Clinical Phenotypes and Molecular Diagnosis in a Hitherto Interaction of Hb E/β Thalassemia Syndrome ($\beta^{E}/\ \beta^{-31,\ A\rightarrow G}$)

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Molecular identification of affected alleles in the index family with rare mutation(s) and/or interaction(s) is an important prerequisite toward a proper genetic counseling. In Thailand, where more than 30% of the populations are heterozygotes for either α or β thalassemia mutation(s). More than 60 different thalassemia syndromes resulting from the interactions of these heterogeneous alleles have been observed.[1] The majority of patients in the hospital based-study are compound heterozygotes for β thalassemia alleles and another hemoglobinopathy namely Hb E, highly prevalent in Thailand, gave rise to Hb E/β thalassemia syndrome. The phenotypes of these syndromes vary from asymptomatic individual to a very severe phenotype mimic that of β thalassemia major.

In this report, we describe a three-year-old Thai girl presenting with mild hypochromic microcytic anemia since birth. She was born prematurely and developed anemia within the first week of life. The cause of anemia was suspected to result from prematurity and low intrauterine iron storage, however hypochromic anemia did not resolve after a three-month of iron supplement therapy. Subsequent studies indicated that the patient had Hb E/β thalassemia disease and the molecular study revealed that the patient was a compound heterozygote for Hb E and a rare β^{+} thalassemia mutation ($\beta^{-31,\ A\rightarrow G}$). This hitherto genotype results in a relatively mild clinical symptom since the patient's baseline Hb values were around 9-10 g/dL with normal weight and height development during the follow-up period.

Keywords: *Hb E/β thalassemia, The promoter mutation (-31), Molecular diagnosis, Genotype-phenotype correlation, Prenatal genetic counseling*

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Hemoglobin is the major intracellular protein of mature red blood cells composed of two α-like chains and two β-like chains, each containing a heme group[2]. The composition of the globin chains can vary, giving rise to several normal and abnormal forms of hemoglobin. Each globin chain (α and β) is expressed from different globin gene clusters. The β globin gene cluster is located on the short arm of chromosome 11 (11p 15.5) consisting of four genes and one pseudogene, in order from the 5 to 3 ; the ε, $^{A}\gamma$, $^{G}\gamma$, ψβ, δ and β genes[2]. The entire β globin gene cluster spans approximately 80kb and is surrounded by a large cluster of olfactory genes. Mutations of the globin genes could result in either a decrease in the expression of one of the globin chains (thalassemia) or a change in the protein structure (hemoglobinopathy) and, thereby, alternating the protein properties and, in some cases, its functions[2, 3].

The molecular basis of β thalassemia (β thal) is usually caused by point mutations due to single

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base substitutions or deletion/insertion of few bases, which prevent the β globin chains from being normally produced[3]. If the mutations completely abolish the globin expression, they are classified as β^0 thalassemia while β^+ thalassemia represents a decrease expression with residual β globin transcripts from affected alleles. Such mutations could be found in the promoter region, the cap site, the splicing regions, exons, RNA cleavage and polyadenylation sites and the 3' untranslated region (UTR)[3]. These mutations interfere with various steps of mRNA transcription and processing, globin translation and post-translational stability. Rarely, β thalassemia is caused by large deletions involved the structural genes or the remote *cis* regulatory elements known as the β globin locus control region (β-LCR)[2].

In Thailand, approximately 30% of the population are either thalassemia or hemoglobinopathy (mainly Hb E) heterozygotes and 3-5% are carriers of the β thalassemia[4]. More than 40 different mutations of β-globin genes have been identified in Thailand. The Hb E (codon 26; GAG -> AAG) mutation is the most common hemoglobinopathy in Southeast Asia, with the β^+ thalassemia phenotype[4]. Interaction of the Hb E with different types of β thalassemia mutation gives rise to heterogeneous clinical phenotype; from asymptomatic individuals to a very severe phenotype mimic that of homozygous β thalassemia or β thalassemia major[5, 6]. A compilation of the clinical data and severity assessment of further patients with Hb E and different β thalassemia mutations is very much needed in order to provide a complete spectrum of the genotype-phenotype interaction this syndrome poses[7]. Such information will be indispensable for an 'evidence based genetic counseling' for future cases. We report here the first interaction of Hb E with a rare β^+ thalassemia; a TATA box mutation ($\beta^{-31, A\rightarrow G}$) in a Thai patient. The clinical presentation and disease progression during the three-year follow up period is described.

