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MOLECULAR MECHANISMS OF THALASSEMIA IN THAILAND

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Abstract

Thalassemia syndrome, resulting from defective synthesis of globin chains, is the most common genetic disorder in Thailand. α-Thalassemia 1 occurs from deletion of both of the duplicated α-globin genes. α-Thalassemia 2 is the result of a deletion leading to loss of one α-globin gene, classified either as leftward (4.2 kb) deletion or rightward (3.7 kb) deletion; the latter is more common in Thai patients. Nondeletion α-thalassemia, so far, is rare or absent in the Thai population. Studies of β-globin gene cluster in β-thalassemia using restriction endonuclease DNA polymorphisms reveal 17 different haplotypes. However, 80.3 %of 158 β-thalassemia chromosomes analysed show two common haplotypes, +−−−−−+and +−−−−++. Point mutations and small deletions or insertions in the nucleotide sequences are responsible for the molecular defects of β-thalassemia. In contrast to β-thalassemias, a wide area of gene deletion involving the $^{A}\gamma$-, δ- and β-globin genes, resulting in $(^{A}\gamma\ \delta\ \beta)^{\circ}$-thalassemia in two unrelated families, have been identified. Hemoglobin (Hb) E and Hb Constant Spring, the two most common abnormal globin variants, also behave like thalassemia.

Introduction

The thalassemias are a group of hereditary anemia characterized by decreased or absent synthesis of one of the globin subunits of the hemoglobin molecule. This is the most common genetic disorder in Thailand. Two major types are α - and β - thalassemia. In addition, two abnormal hemoglobins (Hb), Hb E and Hb Constant Spring, which have the phenotypic expression of thalassemia are also found in this population. The

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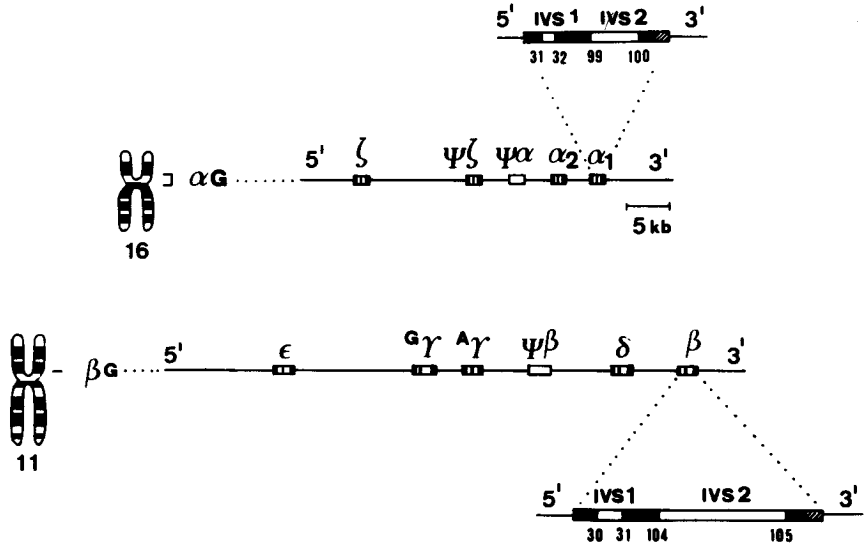

Fig 1. *The arrangement of α - and β -globin gene complex.* For every gene, black boxes represent coding regions (exons), white boxes represent intervening sequences (introns, IVS) and hatched boxes are the 5′ and 3′ untranslated regions. The number of the codons separated by each intron is also shown under the area of the coding sequences.

