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Molecular Characterization and the Severity of Hemoglobin H Disease in Northern Thailand

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ABSTRACT

We investigated the molecular basis for Hb H disease in 41 Thai patients in Northern Thailand by restriction endonuclease analysis with alpha and zeta specific globin probes. The result showed that 36 cases (88%) were due to a combination of thalassemia 1, Southeast Asia type and non-deletion defect type. Five cases (12%) were of a deletion type with 3. 7 kb deletion on one globin gene (rightward type). We did not find any leftward type (4. 2kb) among these patients. In clinical examinations, the patients with the non-deletion defect type of Hb H disease were more severe than the patients with deletion type.

INTRODUCTION

Alpha thalassemia is an inherited disorder of hemoglobin synthesis in which the production of the alpha globin chain in adult ($\alpha_2\beta_2$) and fetal ($\alpha_2\gamma_2$) hemoglobin is impaired. The alpha globin chain gene cluster lies on chromosome 16 arranged in the order 5'-ζ-$\psi\zeta$-$\psi\alpha$-α2-α1-θ-3' within a 30 kb stretch of DNA[1]. The most relevant clinical form of alpha thalassemia is hemoglobin H disease (Hb H disease), the major clinical symptom of which is a microcytic anemia, occurring most frequently in Southeast Asian (SEA) and Mediterranean (MED) populations[2]. The deletion of the alpha globin structural gene is a predominant lesion in alpha thalassemia and the Hb H disease occurs when one chromosome with a deletion of two alpha globin genes (--/), alpha thalassemia 1, combines with another chromosome with a deletion

of one alpha globin gene (– α /), alpha thalassemia 2. This combination results in the presence of a single functional alpha globin gene (– α /––)[3~5]. Globin gene mapping studies and DNA sequencing techniques have indicated that the defective alpha globin synthesis may be a gross deletion involving one, both or part of the alpha genes, smaller deletions of one or more DNA base pairs, or a point mutation but leaves the alpha gene without any deletion[6~8]. It has become the custom to refer to smaller deletions and single point mutation as non-deletion defects ($\alpha^T \alpha$). However, hemoglobin H disease may also be caused by an interaction of deletion alpha thalassemia (––/) with a non-deletion alpha thalassemia determinant ($\alpha^T \alpha$ /)[9] or by a homozygous state of some non-deletion defect ($\alpha^T \alpha / \alpha^T \alpha$)[10].

In Thailand, alpha thalassemia is much more common than beta thalassemia. The frequency of alpha thalassemia is 20-30% of the population, beta thalassemia is 3-9% and hemoglobin Constant Spring (Hb CS) is at least 4%[11]. The population of Northern Thailand has one of the highest frequencies (30%) of alpha thalassemia in the world[12]. Hundrieser, J. et al reported that in Chiang Mai which is located in the northern part of Thailand, alpha globin genotype frequencies are 0.0236 of alpha thalassemia 1 (––SEA), 0.00943 of alpha thalassemia 2 rightward (– $\alpha^{3.7}$), 0.0047 of alpha thalassemia 2 leftward (– $\alpha^{4.2}$) and the expected frequency of hemoglobin H disease was approximately 1: 1800[13]. In rural areas of Chiang Mai the alpha globin genotype frequencies of alpha thalassemia 1 and alpha thalassemia 2 are higher than central area of Chiang Mai. The gene frequency of alpha thalassemia varies in different populations.

This study was undertaken to determine the molecular basis and the severity of hemoglobin H disease in Northern Thailand where the incidence of this disease is high.

PATIENTS AND METHODS

Patients

Forty-one Thai patients with Hb H disease who came for treatment to the thalassemia Clinic in Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand, were analyzed for the alpha globin gene. All of the patients were children from 2 to 14 years of age. The diagnosis of Hb H disease was established by hemoglobin electrophoresis at pH 8.6 and brilliant cresyl blue staining for inclusion bodies[14].

Hematologic studies

Hematologic values indices were measured by a standard procedure. Hemoglobin A_2 (Hb A_2) level was quantified by DEAE-cellulose microcolumn chromatography[15] and hemoglobin F (Hb F) by an alkali denaturation method[16]. Measurement of hemoglobin H (Hb H) was achieved by elution from a cellulose acetate strip.