Material and Method

Hematological study

Routine hematological studies including red blood cell indices were determined by an automated red blood cell counter and the detection of reticulocytes was preformed by staining peripheral blood with methylene blue. Hemoglobin was analyzed by cellulose acetate and starch gel electrophoresis at pH 8.6, iso-electric focusing (IEF) and Low Pressure Liquid Chromatography (LPLC) on an automated hemoglobin analyzer (HB GOLD, Cumbria, Burrow-in-Furness, UK) according to the manufacturer s instruction. Hb F was additionally determined by alkali denaturation.

Molecular characterization of the β globin genes

DNA studies were performed in proband's DNA by extraction of DNA from peripheral leucocytes using a standard-phenol chloroform technique. To determine affected β thalassemia allele, 10 common β thalassemia mutations in Thailand were screened using the reverse dot-blot hybridization technique as previously described[8]. Subsequently, to identify the unknown β thalassemia allele, a direct genomic DNA sequencing of the 2.6 kb fragment covering the whole β-globin gene (from-124 bp upstream of the cap site to +163 bp downstream of the poly A site) including the 5' and 3' UTR in the patient's DNA was performed using polymerase chain reaction (PCR). The amplification primers of the fragment were: forward primer (WB-F) 5'-CGA TCT TCA ATA TGC TTA CCA AG-3' and reverse primer (WB-R) 5'-GGG CCT ATG ATA GGG TAA T-3', respectively. The PCR was performed in 50 ml consisted of 100 ng DNA, 10 mM Tris HCl, 50 mM KCl (pH 8.3), 1.5 mM $MgCl_2$, 200 nM of each dNTP, 25 pmole primers and 1 unit of *Taq polymerase* (Roche Diagnostic, Manheim, Germany). The reaction was carried out with initial denaturation at 95°C for 5 minutes and then 35 cycles of 95°C 30 seconds, 55°C 30 seconds and 72°C 90 seconds and a final extension at 72°C for 10 minutes.

Direct DNA sequencing was performed in the PCR products using their amplification primers and six sequencing primers (sequences are available on request). Sequencing analysis was performed on an ABI 310 automated sequencer (Applied Biosystem, CA, USA) using fluorescent-labeled dideoxy terminator (Perkin-Elmer Biosynthesis, CT, USA).

Results

Case history

A three-year old Thai girl was referred to our department at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, due to anemia at birth. The patient was born prematurely with the gestational age of 35 weeks. Her birth weight was 1,950 grams and the hematocrit was 41%. Due to the respiratory distress, the patient received a single unit of blood transfusion before referral. At one month of age, the patient was investigated at our Department to identify the cause of her anemia. The hematological analyses revealed the patient had mild hypochromic microcytosis without organomegaly or other clinical symptoms suggesting β thalassemia. Therefore the diagnosis of

anemia of prematurity due to possible low iron storage was suspected, and the patient received daily supplement of ferrous sulphate and folinic acid. However, after three month-period of iron supplement, the patient's hemoglobin and hematocrit levels did not increase prompting us to perform the family study. Using hemoglobin analysis, the mother was found to be heterozygote for Hb E (Hb AE) while the father had a phenotype of β thalassemia trait (low MCV, MCH and high Hb A_2) (Table 1). The presence of Hb F, Hb E and Hb A (Table 1) in the patient (age 2 year at the study) was consistent with Hb E/β^+ thalassemia disease. During the three-year follow up period at our department, the patient maintained the levels of baseline hemoglobin of 9-10 g/dL without hepatosplenomegaly and never received blood transfusion. The patient attained a normal weight and height development according to the standard of Thai children.

Molecular analysis

Using the reverse dot-blot hybridization, we could identify only the Hb E mutation in the patient s genomic DNA suggesting that the patient was a compound heterozygote for Hb E and a rare β globin mutation (data not shown). Subsequently, a single nucleotide substitution at the position-31 (A->G) of the β globin gene promoter was detected in a heterozygous pattern in the proband by direct genomic sequencing analysis (Fig. 1). Hb E mutation was also identified as a single nucleotide substitution (*G*AG->*A*AG) at codon 26 using the same technique (data not shown). Therefore, by molecular characterization, the proband was a compound heterozygotes for Hb E and the β globin promoter mutation ($\beta^E/\beta^{-31(A->G)}$).