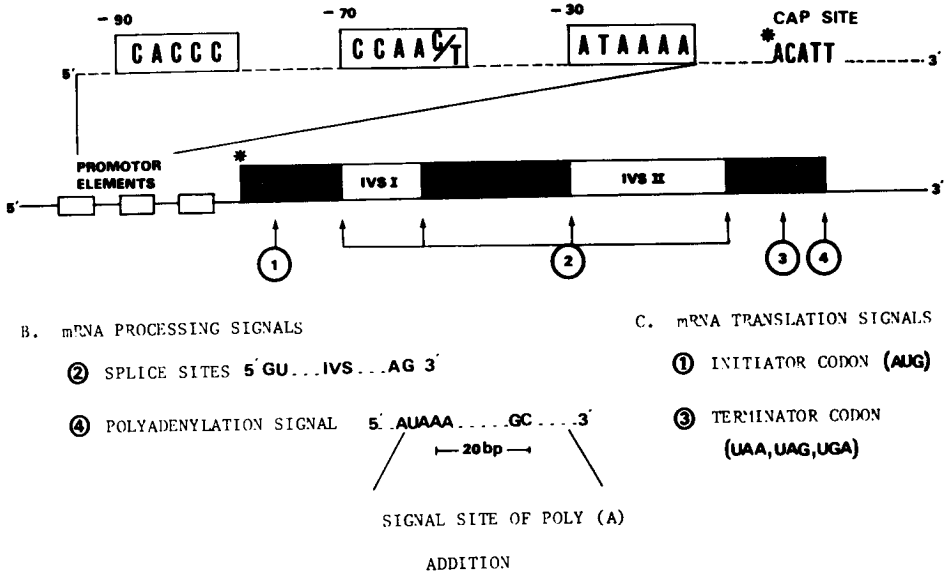

Fig. 2 *Functional anatomy of the globin gene.* Conserved sequences necessary for gene expression, RNA processing and splicing, and translation of the processed mRNA is shown.

frequency is 20-30% for α - thalassemia, 3-9% for β -thalassemia, 13-50% for Hb E and at least 4% for Hb Constant Spring. δβ-Thalassemia and hereditary persistence of fetal hemoglobin (HPFH) are rarely found in the population.

Structure of Human Globin Genes

Hemoglobin is a tetramer that consists of two α -like and two β -like globin subunits. These subunits are encoded by two clusters of genes. The α -gene complex is clustered on the short arm of chromosome 16[2] in a 25 Kb (1 Kb =1000 base pair) region consisting of ζ , ψζ , ψα , α2 and α 1 genes. The β -gene cluster is on the short arm of chromosome 11[3,4] in a 50 Kb region containing ε, $^{G}\gamma$, $^{A}\gamma$, ψβ , δ and β genes. ψζ , ψα and ψβ are pseudogenes which have sequence homology with the active genes but contain mutations that prevent their expression[5-7]. The arrangement of human globin genes is shown in Fig. 1. The α - globin loci are about 3.7 Kb apart and the embryonic ζ-globin gene is about 20 Kb 5′ to the α 2-gene[8-9]. The distance between the β -and δ - gene is about 7 Kb, and that between the δ -and $^{A}\gamma$-gene, 15 Kb. The $^{G}\gamma$ -gene is about 5 Kb "upstream" (5′ relative to start site of gene transcription) to the $^{A}\gamma$ -gene and theε-gene which codes for the embryonic ε chain lies 5′ to the $^{G}\gamma$ -gene[7, 10-12]. Each globin gene is discontinuous in that there are 3 segments of DNA which encode for the polypeptide chain (exons) separated by 2 non-coding intervening sequences (IVS or introns)[13]. The intervening sequences are transcribed in the nucleus of erythroid precursors so that the initial RNA transcript is a mosaic of exons and introns. Within the nucleus, RNA splicing mechanism excises the intron and ligates the exons together resulting in the mature mRNA molecule.

Consensus sequences at the boundaries of the coding and intervening sequences are crucial for the accurate splicing mechanism. The IVS always begin with the dinucleotide GT and end with an AG (called "Chambon rule")[14,15]. Fig. 2 shows the conserved sequences important for gene expression, RNA processing and translation of the processed mRNA. Promotor sequences at the 5′ end of globin gene are essential for RNA polymerase binding and for accurate and efficient RNA transcription. However, the α- and δ -globin genes lack one of the promotor elements, the conserved CACCC sequence, which is found duplicate in the β -globin gene[16,17]. Transcription occurs beyond the DNA strand that corresponds to the 3′ end of RNA coding sequences. Conserved sequences at the 3′ non-coding end of globin gene are responsible for terminating RNA transcription and for the addition of adenosine to form the poly A tract[18].

