–498. doi: 10.1080/03630260701590368

[10] Molchanova TP, Pobedimskaya DD, Huisman TH. The differences in quantities of alpha 2- and alpha 1-globin gene variants in heterozygotes. Br J Haematol. 1994;88 (2):300–306. doi: 10.1111/j.1365-2141.1994.tb05022.x

[11] Uaprasert N, Settapiboon R, Amornsiriwat S, et al. The first validated criteria for effective screening and a new simplified method for α-globin gene sequencing for diagnosis of uncommon α-globin mutations. Int J Hematol. 2017 Jun;105(6):819–827. doi: 10.1007/s12185-017-2186-4

[12] Ahmad NN, Ab C-U, Donoso LA. Modification of standard proteinase K/phenol method for DNA isolation to improve yield and purity from frozen blood. J Med Genet. 1995;32(2):129–130. doi: 10.1136/jmg.32.2.129

[13] Dodé C, Rochette J, Krishnamoorthy R. Locus assignment of human alpha globin mutations by selective amplification and direct sequencing. Br J Haematol. 1990;76 (2):275–281. doi: 10.1111/j.1365-2141.1990.tb07884.x

[14] Panyasai S, Pornprasert S. Hematological characterizations and molecular diagnostic aspects of hb wiangpapao [α44 (CE2)Pro→Ser (α1), CCG>TCG; HBA1: c.133C>T], a new α-globin variant found in a pregnant Thai woman. Hemoglobin. 2017;41(2):73–76. doi: 10.1080/03630269.2017.1338185

[15] Sae-Ung N, Fucharoen G, Sanchaisuriya K, et al. Alpha(0)-thalassemia and related disorders in northeast Thailand: a molecular and hematological characterization. Acta Haematol. 2007;117(2):78–82. doi: 10.1159/000096857

[16] Fucharoen S, Fucharoen G, Sanchaisuriya K, et al. Molecular analysis of a Thai beta-thalassaemia heterozygote with normal haemoglobin A2 level: implication for population screening. Ann Clin Biochem. 2002;39(Pt 1):44–49. doi: 10.1258/0004563021901720

[17] Fucharoen S, Sanchaisuriya K, Fucharoen G, et al. Interaction of hemoglobin E and several forms of alpha-thalassemia in Cambodian families. Haematologica. 2003;88(10):1092–1098.

[18] Fucharoen S, Fucharoen G, Ratanasiri T, et al. A simple non-radioactive assay for hemoglobin E gene in prenatal diagnosis. Clin Chim Acta. 1994;229(1–2):197–203. doi: 10.1016/0009-8981(94)90242-9

[19] Singsanan S, Karnpean R, Fucharoen G, et al. Hemoglobin Q-Thailand related disorders: origin, molecular, hematological and diagnostic aspects. Blood Cells Mol Dis. 2010;45(3):210–214. doi: 10.1016/j.bcmd.2010.06.001

[20] Wang J, Youkharibache P, Zhang D, et al. iCn3d, a web-based 3D viewer for sharing 1D/2D/3D representations of biomolecular structures. Bioinformatics. 2020 Jan 1;36(1):131–135. doi: 10.1093/bioinformatics/btz502

[21] Jiang F, Xu LL, Chen GL, et al. Hematological characteristics of hb constant Spring (HBA2: c.427T>C) carriers in mainland China. Hemoglobin. 2020;44(2):86–88. doi: 10.1080/03630269.2020.1755979

[22] Panyasai S, Satthakarn S, Phasit A. Effective screening of hemoglobin constant Spring and hemoglobin paksé with several forms of α- and β-thalassemia in an area with a high prevalence and heterogeneity of thalassemia using capillary electrophoresis. Heliyon. 2023 12;9(8):e19116. doi: 10.1016/j.heliyon.2023.e19116

[23] Pan L, Qiu Y, Ye L, et al. A clinical update of compound heterozygosity for hemoglobin hekinan II [a27(B8)Glu-Asp; HBA1: c.84G>T] variant in China. Lab Med. 2024:lmae023. doi: 10.1093/labmed/lmae023 Epub ahead of print.

[24] Sanchaisuriya K, Fucharoen G, Sae-ung N, et al. Molecular and hematologic features of hemoglobin E heterozygotes with different forms of alpha-thalassemia in Thailand. Ann Hematol. 2003;82(10):612–616. doi: 10.1007/s00277-003-0689-y

[25] Charoenkwan P, Wanapirak C, Thanarattanakorn P, et al. Hemoglobin E levels in double heterozygotes of hemoglobin E and SEA-type alpha-thalassemia. Southeast Asian J Trop Med Public Health. 2005;36(2):467–470.

[26] Fucharoen G, Trithipsombat J, Sirithawee S, et al. Molecular and hematological profiles of hemoglobin EE disease with different forms of alpha-thalassemia. Ann Hematol. 2006;85(7):450–454. doi: 10.1007/s00277-006-0093-5

[27] Chui DH. Alpha-thalassaemia and population health in Southeast Asia. Ann Hum Biol. 2005;32(2):123–130. doi: 10.1080/03014460500075084

[28] Sanchaisuriya K, Fucharoen S, Fucharoen G, et al. A reliable screening protocol for thalassemia and hemoglobinopathies in pregnancy: an alternative approach to electronic blood cell counting. Am J Clin Pathol. 2005 Jan;123 (1):113–118. doi: 10.1309/fuf9evgq24v1pktp

[29] Luo S, Chen X, Chen L, et al. Analysis of hb levels and degree of anemia in relation to genotype in 615 patients

with hemoglobin H disease. Expert Rev Hematol. 2020;13 (9):1027–1033. doi: 10.1080/17474086.2020.1803736

[30] Sack JS, Andrews LC, Magnus KA, et al. Location of amino acid residues in human deoxy hemoglobin. Hemoglobin. 1978;2(2):153–169. doi: 10.3109/03630267809074782

[31] Srivorakun H, Singha K, Fucharoen G, et al. Novel interactions of two α-Hb variants with SEA deletion α0-thalassemia: hematological and molecular analyses. Hematology. 2018;23(3):187–191. doi: 10.1080/10245332.2017.1380930

[32] Chihchuan L, Hainan T, Hweiyuen L, et al. Hemoglobin shuangfeng (alpha 27 (B8) glu substituting for lys): a new unstable hemoglobin variant. Hemoglobin. 1981;5(7–8):691–700. doi: 10.3109/03630268108991837

[33] Carstairs KC, Raulfs A, Kutlar A, et al. Hb Fort Worth or alpha2 27(B8)Glu—gly beta2 in a black family from Canada. Hemoglobin. 1985;9(2):201–205. doi: 10.3109/03630268508997005

[34] Faustino P, Picanço I, Miranda A, et al. L. Compound heterozygosity for hb Spanish town [alpha27(B8)Glu–>val], hb S [beta6(A3)Glu–>val] and the -alpha(3.7kb) thalassemia deletion. Hemoglobin. 2002;26(2):185–189. doi: 10.1081/hem-120005458