DNA analysis

DNA was extracted from the buffy coat of 5 ml of blood by phenol-chloroform and ethanol precipitation[17]. DNA (10 μg) was digested with 20 units of restriction endonuclease of *Eco* R I, *Bam* H I and *Bgl* II (Takara Shuzo Co., Japan) for 5 hours under conditions recommended by the manufacturer. The digested DNA was fractionated in 0.7% agarose gel (Sea Kem agarose, FMC BioProducts, U. S. A). The gel was blotted on a nitrocellulose membrane (Schleicher and Schuell, Inc., Germany). Identification of the alpha globin gene restriction fragment was started by prehybridization in 5× Denhard' s, 0.5% SDS, 50% formamide, 6× SSPE and 100 μg/ml of salmon sperm DNA for 3 hours, detail of which are given in the review by Old and Higgs[18].

The probes were labelled with [$\alpha - {}^{32}$P] dCTP (3000 Ci/mmole) under conditions recom-

mended in the Multiprimer DNA Labelling Kit (Amersham). The alpha globin gene probe was 1.5 kb of *Pst* I fragment of the cloned pEMBL α. The zeta probe was 400 kb of *Pst* I/*Hind* III fragment of the cloned pEMBL ζ. Labelled probes were made single stranded by boiling 5 min and then made up to 10×10^{8} cpm/ml of the above prehybridization mixture. Hybridization of the probe and nitrocellulose membrane was undertaken overnight at 42℃. The excess probe was then removed by washing with a mixture of 2 × SSC and 0.1% SDS twice at 37℃ for 60 minutes and with a mixture of 0.1 × SSC and 0.1% SDS twice for 30 minutes at 56℃ for alpha probe and at 45℃ for zeta probe respectively. After washing, radiolabelled bands were detected by autoradiography using Fuji RX X-ray film for 3-7 days. The band size was determined by reference to Lambda DNA digested with *Hind* III.

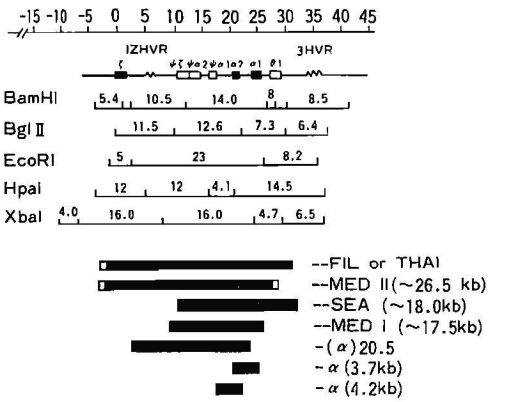

Fig. 1. A Comparison of five types of alpha thalassemia 1 and two types of alpha thalassemia 2 that are present in the patients with Hb H disease. The genes are represented by solid boxes, pseudogenes are shown as open boxes and hypervariable regions as zig-zag lines. The scale is indicated in kilo bases (Kb).

RESULTS

DNA of each of the 41 hemoglobin H patients was extracted and digested with restriction enzyme *Bam* H I, *Bge* II and *Eco* R I. The restriction DNA fragments were fractionated by agarose gel electrophoresis. After blotting on a nitrocellulose membrane, the restriction fragments were detected by hybridization with a ^{32}P labelling alpha globin specific probe. The radiolabelled bands were detected by autoradiography and the size of each band was determined by reference to Lambda DNA digested with *Hind* III. Normal individual (N) produces a restriction fragment of 14.0 kb with *Bam* H I, 23.0 kb with *Eco* R I and 2 restriction fragments of 12.6 kb and 7.3 kb with *Bge* II (Fig. 1, Fig. 2). Patients E and R showed restriction fragments of 10.5 kb, 19.0 kb and 16.0 kb with *Bam* H I, *Eco* R I and *Bgl* II, respectively (Fig. 3a, b, c). The shortened *Bam* H I and *Eco* R I alpha-specific fragments, 10.5 kb and 19. 0 kb