Translation of the processed mRNA is on polyribosomes in the cytoplasm. Initiation of translation starts at the codon AUG and terminates at codon UAA, UAG or UGA. α- and ζ -Globin chains have 141 amino acids whereas β-, δ-, γ- and ε-chains

have 146 amino acids. In the normal individual, production of α- and non α-globin chains is equal[19]. Gene deletions or point mutations in the nucleotide sequences necessary for transcription, RNA processing and translation are found to be causes of thalassemic diseases.

Alpha Thalassemia

Alpha thalassemia is most often due to gene deletion[19]. A summary of deletions in the α-globin gene cluster leading to α-thalassemia 1 and α-thalassemia 2 in Thai population is shown in Fig. 3. α-Thalassemia 1 occurs from deletion of both of the duplicated α-globin genes. The 5′ start of the deletion in α-thalassemia 1 in Thais may exist within the third exon of the ψζ-gene and the 3′ end of this deletion terminates within the hypervariable region located at the 3′ end of the α-globin gene complex, removing about 17.5 Kb of DNA from the α-globin gene cluster[20] (--SEA in Fig. 3.).

Deletions of the α-globin complex that cause α-thalassemia 2 remove one α-globin gene, thus reducing the output from that chromosome by about one-half. Two types of α-thalassemia 2 have been observed, one involving a deletion of 4.2 Kb of DNA (leftward type, $-\alpha^{4.2}$) and another removing 3.7 Kb (rightward type, $-\alpha^{3.7}$)[11]; the latter is more common in many populations including Thais[20].

The 4 Kb α-globin duplication unit is shown to contain three highly conserved X, Y and Z, homology blocks (Fig.3)[6,22]. Thus misaligned reciprocal recombination between the duplicated α-globin genes can lead to a deletion of one of the two α-genes. The $-\alpha^{4.2}$ involves the X homology block 3′ to the ψα-gene on one chromosome and the block 3′ to the α 2-gene on the other, whereas the $-\alpha^{3.7}$ defect results from either inter- or intrachromosomal recombination between the two homologous Z segments[23]. Higgs *et al.* have shown that the point of crossover giving rise to the $-\alpha^{3.7}$ defect in Thais is located within the homologous region 5′ to the third exon of the α-globin genes in Z segments, of which the homology is more than 99%[24]. However, deletions that involve the α-thalassemia 1 do not seem to occur between these highly homologous regions of DNA.

Non-deletion α-thalassemia described in Mediterraneans and Saudi Arabians, so far, is rare or absent in Thai population[20]. However, some α-globin chain variants can have the thalassemia-like expression, for example Hb Suan-Dok which is the highly unstable abnormal α-globin chain[25]. Hb Constant Spring, the elongated α-chain variant commonly found in Thailand, also has the α-thalassemia 2 like effect. This abnormal globin occurs from the mutation of the termination codon in the α 2-gene, from UAA to CAA, resulting in a longer mRNA which is translated to the next in-phase terminator to give a globin with 172 rather than normal 141 amino acids[26,27]. Hb Constant Spring α mRNA is quantitatively reduced, which may reflect its instability, thus resulting in minute amounts of Hb Constant Spring production. The other α-chain variant, Hb Mahidol (Q) is associated with the leftward deletion type of α-thalassemia 2

