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Non-transfusion dependent HbE/β^{O}-thalassemia as the results of co-existent SEA-α^{O} thalassemia, Hb Constant Spring, and XmnI-$^{G}\gamma$ site: Thai family studies

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ABSTRACT

Background: Four university students of northern Thai descent were found to be HbE/β^{O}-thalassemia. However, they all had a mild form of this disease, categorized as Non-Transfusion Dependent Thalassemia.

Objectives: To analyze involvement of types of β-globin mutations, α-thalassemia, and *Xmn*I-$^{G}\gamma$ site in mild clinical symptoms observed in four Thai non-transfusion dependent HbE/β^{O}-thalassemia cases.

Materials and methods: EDTA blood samples were collected from the patients and their family members after signing the informed consent. Automated complete blood count with blood smear examination, hemoglobin typing, molecular analysis for α and β-globin mutations, β-globin gene haplotypes, and *Xmn*I-$^{G}\gamma$ site were performed on all blood samples. In addition, nucleotide sequencing of β-globin gene and globin chain separation were performed for patient#3 and their parents.

Results: The first three patients had hemoglobin levels ranging 8.5-11.2 g/dL, while the fourth patient had hemoglobin level of 6.7 g/dL. The first and fourth patients were compound heterozygote for β^{E} (HBB:c.79G>A) and β^{17} (HBB:c.52A>T) alleles with typical hemoglobin pattern of EF. The second patient was compound heterozygote for β^{E} and $\beta^{41/42}$ (HBB:c.126_129delCTTT) alleles also with typical hemoglobin pattern of EF. The third patient was compound heterozygote of β^{E} and $\beta^{IVS1\text{-}1}$(HBB:c.92+1G>T), however, with atypical hemoglobin pattern of EE. Family analysis found co-inheritance of Hb Constant Spring (HBA2:c.427T>C) and the *Xmn*I-$^{G}\gamma$ site (T at rs7482144) in the first two patients, of SEA-α^{O} thalassemia (NG_000006.1:g.26264_45564del19301) and *Xmn*I-$^{G}\gamma$ site in the third patient, and of only *Xmn*I-$^{G}\gamma$ site in the fourth patient.

Conclusion: These family studies proved the fact that co-existence of SEA-α^{O} thalassemia and Hb Constant Spring in HbE/β^{O}-thalassemia could lead to mild clinical severity. Minimal effect of *Xmn*I-$^{G}\gamma$ site on clinical symptoms of this disease was emphasized. This information should be useful in prenatal diagnosis of HbE/β-thalassemia.

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Introduction

HbE/β-thalassemia is the syndrome resulting from co-segregation of β^{E}-gene (HBB:c.79G>A) and β-thalassmia gene.[1] Surveys in Thailand have shown that the affected patients had different clinical symptoms. Some patients had anemia, requiring regular blood transfusions while others in contrast are not anemic and do not need any blood

transfusion throughout their life.[2] At least 3 determinants have been shown to be linked to mild clinical symptoms of the HbE/β-thalassemia. These include 1). types of β-globin gene mutations, 2). co-existence of α-thalassemia, and 3). co-inheritance of loci involved in increased γ-globin gene expression such as the *Xmn*I-^{G}γ site (T at *rs7482144*).[3] Four university students of northern Thai descent were found to be HbE/β^{O}-thalassemia following routine blood tests. However, they all had a mild form of this disease as they required either no or occasional blood transfusion which was categorized as Non-Transfusion Dependent Thalassemia.[4] This study was therefore aimed to analyze involvement of these three genetic determinants in mild clinical symptoms observed in these propositi. This finding would be useful in predicting the clinical symptoms of *in-utero* fetuses prenatally diagnosed to be HbE/β^{O}-thalassemia.

Materials and methods

Subjects and blood samples

Four university students of northern Thai descent and their family members were collectively analyzed. EDTA blood samples were collected from all subjects after signing the informed consents. The protocol of this study was reviewed and approved by the Ethic Committee of Faculty of Medicine, Chiang Mai University, Thailand with the approval number 205/2012.

Blood analysis and Hb typing

Automated complete blood count (Sysmex KX-21 Hematology Analyzer, Sysmex Corporation, Kobe, Japan) with blood smear examination was performed. Hb typing was determined by cation exchange high-performance liquid chromatography (HPLC) using the Primus Variant System 99 (Primus Corporation, Kansas City) and VARIANT II Hemoglobin Testing System (Bio-Rad Laboratories Ltd., Hercules, California).

Molecular analysis

The α- and β-globin gene mutations were identified by multiplex allele-specific PCR routinely performed in our laboratory.[5,6] The *Xmn*I-^{G}γ site was identified by PCR-RFLP analysis also routinely performed in our laboratory.[7,8] The β-globin gene RFLP haplotypes (*Hind*II-ε , *Hind*III-^{G}γ, *Hind*II-^{A}γ , *Hind*II-5'ψβ, *Hind*II-3'ψβ, *Ava*II-β and *Hinf*I-β) were determined following the procedure described elsewhere.[9,10]

Results

Family 1

Family 1 consisted of a father, mother, brother, and patient (Supplementary Figure 1). Father was β-thalassemia carrier with Hb type of A_2A (Supplementary Figure 2), not anemic with mild change of RBC morphology (Figure 1). He was heterozygous for β^{17} mutations (HBB:c.52A>T) (β^{17}/β^{N}) and had no co-existence of α thalassemia, Hb Constant Spring (Cs) and *Xmn*I-^{G}γ site (T at *rs7482144*). The mother was HbE carrier with Hb type of AE (Supplementary Figure 2). She was mildly anemic with mild change of RBC morphology (Figure 1). She was heterozygous for β^{E} mutation (HBB:c.79G>A) (β^{E}/β^{N}) with compound heterozygote of 3.7kb-α thalassemia 2 (NG_000006.1:g.34164_37967del3804) and Hb Constant Spring (ABA2:c.427T>C) (-α$^{3.7}$/α ^{Cs}α). The *Xmn*I-^{G}γ site was in homozygous state (*Xmn*I-^{G}γ : +/+). Brother was HbE carrier with Hb type of AECs (Supplementary Figure 2). He was not anemic with normal RBC morphology (Figure 1). He was heterozygous for β^{E} mutation, Hb Constant Spring and for *Xmn*I-^{G}γ site, having genotypes as β^{E}/β^{N}, α^{Cs}α/αα, *Xmn*I-^{G}γ:+/-, respectively. The patient had HbE/β^{O} thalassemia with Hb type of EF (Supplementary Figure 2). She was mildly anemic with a thalassemia blood picture (Figure 1). She had Mahidol score for clinical severity of zero [11]. She was compound heterozygous for β^{17} and β^{E} mutations (β^{17}/β^{E}), inherited from her father and mother, respectively. She was also heterozygous for Hb Constant Spring (α^{Cs}α/αα) and *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ:+/-), inherited from her mother. Details of the hematologic parameters of this family are shown in Table 1.