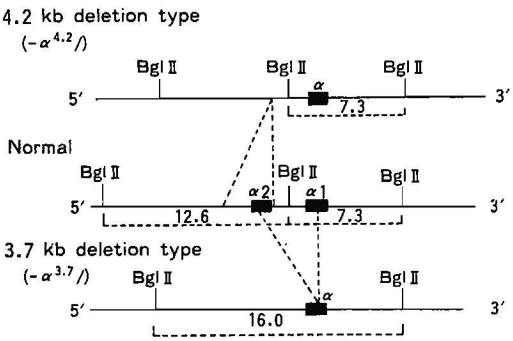

Fig. 2. *Bgl* II restriction endonuclease maps of the area around the alpha globin gene cluster in normal and both alpha thalassemia 2 genotypes.

are shown in Fig. 3 a and b, are characteristic of a single gene deletion which results in an alpha thalassemia 2 haplotype (-α /). To characterize this haplotype, DNA was digested with *Bgl* II, which distinguishes between the rightward deletion (-$\alpha^{3.7}$) and leftward deletion

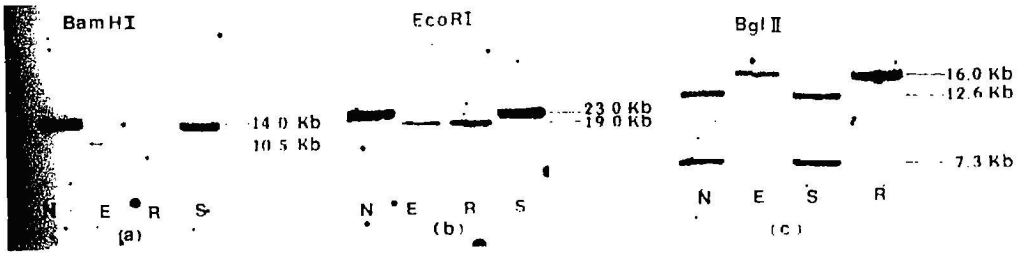

Fig. 3.(a, b, c) Autoradiograph of DNA from normal control (N) and Hb H disease patients E,R and S after digestion with *Bam* HI, *Eco* RI and *Bgl* II and hybridization with alpha globin probe.

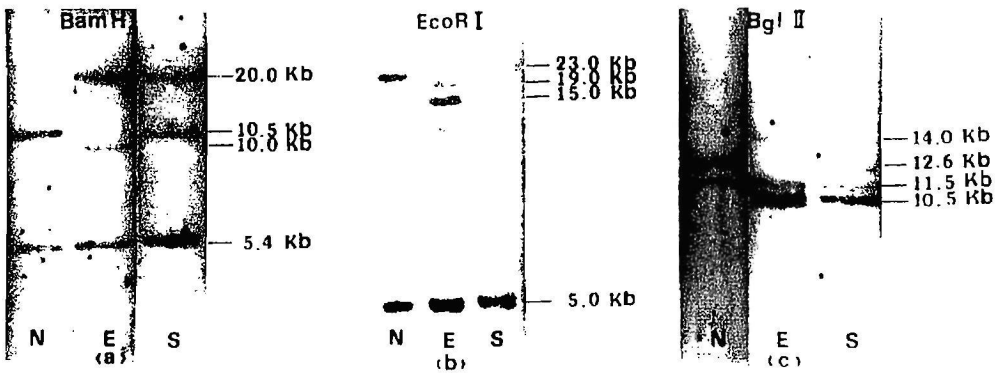

Fig. 4.(a, b, c) Autoradiograph of DNA from normal control (N) and Hb H disease patients E and S after digestion with *Bam* HI, *Eco* RI, and *Bgl* II and hybridization with zeta globin probe.

($-\alpha^{4.2}$). These two types showed 16 kb and 7.3 kb with *Bgl* II alpha specific fragments respectively (Fig. 2). Only 16 kb has been observed in 5 patients (Fig. 3c), including patients E and R. Therefore their genotype was $--/-\alpha^{3.7}$ or $-\alpha^{3.7}/-\alpha^{3.7}$. The remaining 36 patients, including patient S, produced the normal fragments with *Bam* H I, *Eco* R I and *Bgl* II, suggesting that the haplotypes in the patients were the non-deletion type. The genotypes should be $\alpha^{T}\alpha/\alpha^{T}\alpha$ or $--/\alpha^{T}\alpha$. The possibilities, $-\alpha^{3.7}/-\alpha^{3.7}$ and $\alpha^{T}\alpha/\alpha^{T}\alpha$, were ruled out by further examination by hybridization with ^{32}P labelling zeta globin specific probe.