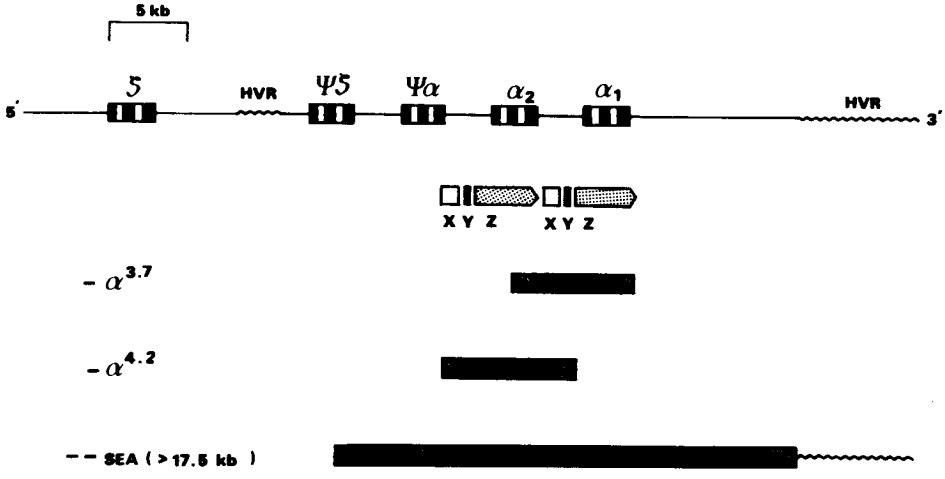

Fig. 3 *Summary of deletions in the α-globin gene cluster leading to α-thalassemia in Thailand.* ⩙ represents the hypervariable region on the α-globin gene cluster. The homology blocks, X, Y and Z, are shown under the physical map. The extents of the deleted segments are shown by the black boxes and the hatched boxes indicate the possible limit of deletion.

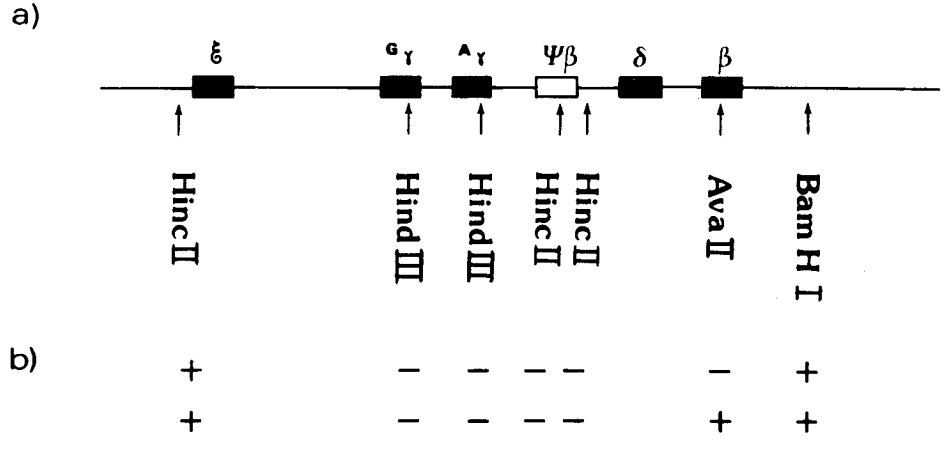

Fig. 4 *The polymorphic loci used to define the haplotype (a) and two common haplotypes of the β-globin gene complex (b).* + indicates that the enzyme cuts at this site, — indicates that the enzyme does not cut at this site.

which can lead to Hb H disease when it interacts with α-thalassemia 1[20].

In summary, four varieties of α-thalassemia can be defined depending on the number of α-globin gene deletion in the diploid genome. Thus deletion of 1, 2, 3 and 4 -globin genes results in α-thalassemia 2 (-α / αα), α-thalassemia 1 (--/ αα), Hb H disease (--/-α) and Hb Bart's hydrops fetalis (--/--), respectively. Deletions of 1 or 2 α-genes do not give rise to clinical disease, whereas deletion of four α-genes leads to the most severe form of thalassemia, Hb Bart's hydrops fetalis, in which the fetus dies *in utero* or soon after birth. Two genotypes of Hb H disease are commonly found in Thailand: α-thalassemia 1/α-thalassemia 2 (--/-α) and α-thalassemia 1/Hb Constant Spring (--/ $\alpha^{CS}\alpha$). Rare Hb H diseases can occur from the interaction between α-thalassemia 1 and Hb Mahidol or Hb Suan-Dok.

Beta Thalassemia

In β-thalassemia, gene deletion does not appear to be the underlying abnormality. It is a very heterogeneous disorder caused by many different defects in the β-globin gene. Recombinant DNA technology has enabled a number of different β-thalassemia genes to be cloned and sequenced. It has been found that point mutations and small deletions or insertions in the nucleotide sequences are responsible for the molecular defects of β-thalassemia[17].