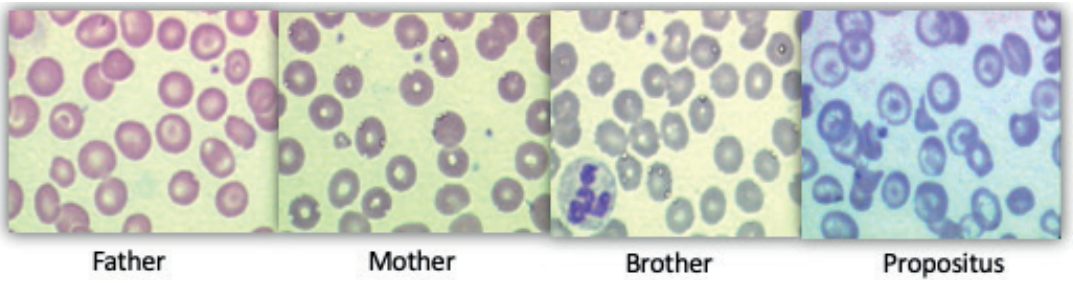

Figure 1. *RBC morphology of Family 1 members. Father and Mother had mild changes in RBC morphology. Brother had normal RBC morphology. The patient had thalassemic RBC morphology.*

Table 1. Hematological parameters, Hb typing results, globin gene genotype, and *Xmn*I-^{G}γ genotype of members in Family 1.

	Father	Mother	Brother	Patient
RBC(x10^{6}/μL)	5.72	5.09	5.55	5.17
Hb (g/dL)	12.0	10.8	13.8	8.9
Hct (%)	39.9	36.6	43.5	29.9
MCV (fL)	69.8	71.9	78.4	57.8
MCH (pg)	21	21.2	24.9	17.2
MCHC (g/dL)	30.1	29.5	31.7	29.8
RDW (%)	14.8	13.8	14.4	27.3
RBC morphology	Sl. change	Sl. Change	Normal	Thalassemia
Cation-exchange HPLC	A_2A [A_0 65.6%, A_2 5.4%]	EA [A_0 68.8%, E 19.0%]	EA [A_0 64.8%, E 23.6%]	EF [E 61.3%, F22.1%]
α-globin gene	αα/αα	-α$^{3.7}$/α^{Cs}α	α^{Cs}α/αα	α^{Cs}α/αα
β-globin gene	β^{17}/β^{N*}	β^{E}/β^{N*}	β^{E}/β^{N*}	β^{E}/β^{17}
*Xmn*I-^{G}γ site	-/-	+/+	+/-	+/-

Note: β^{N} is normal β-globin gene.

Family 2

Family 2 consisted of a father, mother, brother, and patient (Supplementary Figure 1). The father was HbE carrier with Hb type of AE (Supplementary Figure 3), not anemic, and had normal RBC morphology (Figure 4). He was heterozygous for Hb Constant Spring (α^{Cs}α/αα) and homozygous for the *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ:+/+). The mother was β-thalassemia carrier with Hb type of A_2A (Supplementary Figure 3). She was mildly anemic with moderate change of RBC morphology (Figure 2). She was heterozygous for β$^{41/42}$ mutation (β$^{41/42}$/β^{N}) without co-existence α thalassemia, Hb Constant Spring, and the *Xmn*I-^{G}γ site. Brother was normal with Hb type of A_2A (Supplementary Figure 3). He was not anemic with normal RBC morphology (Figure 2).. He was heterozygous for Hb Constant Spring (α^{Cs}α/αα) and *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ:+/+). The patient had HbE/β^{O} thalassemia with Hb type of EF (Supplementary Figure 3). He was mildly anemic with thalassemia blood picture (Figure 2). His Mahidol score for clinical severity was zero.[11] He was compound heterozygous for β^{E} and β$^{41/42}$ mutations (HBB:c.126_129delCTTT) (β$^{41/42}$/β^{E}), inherited from his father and mother, respectively. He was also heterozygous for Hb Constant Spring (α^{Cs}α/αα) and *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ:+/+), inherited from his father. Details of the hematologic parameters of this family are shown in Table 2.

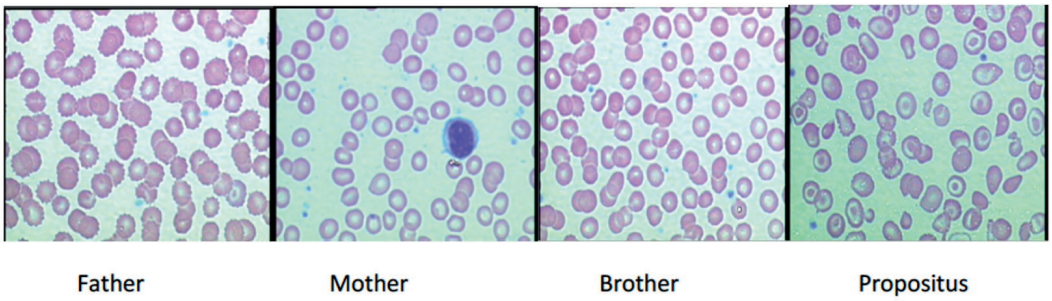

Figure 2*. RBC morphology of Family 2 members. Mother had a moderate change in RBC morphology. Father and Brother had normal RBC morphology. The creation seen in Father RBC was RBC shrinkage due to prolonged storage in EDTA. The patient had thalassemic RBC morphology.*

Table 2. Hematological parameters, Hb typing results, globin gene genotype, and *Xmn*I-^{G}γ genotype of members in Family 2.