DNA of normal individuals (N) when digested with *Bgl* II and hybridized with zeta specific probe, gives fragments of 12.6 kb (pseudo zeta) and 11.5 kb (zeta) and with *Bam* HI, 10.5 kb (pseudo zeta) and 5.4kb (zeta) (Fig. 4a, c). The size of the zeta fragment varies in normal individuals due to the hypervariable region[10]. Southeast Asia and Mediterranean infants having Hb Bart' s hydrops fetalis syndrome produce none of these normal *Bgl* II specific fragments. In Southeast Asian hydrops fetalis infants (--SEA/--SEA), the remaining zeta gene is present in 10.5 kb fragment digested with *Bgl* II and in 20 kb fragment digested with Bam HI[19]. All of the 41 cases, including patients E and S, have smaller 10.5 kb

Table 1. Alpha and zeta globin specific fragment sizes (kb) and the genotypes in 41 cases of Hb H disease

Subjects	Alpha probe			Zeta probe			Genotypes
	*Bam*H I	*Eco*R I	*Bgl* II	*Bam* HI	*Eco* RI	*Bgl* II	
Normal	14.0	23.0	12.6, 7.3	5.4 , 10.5	23.0 , 5.0	12.6 , 11.5	($\alpha\ \alpha / \alpha\ \alpha$)
Hb H patients (5 cases)	10.5	19.0	16.0	20.0,10.0,5.4	19.0,15.0,5.0	14.0,11.5,10.5	(- - /- $\alpha^{3.7}$)
Hb H patients (36 cases)	14.0	23.0	12.6, 7.3	20.0,10.5,5.4	23.0,15.0,5.0	12.6,11.5,10.5	(- - / $\alpha^{T}\alpha$)

Fragment sizes in kb
Non-deletion = T

Table 2. Hematologic data of two genotypes of Hemoglobin H disease

Genotype	Hb g/dl	Hct %	MCHC g/dl	MCH pg	Hb A_2 %	Hb F %	Hb H %	Inclusion bodies %
Normal (N=12) $\alpha\ \alpha / \alpha\ \alpha$	13.9±0.5	41.5±0.3	33.5±0.7	27.8±0.6	1.6±0.6	0.7±0.1	Not found	Not Found
Deletion (N=5) --/- $\alpha^{3.7}$	9.0±0.9	28.0±0.4	32.1±2.2	21.4±2.1	2.2±0.4	0.9±0.2	9.0±1.5	55.0±9.0
Non-deletion (N=36) --/ $\alpha^{T}\alpha$	8.1±1.1	26.0±0.5	31.1±1.8	22.5±1.6	1.9±0.9	3.1±0.6	17.6±3.4	78.0±11

Hb = Hemoglobin coccentration
Hct = Hematocrit
MCHC= Mean corpuscular hemoglobin concentration
MCH = Mean corpuscular hemoglobin

fragment (Fig. 4c) and 20 kb fragment (Fig. 4a). Therefore all of the 41 patients carried the haplotype of (−−SEA), showing that the genotype of 5 patients, including patient E, is due to the interaction of alpha thalassemia 1 Southeast Asia type and alpha thalassemia 2 rightward type (−−SEA / $-\alpha^{3.7}$).

That of the remaining 36 patients, including patient S, is due to the interaction of non-deletion haplotype with the Southeast Asia type of alpha thalassemia 1 and the genotype was --SEA / $\alpha^{T}\alpha$. To confirm that the remaining 36 patients have a zeta/alpha gene complex intact, *Eco* R I restricted DNA from these patients were hybridized with zeta DNA globin specific probe. The normal 5.0 kb and 23.0 kb which contain zeta gene and 5'− $\psi\ \zeta - \psi\ \alpha\ 1 - \alpha\ 2 - \alpha\ 1 - 3'$ complex in these patients were shown (Fig. 4b). The data concerning the alpha and zeta globin specific fragment size (kb) and the genotype in all 41 cases of Hb H disease are summarized in Table 1.