Study of the β-globin gene cluster using sequence analysis of cloned DNA fragments or restriction endonuclease DNA mapping has also revealed the common silent or neutral variation in DNA. If two or more alleles are present at any given locus at frequencies greater than 0.01, this is called DNA polymorphism. These nucleotide substitutions may either introduce or remove cleavage sites for restriction endonucleases which recognize specific DNA sequences. Thus each polymorphic site can either be present (+) or absent (−) in a particular chromosome. An ordered sequence of linked DNA polymorphisms is called a haplotype[28,29]. Fig. 4 shows the polymorphic restriction enzyme sites commonly used in haplotyping studies. A close association of DNA polymorphism haplotypes and specific mutations has been reported by Orkin[28]. However, the mutations are generally different in different ethnic groups even when the DNA polymorphisms surrounding the β-globin gene are the same and one mutation can be present on more than one haplotype (Table 1)[28,30,31].

Study of the β-globin gene cluster in Thai β-thalassemic patients revealed 17 different haplotypes from 158 chromosomes[32]. However, 80.3% of the chromosomes analysed showed two common haplotypes, +−−−−−+ and +−−−−++ (Fig.4 and Table 1). Three Indian patients, immigrants into Thailand, who have the +−−−−−+ haplotype were found to have a deletion of 619 base pairs, starting in the IVS 2 of the β-globin gene (Fig.5). This deletion has previously been reported with a high incidence amongst Asian Indians who also have the same haplotyping data[30]. In another study by

TABLE 1 EXAMPLES OF HAPLOTYPES AND THEIR MUTATIONS FOUND IN β- THALASSEMIC PATIENTS IN VARIOUS ETHNIC GROUPS[28, 30-32].

Haplotype	Thai		Chinese		Indian		Mediterranean	
	Frequency (%)	Mutation	Frequency (%)	Mutation	Frequency (%)	Mutation	Frequency (%)	Mutation
+−−−− −+	41.1	619 bp deletion 4NT deletion at 41-42	48.7	A insert at 71-72	63.6	619 bp deletion IVS-1 p.5, G→C	6.0	IVS-2 p.745, C →G
+−−−− ++	39.2	?	37.8	IVS-2 p. 654, C →T	7.0	G insert at 8-9	47.0	IVS-1 p. 20 from 3′ splice-site, G →A
+−−−− −−	1.3	3.4 Kb deletion	0	—	0	—	0	—
+−−−− +−	1.3	?	0	—	11.4	TCTT deletion at 41-42 C deletion at 6	12.0	IVS-1, G →A
−++−+ −+	4.4	?	10.8	TATA box, A →G	2.3	IVS-1 p.5, G →C	0	—
−++−+ ++	1.2	?	0	—	2.3	nonsense mutation at codon 15	17.0	nonsense mutation at codon 39
−+−++ −+	1.3	?	*	nonsense mutation at codon 17	0	—	1.0	AA deletion at 8
−+−++ ++	1.8	?	0	—	9.0	25 bp deletion IVS-1, G →T	3.0	nonsense mutation at codon 39
−+−++ +−	0	—	0	—	2.3	TCTT deletion at 41-42	8.0	IVS-2, G →A
−+−+− −+	1.9	nonsense mutation at codon 17	0	—	0	—	0	—

? = not known, being examined

* = unreported haplotype[32]

Fukumaki *et al.* a patient who shares the same haplotype, +−−−−−+ , was found to have a four-nucleotide deletion in codons 41 and 42 of the β -globin gene[33]. This deletion shifts the reading frame resulting in an in-phase terminator in codon 59. The same mutation has been reported in Indian and Taiwan β-thalassemic patients who have different haplotypes[30,33].

Cloning and sequencing analysis were also carried out in a patient who showed the unique haplotype, −+−+−−+ (Table 1). It was found that the β -globin gene had a single nucleotide substitution producing a terminator codon at position 17[32]. This mutation was first reported in a Chinese β -thalassemic patient[34]. The other unique haplotype, +−−−−−−, was found to have a 3.4 Kb deletion of DNA including the entire β -globin gene (Fig.5). This deletion, and its associated haplotype, has not been reported before and surprisingly the patient is a homozygote for the same haplotype[32].

All mutations described above lead to β^0-thalassemia in which no β -globin chain is synthesized. Work is in progress to identify the molecular defects of the other patients with the common and rare haplotypes described in Table 1.