	Father	Mother	Brother	Patient
RBC(x10^{6}/μL)	4.81	4.12	5.6	5.54
Hb (g/dL)	14.3	8.2*	14	11.2
Hct (%)	44.2	27.4	43.8	36.4
MCV (fL)	91.9	66.5	78.2	56.9
MCH (pg)	32.4	19.9	25	17.3
MCHC (g/dL)	32.4	29.9	32	30.5
RDW (%)	13.6	15.9	12.9	27.5
RBC morphology	Normal	Mod Change	Normal	Thalassemia
Cation-exchange HPLC	AE [A_0 65.2%, E 25.2%]	A_2A [A_0 71.4%, A_2 4.8%]	A_2A [A_0 86.8%, A_2 2.5%]	EF [E 66.6%, F20.9%]
α-globin gene	$\alpha^{Cs}\alpha/\alpha\alpha$	αα/αα	$\alpha^{Cs}\alpha/\alpha\alpha$	$\alpha^{Cs}\alpha/\alpha\alpha$
β-globin gene	β^{E}/β^{N**}	$\beta^{41/42}/\beta^{N*}$	β^{N}/β^{N*}	$\beta^{E}/\beta^{41/42}$
*Xmn*I-^{G}γ site	+/+	-/-	+/-	+/-

*Note: Co-occurrence of iron deficiency anemia was suspected, **β^{N} is normal β-globin gene.*

Family 3

Family 3 consisted of a father, mother, and patient (Supplementary Figure 1). The father was HbE carrier with Hb type of AE (Supplementary Figure 4), not anemic, and normal RBC morphology (Figure 3). He was heterozygous for β^{E}-mutation (β^{E}/β^{N}) and *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ:+/-), without co-existence of α-thalassemia and Hb Constant Spring. The mother was β-thalassemia carrier with Hb type of A_2A (Supplementary Figure 4). She was mildly anemic with moderate change of RBC morphology (Figure 3). She was heterozygous for β^{IVS1-1} mutation (HBB:c.92+1G>T) (β^{IVS1-1}/β^{N}) with co-existence of SEA-α^{O} thalassemia (- -^{SEA}/αα). The patient had HbE/β^{O} thalassemia with Hb type of EE (Supplementary Figure 4). She was mildly anemic with thalassemia blood picture (Figure 3). Her Mahidol score for clinical severity was zero.[11] She was compound heterozygous for β^{E} and β^{IVS1-1} mutations (β^{E}/β^{IVS1-1}), inherited from her father and mother, respectively, and typical for HbE/β^{O}-thalassemia (Supplementary Figure 5). She was also heterozygous for the SEA-α^{O} thalassemia (- -^{SEA}/αα) and the *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ:+/-), inherited from her mother and father, respectively. As no HbF peak was seen in HPLC, the Acid-Urea-Triton X (AUT)-Polyacrylamide Gel Electrophoresis (PAGE) of globin chain analysis was performed and found no γ-globin chain band, confirming the Hb typing result of EE without HbF peak (Supplementary Figure 6). Analysis of RFLP haplotype of β-globin gene cluster found that the patient carried haplotypes III and I, inherited from her father and mother, respectively. Details of hematologic parameters of this family are shown in Table 3.

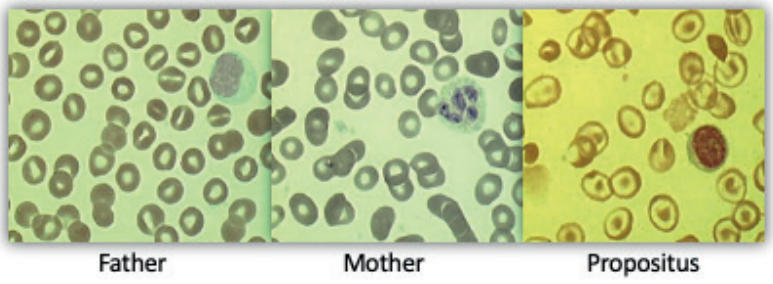

Figure 3. *RBC morphology of Family 3 members. Father and Mother had moderate changes in RBC morphology. The patient had thalassemic RBC morphology.*

Table 3. Hematological parameters, Hb typing results, globin gene genotype, and *Xmn*I-^{G}γ genotype of members in Family 3.

	Father	Mother	Patient
RBC(x10^{6}/μL)	4.95	4.3	5.3
Hb (g/dL)	13.0	10.3	8.5
Hct (%)	40.7	33.9	29.4
MCV (fL)	82.2	78.8	55.5
MCH (pg)	26.3	24.0	16.0
MCHC (g/dL)	31.9	30.4	28.9
RDW (%)	13.8	17	22.7
RBC morphology	Mild change	Mild change	Severely change
Cation-exchange HPLC	AE [A_0 62.2%, E 25.3%]	A_2A [A_0 83.5%, A_2 4.4%]	EE [E 89.9%]
α-globin gene	αα/αα	--^{SEA}/αα	--^{SEA}/αα
β-globin gene	β^{E}/β^{N*}	β$^{IVS1-1(G>T)}$/β^{N*}	β$^{IVS1-1(G>T)}$/β^{E}
*Xmn*I-^{G}γ site	+/-	-/-	+/-
β-globin gene haplotype	I/III	I/VII	I/III

β^{N} is normal β-globin gene.

Family 4

Family 4 consisted of mother, brother, and patient (Supplementary Figure 1). Mother was HbE carrier with Hb type of AE (Supplementary Figure 7), not anemic and with normal RBC morphology (Figure 4). She was heterozygous for β^{E}-mutation (β^{E}/β^{N}) and the *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ:+/-). Brother was otherwise normal. The patient was HbE/β^{O} thalassemia with Hb type of EF (Supplementary Figure 7). She was mildly anemic with thalassemia blood picture (Figure 4). Her Mahidol score for clinical severity was 3.5, indicating moderate degree of clinical severity.[11] She was compound heterozygous for β^{17} and β^{E} mutations (β^{E}/β^{17}), inherited from her deceased father and mother, respectively. She had no co-inherited α-thalassemia and was only heterozygous for *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ:+/-). Details of hematologic parameters of this family are shown in Table 4.