Discussion

To the best of our knowledge, this is the first report of the promoter mutation ($\beta^{-31A\rightarrow G}$) in Southeast Asian population and hitherto found in a combination with Hb E. This rare β^+ thalassemia has been first characterized in a Japanese patient in 1986[9]. The single base substitution (A→G) at position -31 (from the ATG initiation codon) resides within the highly conserved proximal promoter element, the TATA box, and it is expected to down regulate the β globin gene transcription. The TATA box consensus sequences (highlighted) read, from the 5'-GGGC**ATAAA**AG-3' coordinating the position-31 to -28 from the ATG signal of the β globin genes[10, 11]. Transient expression system of the mutant allele in COS cells showed that this TATA box mutation results in 45% of normal β globin mRNA output, a level compatible with the relatively mild phenotype of β^+ thalassemia[9]. This mutation has provided a supporting evidence of the functional significance of the TATA box for *in vivo* transcription of the human β globin gene. In general, accurate transcriptional initiation requires the presence of the TATA box sequences,

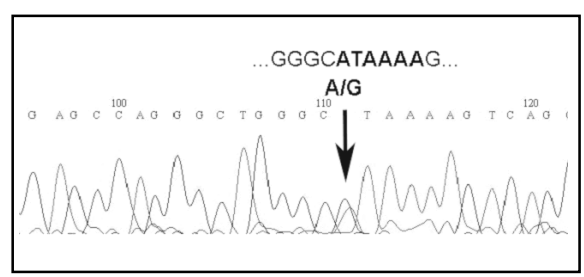

Fig 1. Direct genomic sequencing of the β globin genes in the patient demonstrating a nucleotide substitution (A→G) at the position -31 from the transcription start site (TATA box signal (ATAAAA) is remarked over the sequence)

Table 1. Hematological parameters in the index family and four unrelated heterozygotes with this rare β^{-31} mutation (M= male, F = female, NA = not available)

Cases	Age (yrs)	Hb (g/dL)	Hct (%)	MCV (fl)	MCH (pg)	Hb Typing (%)			β globin genotypes
						A	A_2/E	F	
Proband	3	10	30.0	63.0	19.6	36.8	42.7	20.5	β^{-31}/β^E
Mother	30	12.8	39.0	78.4	25.7	73.0	27.0	0	β^N/β^E
Father	32	13.5	39.7	75.7	15.5	93.9	4.7	1.4	β^N/β^{-31}
T1 (M)	35	15.6	46.0	80.3	27.3	93.8	4.7	1.5	β^N/β^{-31}
T2 (F)	22	10.7	31.4	80.0	27.3	93.8	4.8	1.4	β^N/β^{-31}
T3 (F)	30	10.3	31.0	72.0	23.2	93.7	6.3	0	β^N/β^{-31}
T4 (F)	29	11.0	33.0	78.0	24.8	95.8	4.2	0	β^N/β^{-31}

and other elements locating around the start site form the core promoter and initiator (Inr) elements[11]. Using artificial constructs, there has been shown that the TATA box is the major selector of the site of transcription initiation and that the Inr elements contribute to the magnitude of the process[11]. So far, nine different mutations including 1) -32 (C→A)[12], 2) -31 (A→C) [3], 3) -31 (A→G) [9], 4) -30 (T→C) [13], 5)-30 (T→A) [14], 6) -29 (A→G) [15], 7) -28 (A→C) [16], 8) -28 (A→G) [17] and 9) -27 (A→T)[18] from different populations have been reported to affect the conserved sequences that form the β globin promoter. In transient transfection assays using Hela and mouse erythroid leukemic (MEL) cells, this group of mutations resulted in decreased expression of the mutant constructs to approximately 25-30% of the wild type[17, 19]. It was proposed that the TATA box mutations destabilize and/or weaken the TFIID (the basal transcription factor-D) binding resulting in the partial loss of transcription in the β globin genes[19]. It appears that, by and large, point mutations involving the β globin gene promoters including the TATA box mutations are associated with slightly higher levels of Hb F than other mechanisms causing β thalassemia[2]. However during recent years, we have identified four additional individuals as being heterozygotes for this rare mutation (β^{-31}/β^{N}), surprisingly a modest increased Hb F was observed in some, but not all, individuals (Table 1, T1-T4). It might suggest a different chromosomal environment, most likely a link between the mutations and the *Xmn* I polymorphism at the $^{G}\gamma$ globin gene promoter,[20] between our cases from those reported in the literatures.