Hematologic studies

The mean and standard deviation (S. D.) of hematologic data of two genotype of Hb H dis-

ease were shown in Table 2. The level of Hb H, Hb F and inclusion bodies are significantly higher ($P<0.001$) in the patients of genotype--SEA/ $\alpha^T\alpha$ than ——SEA/— $\alpha^{3.7}$. The average hemoglobin concentration and the percentage of Hb A_2 are lower in the non-deletion type group than in the deletion type group but these values are not statistically significant. These data suggest that patients with the non-deletion type are more severely anemic than those with the deletion type .

DISCUSSION

All of the patients with Hb H disease in this study had an extensive deletion in both α 1 and α 2 globin genes on one chromosome. DNA mapping patterns in all cases demonstrated one type of alpha thalassemia 1 with the same molecular defect (——SEA /) (Fig. 1). However, two different alpha thalassemia defects were found in the alpha globin gene locus on the second chromosome 16. First we found 5 patients in which the defect resulted from a deletion of 3. 7 kb of DNA. Therefore, the genotype for this group is ——SEA/— $\alpha^{3.7}$. The second defect, present in 36 patients, was non-deletional because gene maps were normal showing the $\alpha^T\alpha$ /haplotype. Genotype for Hb H disease in this group was ——SEA/ $\alpha^T\alpha$ (Table 1).

We found that the non-deletion type of the Hb H disease was more common (88%) in the northern part of Thailand. This trend is different from central Thailand (Bangkok) where the non-deletion Hb H is rare. The molecular basis for the non-deletion type has been described with different mechanisms[13]. There are some non-deletional alpha thalassemias which resulted from a single base or an oligonucleotide mutation, affecting either α 2($\alpha^T\alpha$) or α 1 ($\alpha\alpha^T$). Mutation of the dominant α 2 gene produces a more severe phenotype than those affecting the α 1 gene[20] because the α 2 globin gene produces 1.5-2.8 times more mRNA than the α 1 globin gene[21]. Many types of non—deletion mutations that cause thalassemia in Southeast Asia were discovered[22]. They include such types as a termination codon mutation at α 2(142 TAA→CAA)called hemoglobin Constant Spring[23], Hb Quong Sze, an extremely unstable α 2 globin structural variant (125 Leu → Pro)[24,25] and hemoglobin Suan-Dok, an unstable α 2 globin structure (109 Leu → Pro)[26]. There is strong genetic and biosynthetic evidence that the Hb CS mutation results in an overall defect in the alpha chain in the affected red blood cell[27] and that its interaction with the severe alpha thalassemia gene (alpha thalassemia 1) causes about half the cases of Hb H in Southeast Asia[28,29]. Laig, M. *et al.*[30] showed that Hb CS occurs at a high frequency in the North and Northeast of Thailand. In the North the α^{CS} gene was found at a frequency of 0.033, and between 0.05-0.06 was observed in the Northeast. Hemoglobin CS is unstable and present in a small amount in the red blood cell. It is difficult to detect it with a conventional electrophoretic method, especially if other slow-moving hemoglobins e. g., Hb E, are present. Although Hb CS is very common in the North, other α 2 globin gene mutations may also be present in Thailand. Additional experiments involving hybridization of genomic DNA with an oligonucleotide specific probe for Hb CS[31], gene cloning and nucleotide sequence may solve this problem.

We compared the clinical and hematologic phenotype of the two groups of patients with Hb H disease resulting from these different molecular mechanisms. Because of the fluctuation of Hb levels during the development of hemolytic crisis, we compared the mean values of repeated determination carried out during follow-up. Our results clearly show that the Hb H disease phenotype produced by the combination of the alpha thalassemia 1 (——SEA) with

the non-deletion ($\alpha^{T}\alpha$) determinants is more severely anemic than that resulting from the deletion genotype (−−SEA/− $\alpha^{3.7}$). Characterization of these defects at the molecular level will facilitate a more accurate correlation of phenotype with genotype in patients with Hb H disease. Thus it is becoming possible to use this information to provide useful genetic counseling and prenatal testing service for those at risk of producing offspring with the most severe form of alpha thalassemia.

Note: Informed consent was obtained from all subjects and the guideline for human experimentation in the author's institution was followed in the conduct of the clinical research.

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