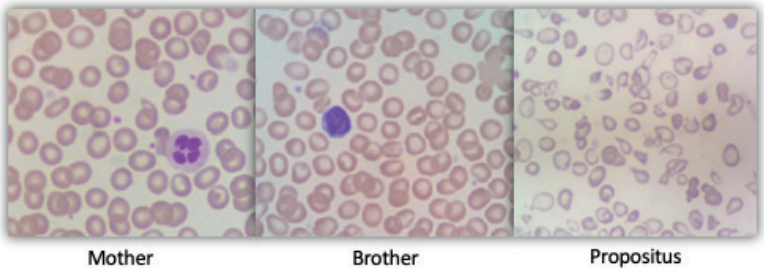

Figure 4. *RBC morphology of Family 4 members. Mother and Brother had mild changes in RBC morphology. The patient had thalassemic RBC morphology.*

Table 4. Hematological parameters, Hb typing results, globin gene genotype and *Xmn*I-^{G}γ genotype of members in Family 4.

	Mother	Brother	Patient
RBC(x10^6/μL)	5.24	6.37	4.66
Hb (g/dL)	12.7	16.3	6.7
Hct (%)	40.4	51.7	22.9
MCV (fL)	77.1	81.2	49.1
MCH (pg)	24.2	25.6	14.4
MCHC (g/dL)	31.4	31.5	29.3
RDW (%)	12.0	14.6	26.4
RBC morphology	Normal	Normal	Thalassemia
Cation-exchange HPLC	AE [A_0 63.4%, E 26.5%]	A_2A [A_0 86.7%, A_2 3.2%]	EF [E 62.8%, F30.4%]
α-globin gene	αα/αα	αα/αα	αα/αα
β-globin gene	β^E/β^{N*}	β^N/β^{N*}	β^E/β^{17}
*Xmn*I-^{G}γ site	+/-	-/-	+/-

Note: β^N is normal β-globin gene.

Discussion

HbE/β-thalassemia is genotypically a compound heterozygote of β^E and $\beta^{Thalassemia}$ alleles. There are broadly two groups of $\beta^{Thalassemia}$ genes, including the severe β^0-and mild β^+-genes. The β^{17}, $\beta^{41/42}$, and β^{IVS1-1} mutations observed in the propositi of this study were all β^0-mutations, in which no functional β-globin chain is produced. The β^{17} is a nonsense mutation (A>T) at codon 17, causing premature stopping of β-globin mRNA translation at this codon. In addition, the $\beta^{41/42}$ is a frameshift mutation caused by a 4 base pair (bp) deletion at codons $^{41/42}$ of the β-globin gene. This 4-bp deletion causes a shift of triplet codons that leads to the formation of a premature stop codon at codon 59. In contrast, the IVS1-1 is the G>T mutation at the first nucleotide of intron or intervening sequence (IVS)#1 of the β-globin gene. This mutation abolishes normal splicing of the β-globin mRNA and, as a result, no normal β-globin mRNA is formed, causing the β^0-type of thalassemia.[1] Compound heterozygote of these β^0 alleles with the β^E allele can lead to disorder of HbE/β^0-thalassemia.[2]

The clinical phenotype of HbE/β^0-thalassemia varies widely with steady-state hemoglobin levels ranging from 3.0 g/dL to 11.0 g/dL.[2] Types of β-globin mutations, co-existence of α-thalassemia and co-inheritance of loci linked to increased γ-globin gene expression were shown to ameliorate clinical symptoms of β-thalassemia and HbE/β-thalassemia. [2,3,12-14] The patients in this report were compound heterozygote for β^E allele and β^{17} or $\beta^{41/42}$ or β^{IVS1-1}, all of which were β^0-thalassemia genes. Based on the β-globin mutations, all these propositi should be clinically severe with thalassemia major or transfusion-dependent types.[15-19] However, the clinical symptoms of these propositi were only of intermedia types, requiring no or only occasional blood transfusion.

For patients 1 and 2, Hb Constant Spring combined with the *Xmn*I-^{G}γ site could account for their mild clinical symptoms. The reduction of free α-globin chain in Hb Constant Spring and the increased formation of HbF in the presence of the *Xmn*I-^{G}γ site lessened the chance of α-globin aggregation which can destroy red blood cells. Hb Constant Spring is an α-structural variant (α-Constant Spring) that originated from a point mutation (T>C) at a stop codon of α2-globin chain, leading to instability of the α-Constant Spring mRNA.[20,21] In addition, expression of the downstream α1-globin gene is reduced due to the α-Constant Spring mutation in the α2-globin gene. [22] The combination of these two mechanisms leads to the α-globin chain being markedly decreased in Hb Constant Spring, causing severe α^+-thalassemia. The severe characteristics of Hb Constant Spring were observed in clinical analysis of HbH disease in Thailand, which clearly showed that HbH-Constant Spring disease (- -/$\alpha^{Cs}\alpha$) was more severe than deletional HbH disease (- -/-α).[23,24]

The *Xmn*I-^{G}γ polymorphism, co-inherited in patients 1, and 2, was shown to be the major *cis*-acting factor for re-activating γ-globin gene expression and HbF/F cells in human adults.[25-28] It has also been found to reduce clinical symptoms in sickle cell disease, β-thalassemia, and HbE/β thalassemia.[18,19,29,30] Therefore, a co-occurrence of the *Xmn*I-^{G}γ site could contribute to reducing even more the clinical symptoms in these two propositi.