Interaction of this TATA box mutation, as being β^{+} thalassemia allele, with Hb E mutation gives rise to a rather mild phenotype since the patient did not require transfusion after her stormy neonatal period and did maintain her levels of hemoglobin at around 9-10 g/dL without any detrimental effect on growth and height development. This finding is not surprising since, as we have shown before, the type of β thalassemia mutation, either β^{0} or β^{+} thalassemia, plays an important role as a primary genetic factor determining the resulting phenotype in Hb E/β thalassemia[6]. Patients with Hb E/β^{+} thalassemia usually have a milder phenotype compared to that of Hb E/β^{0} thalassemia[6]. It remains to be seen whether how disease associated complications such as chronic anemia, iron overload and possible risk of idiopathic pulmonary hypertension in long-term manipulate the natural history and prognosis and the life span in this group of patients comparing with the Hb E/β^{0} thalassemia and normal individuals.

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อาการทางคลินิกและการวินิจฉัยในระดับอณูวิทยา ในผู้ป่วยฮีโมโกลบิน อี/เบต้าธาลัสซีเมีย ($\beta^E/\beta^{-31, A\rightarrow G}$)

ณัฐศวีร์ วัฒนา, วิปร วิประกษิต, กลีบสไบ สรรพกิจ, วรวุฒิ จีนช้าง, กวิวัณณ์ วีรกุล, วรวรรณ ตันไพจิตร

การศึกษาทางอณูวิทยา เพื่อตรวจการกลายพันธุ์ของธาลัสซีเมีย มีความสำคัญและจำเป็นในการพยากรณ์ความรุนแรงของโรคและการให้คำปรึกษาแนะนำทางพันธุศาสตร์ในครอบครัวผู้ป่วยธาลัสซีเมียมาก ในประเทศไทยซึ่งมี พาหะแอลฟ่าและเบต้าธาลัสซีเมียรวมกันถึงร้อยละ 30 ของประชากร ปฏิสัมพันธ์ของยีนเหล่านี้ ทำให้เกิดโรคธาลัสซีเมียได้ถึงมากกว่า 60 แบบ ผู้ป่วยเบต้าธาลัสซีเมียส่วนใหญ่เกิดจากปฏิสัมพันธ์ของยีนเบต้ากับยีนฮีโมโกลบิน อี ซึ่งเป็นฮีโมโกลบินผิดปกติที่พบมากที่สุดในประเทศไทย เกิดเป็นโรคเบต้าธาลัสซีเมีย/ฮีโมโกลบิน อี ซึ่งมีความรุนแรงตั้งแต่ไม่มีอาการจนถึงอาการมากแบบเบต้าธาลัสซีเมียเมเจอร์ ทั้งนี้ขึ้นอยู่กับลักษณะการกลายพันธุ์ของยีนเบต้าเป็นปัจจัยสำคัญ

รายงานนี้กล่าวถึงเด็กหญิงไทยอายุ 3 ปี ซึ่งมีอาการซีดแรกเกิดที่คาดว่ามีปัญหาจากการเสียเลือดและขาดเหล็ก ซึ่งแม้ได้รับการรักษาด้วยยาธาตุเหล็กจนถึงอายุ 3 เดือน ก็ยังมีอาการซีดอยู่ จากการศึกษาระดับโปรตีนพบว่าเป็นโรคเบต้าธาลัสซีเมียชนิดไม่รุนแรง และการศึกษาเพิ่มเติมทางอณูวิทยาพบว่าโรคธาลัสซีเมียในผู้ป่วยรายนี้เกิดเนื่องมาจากมีปฏิสัมพันธ์ของยีนฮีโมโกลบิน อี กับยีนเบต้าธาลัสซีเมียชนิดไม่รุนแรง ($\beta^{-31, A\rightarrow G}$) มีผลทำให้ผู้ป่วยมีอาการซีดเพียงเล็กน้อยเท่านั้น แต่การเจริญเติบโตปกติ การกลายพันธุ์แบบนี้พบน้อยและปฏิสัมพันธ์ระหว่างเบต้า ธาลัสซีเมียชนิดนี้ กับฮีโมโกลบิน อี ดังเช่นในผู้ป่วยรายนี้ คาดว่ายังไม่มีผู้ใดรายงานมาก่อน