So far, the molecular defect leading to β^+- thalassemia in Thailand has not been identified. Abnormal transcription and splicing of RNA have been found to be responsible for β^+-thalassemia in many populations. However, Hb E, a β -globin variant ($\beta^{26\ glu \rightarrow lys}$)commonly found in S.E. Asia, behaves as a mild β^+-thalassemia. It was found that the codon change GAG to AAG at position 26 activated an alternative site for β^E-mRNA splicing at codon 25 (Fig. 6)[35]. Thus, in addition to normally spliced mRNA, an abnormal mRNA was present which utilized this alternative site resulting in a deletion of a portion of the first exon. This mechanism results in reduced β^E-mRNA[36,37] and thus reduced synthesis of β^E-globin chains[38] accounting for the β^+-thalassemia phenotype of Hb E.

Hb Lepore and δβ-Thalassemias

Hemoglobin Lepore is another structural variant associated with the thalassemia phenotype. The crossover event deletes the DNA between δ - and β -globin genes (Fig.5). This will result in a δβ -fusion gene which can produce a δβ -fusion chain or Hb Lepore[39]. The abnormal globin chain is synthesized in a reduced amount leading to the feature of β -thalassemia trait.

In δβ -thalassemia , gene deletions of different extents including δ - and β -globin genes have been found to be a common underlying molecular defect[40]. $^G\gamma\ (^A\gamma\delta\beta)^0$-Thalassemia detected in two unrelated Thai families, who have an increased amount of Hb F, also occur from gene deletions involving the $^A\gamma$-, δ- and β -genes (Fig. 5)[41]. However, non-deletion of the β -globin gene complex was also demonstrated in some patients with increased Hb F (unpublished data). In these cases gene cloning and sequencing are needed to define their molecular defects.

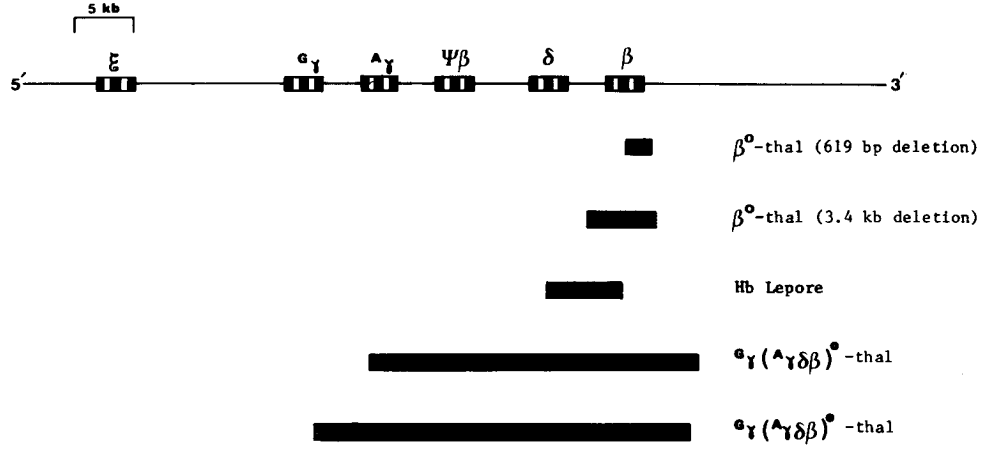

Fig. 5 *Summary of deletions in the* β-globin *gene cluster leading to* β^0-thalassemia, δβ-thalassemia, *and Hb Lepore in Thai patients.* Black areas indicate the known extent of deletion whereas the hatched areas indicate the possible limit of deletion.