However, patient 4, who only had *Xmn*I-^{G}γ site in the heterozygous form, had more severe clinical symptoms than patients 1, and 2. It was clear from the results that the patient 4 was a compound heterozygote for β^E and β^{17} alleles. The β^{17} is nonsense mutation (A-T), resulting in the complete absence of β-globin chain synthesis.[19] The β^E is missense mutation (glutamic – lysine) that also causes reduction of β^E globin chain due to random usage of cryptic splice site at codon 25 of β-globin gene during β^E-mRNA production.[33] As a result, synthesis of β-globin chain in patient 4 was markedly decreased, leading to

an overwhelmingly increased quantity of free α-globin chain and α/β-globin chain synthetic ratio. However, It has been known that the γ-globin gene reactivation efficiency of the *Xmn*I-^{G}γ site alone is limited and that the resulting γ-globin chain produced cannot assemble all the free α-globin chain to produce HbF. As a result, the free α-globin chain remaining can aggregate, precipitate, and damage red blood cells.[3] In fact, previous surveys in Thailand had reported minimal impact of the heterozygous *Xmn*I-^{G}γ site in reducing the clinical severity of HbE/β-thalassemia.[7,18,19,30,32]

Patient 3, who also had very mild HbE/β^{O}-thalassemia with an atypical pattern of Hb typing of EE instead of EF as determined by cation-exchange HPLC (Supplementary Figure 5) had both SEA-α^{O}-thalassemia and *Xmn*I-^{G}γ site. Her γ-globin chain was not reactivated as shown by AUT-PAGE (Supplementary Figure 8). Therefore, this atypical Hb pattern could be explained by non-upregulated γ-globin gene expression and mild clinical symptoms of this patient would be attributed to the SEA-α^{O}-thalassemia alone. In fact, it has been established that the SEA-α^{O}-thalassemia is a severe form of α-thalassemia producing no α-globin chain.[22,34,35] Thus, reduced chance of α-globin aggregation and precipitation occurred in this patient and her clinical symptoms improved. Further studies on the exact mechanism controlling the expression of the γ-globin gene should clarify the low γ-globin gene expression in this HbE/β^{O}-thalassemia patient.

Analysis of 925 Thai HbE/β-thalassemia patients in 2008 showed that the patients having co-existent SEA-α thalassemia and Hb Constant Spring had later disease onset, less frequent requirement for blood transfusion, fewer episodes of hepatosplenomegaly than those having no α-thalassemia. The hematological parameters were better in the HbE/β-thalassemia patients having SEA-α^{O}-thalassemia and Hb Constant Spring than those having normal α-globin gene.[36] In addition, the study of 240 Asian Indian HbE/β-thalassemia patients by Sharma V and colleagues demonstrated ameliorating effect of α-thalassemia, including Hb Constant Spring, on clinical severity.[37] They concluded that the α-thalassemia was the major clinical modifying factor of their HbE/β-thalassemia patients. The findings in this study supported those raised by these two studies.

In conclusion, these family studies proved the findings of previous cohort studies that SEA-α^{O} thalassemia and Hb Constant Spring had the potential in alleviating the clinical symptoms of HbE/β^{O}-thalassemia. The minimal effect of the *Xmn*I-^{G}γ site on clinical severity of HbE/β^{O}-thalassemia was evident. This information may be useful for family counseling during prenatal diagnosis of β-thalassemia syndrome.

Conflict of interest

None

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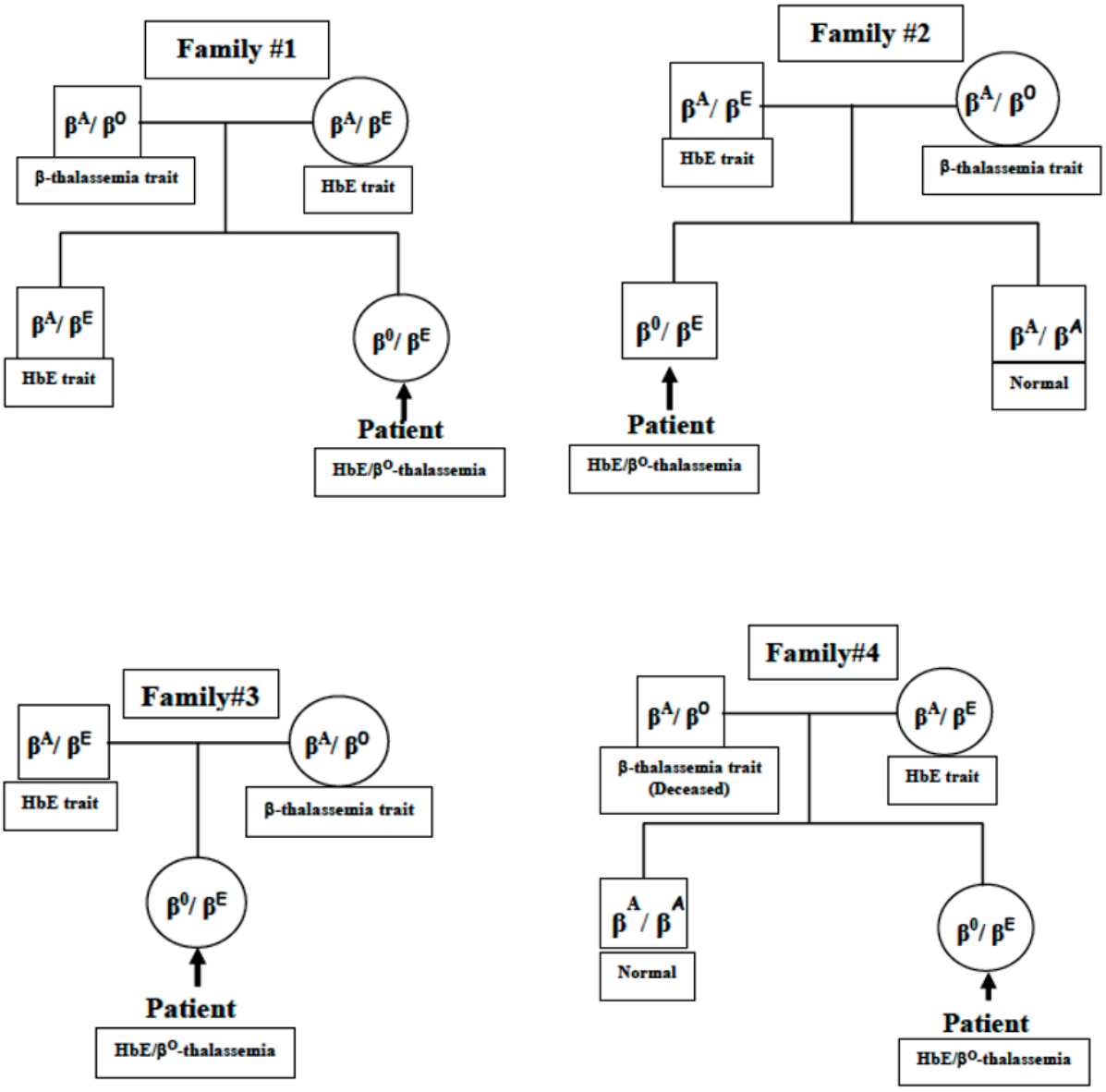
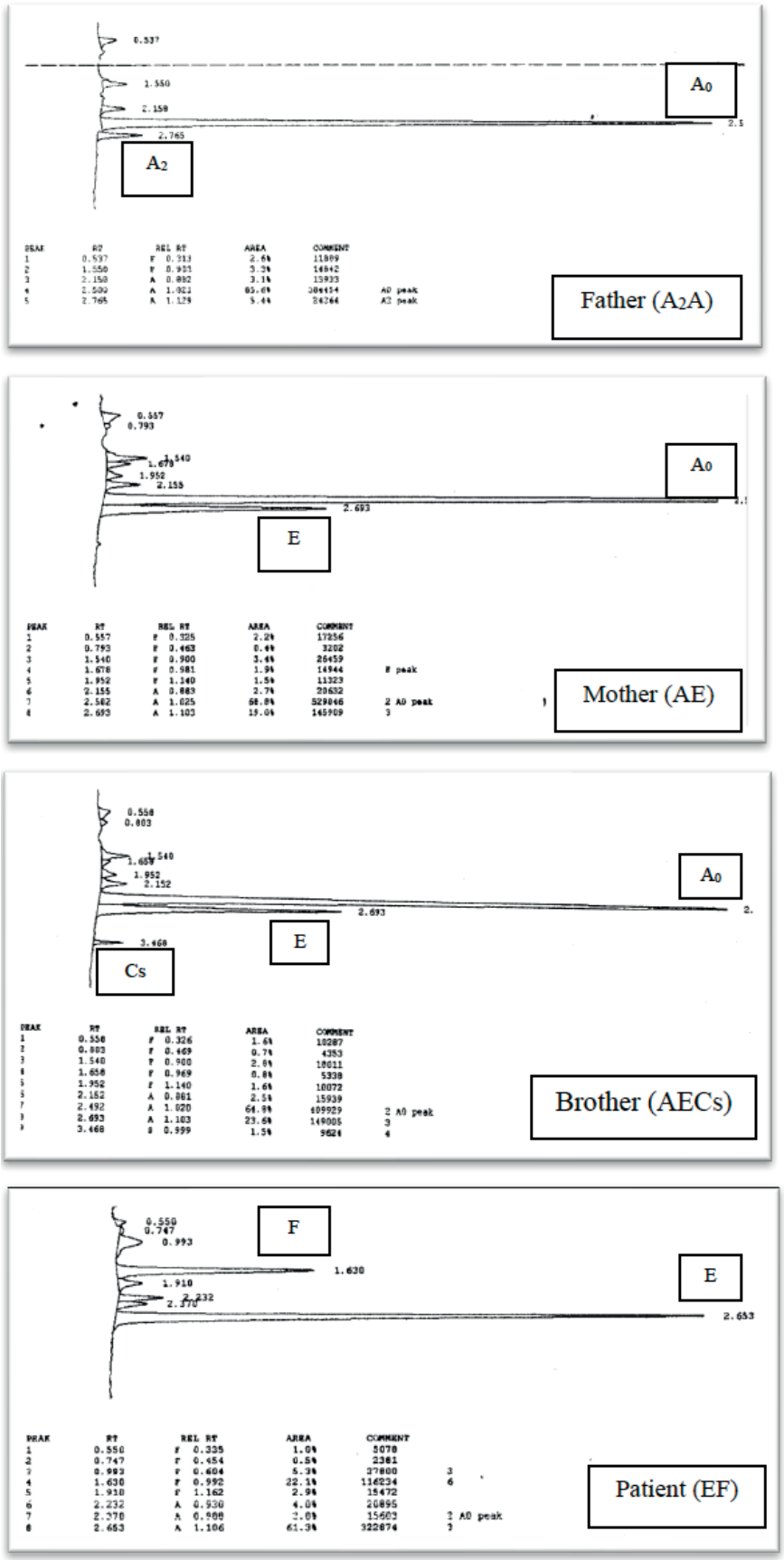
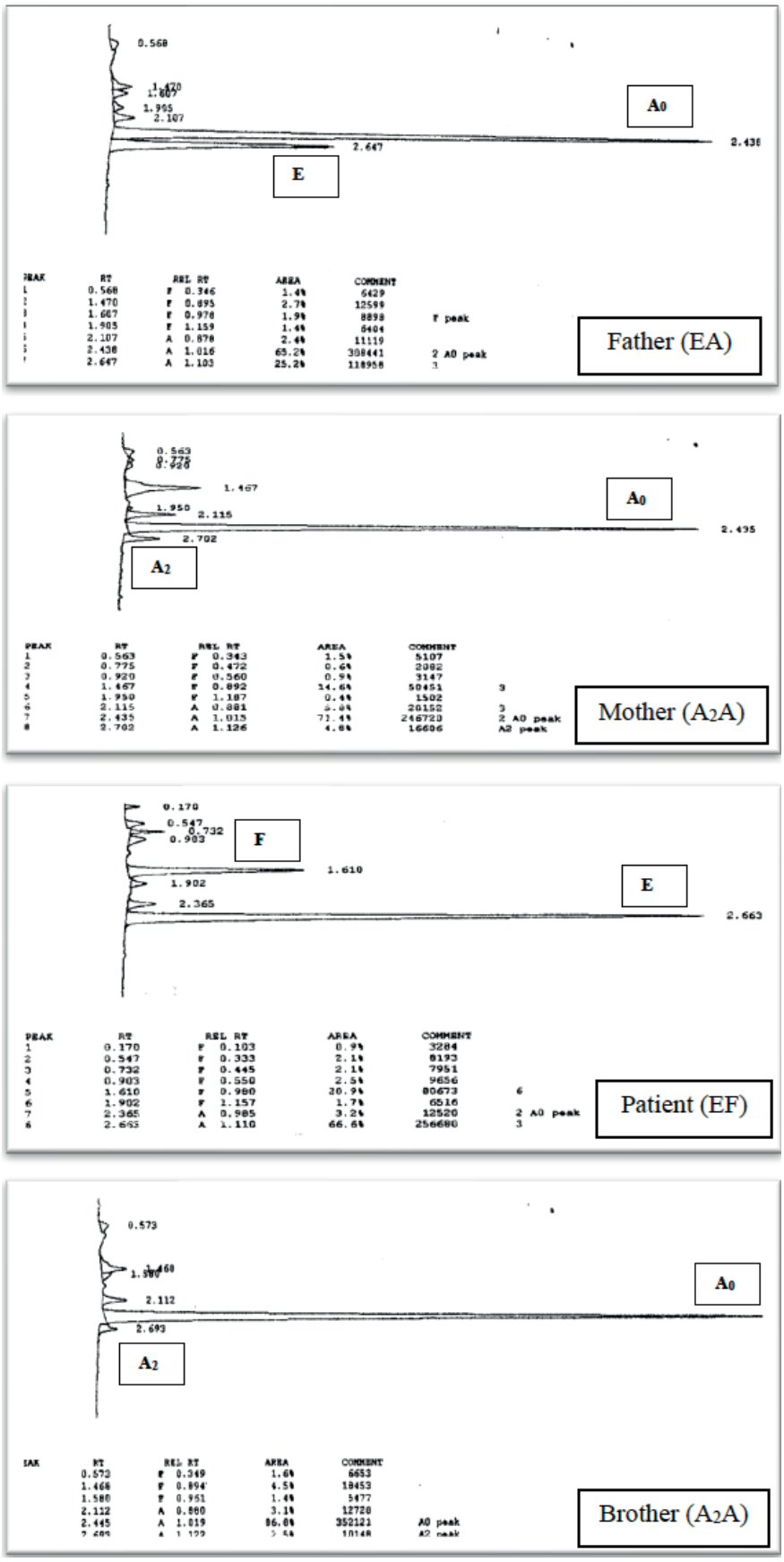
Supplementary Figures