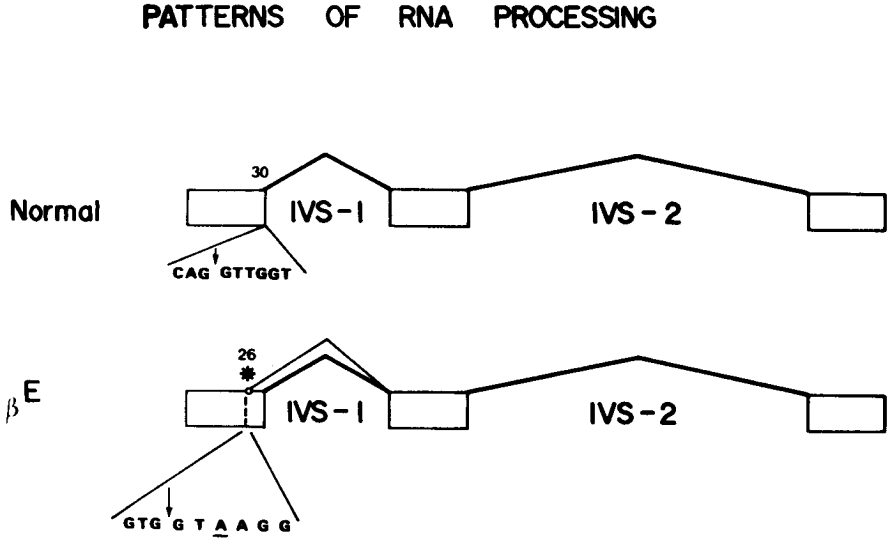

Fig. 6 *The alternative splice site for the* β^E*-mRNA (*) and the normal splice junction (CAG↓GTTGGT) at codon 30 of* β*-globin gene.* (see text for details).

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บทคัดย่อ

ธาลัสซีเมียเป็นโรคกรรมพันธุ์ที่พบบ่อยที่สุดในประเทศไทย อุบัติการของอัลฟ่า– ธาลัสซีเมียพบประมาณ 20-30 % เบต้า-ธาลัสซีเมีย 3-9 % ฮีโมโกลบิน อี (Hb E) 13-50 % และ Hb Constant Spring อย่างน้อยประมาณ 4% ธาลัสซีเมียเกิดจากความผิดปกติในระดับโมเลกุลทำให้มีการสังเคราะห์สายโกลบินน้อยลง ธาลัสซีเมียที่พบบ่อยคือ อัลฟ่า-และเบต้า-ธาลัสซีเมีย เกิดจากการสังเคราะห์สายอัลฟ่าและเบต้าโกลบินน้อยลง อัลฟ่า-ธาลัสซีเมียส่วนใหญ่เกิดจากยีนแหว่ง อัลฟ่า-ธาลัสซีเมีย 1 เกิดจากการแหว่งหายไปของยีนอัลฟ่า-โกลบินทั้งสองอันที่อยู่บนโครโมโซมข้างเดียวกัน สำหรับอัลฟ่า - ธาลัสซีเมีย 2 ส่วนใหญ่เกิดจากการแหว่งหายไปของเนื้อยีนขนาด 3.7 กิโลเบสที่อยู่ระหว่างยีนอัลฟ่าโกลบินทั้งสองอัน ส่วนน้อยของอัลฟ่า-ธาลัสซีเมีย 2 เกิดจากการแหว่งหายไปของเนื้อยีนขนาด 4.2 กิโลเบส ซึ่งรวมทั้ง 5'อัลฟ่าโกลบินยีนด้วย ในประเทศไทย อัลฟ่า-ธาลัสซีเมียที่ไม่ได้เกิดจากยีนแหว่งนั้นพบได้น้อยหรือไม่พบเลย.

จากการศึกษา DNA polymorphism ของยีนเบต้า-โกลบิน และยีนโกลบินที่อยู่ในกลุ่มเดียวกันด้วย restriction enzyme 7 ชนิด ในผู้ป่วยโรคเบต้า-ธาลัสซีเมีย พบว่ามีถึง 17 แบบ (haplotype) แต่ที่พบบ่อยที่สุดคือ แบบ +----−+ และ + ---- ++

เดลต้า-เบต้า ธาลัสซีเมีย เกิดจากการแหว่งของเนื้อยีนในบริเวณที่มากกว่าเบต้า–ธาลัสซีเมีย เท่าที่ตรวจพบในขณะนี้จากผู้ป่วยไทย 2 ราย พบว่ามีการแหว่งของยีน แกมม่า-เอ, เดลต้า และเบต้า โกลบิน ส่วนฮีโมโกลบินผิดปกติที่พบบ่อยในประเทศไทย คือ Hb E และ Hb Constant Spring พบว่าเกิดความผิดปกติทั้งโครงสร้างและการสังเคราะห์สายโกลบิน