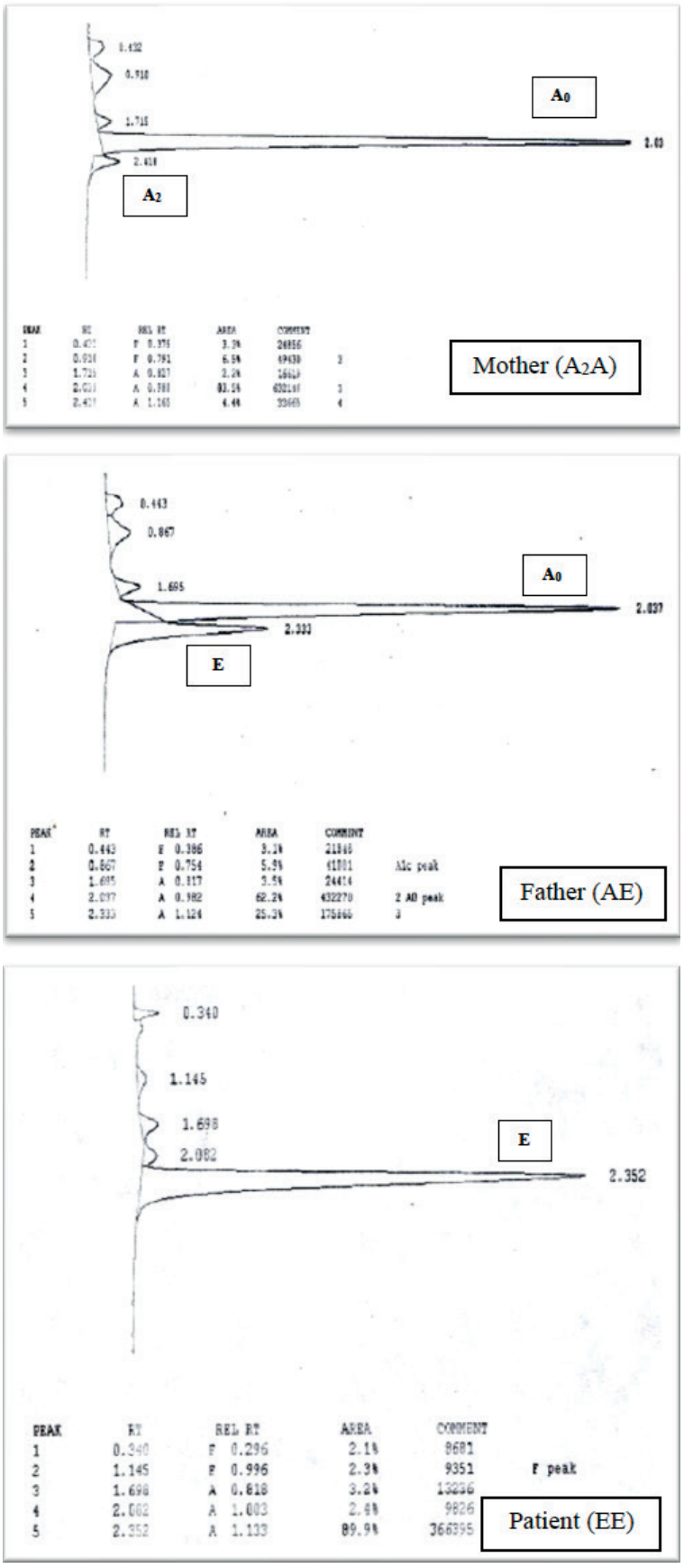
Supplementary Figure 1. *Pedigree of 4 families analyzed in this family study.*



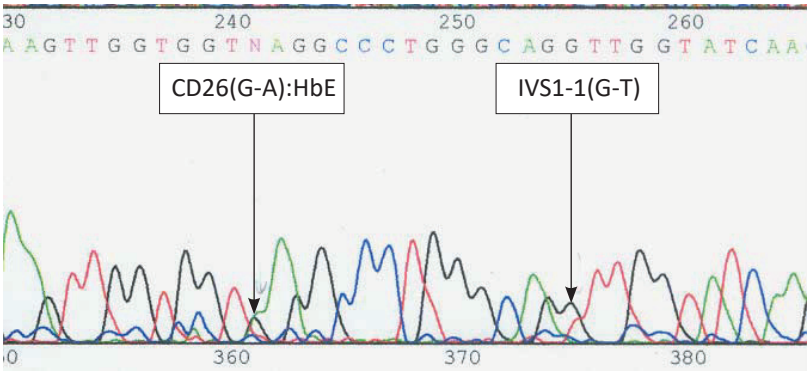
Supplementary Figure 2. *HPLC chromatogram (Primus Variant System 99) of hemoglobins of Family 1. The father was β-thalassemia heterozygote with HbsA$_2$A. Mother and brother were HbE heterozygote with Hbs EA. The patient was HbE/β^0-thalassemia with Hbs EF. The major HbA is HbA$_0$. The number above each peak is the retention time.*



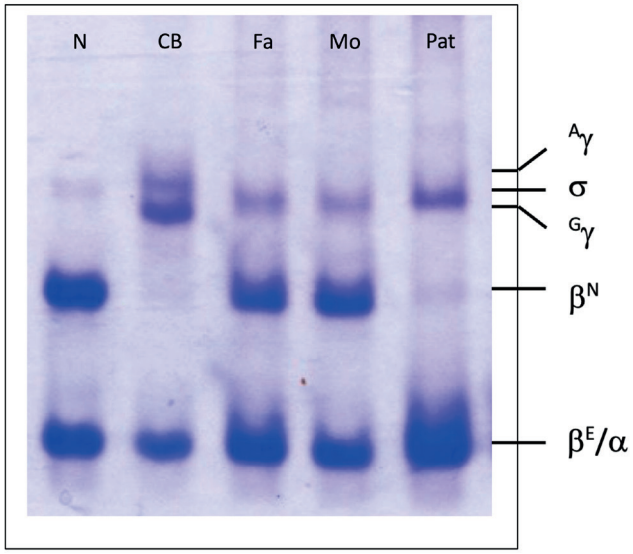
Supplementary Figure 3. *HPLC chromatogram (Primus Variant System 99) of hemoglobin in Family 2. The father was HbE heterozygote with Hbs EA. Mother was β-thalassemia heterozygote with Hbs A_2A. Brother was normal with Hbs A_2A. Patient was HbE/β^0-thalassemia with Hbs EF. The major HbA is HbA_0. The number above each peak is the retention time.*



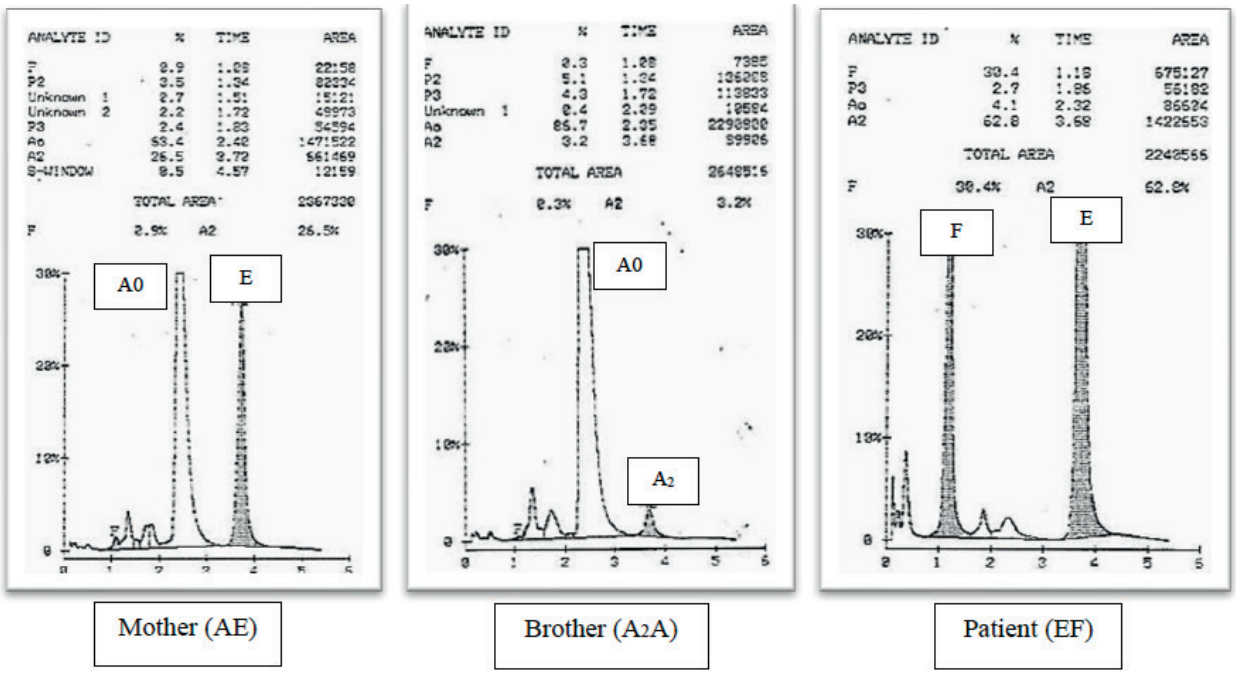
Supplementary Figure 4. *HPLC chromatogram (Primus Variant System 99) of hemoglobin in Family 3. The father was HbE heterozygote with Hbs EA. Mother was β-thalassemia heterozygote with Hbs A_2A. Patient was HbE/$β^0$-thalassemia with atypical Hb pattern of EE. The major HbA is HbA_0. Number above each peak is the retention time.*



Supplementary Figure 5. *Nucleotide sequencing of β-globin gene in patient of Family 3. Overlapped peaks are seen at codon 26 and IVS1-nucleotide 1, indicating heterozygous state of these base substitutions.*



Supplementary Figure 6. *AUT-PAGE for globin chain separation in Family 3. Note: No γ globin bands are seen in the patient. (N: normal, CB: cord blood, Fa: father, Mo: mother, Pat: patient)*



Supplementary Figure 7. *HPLC chromatogram (BioRad VARIANT II Hemoglobin Testing System) of hemoglobin in Family 4. The mother was HbE heterozygote with Hbs EA. Brother was normal with Hbs A_2A. The patient was HbE/β^0-thalassemia with Hbs EF. The major HbA is HbA_0.*