CHAPTER IV

DISCUSSION

Alpha-thalassemia is the most prevalent hemoglobinopathy in Thailand. Population studies of the various anomalies of the alpha globin chain production and structure on the protein level are problematic because the available methods do not differentiate between the various forms of alpha-thalassemia. The methods employed in previous studies were the determination of alpha/beta globin synthesis ratio and the concentration of Hb Bart's.

The first method is not suitable to identify alpha-thalassemia traits, because the alpha/beta globin chain production ratio is significantly reduced in many alpha-thalassemias, and there is a marked overlap between alpha-thal-1 and alpha-thal-2,and between alpha-thal-2 and the normal ratio(29).

The second method is unsuitable for the detection of alpha-thalassemia for several reasons , such as: it can be carried out only in the newborn, the ranges of this value in alpha-thal-1 and alpha-thal-2 probably overlap, not all infants with alpha-thal-2 have detectable amounts of Hb Bart's, and the newborn infants carrying the Hb Constant Spring gene have variable Hb Bart's concentration (29,30).

For the detection of nondeletional alpha-thalassemia, especially the common Hb Constant Spring,gel electrophoresis was usually employed,

but there are also problems: Hb Constant Spring is unstable and present in small percentages. Furthermore, it remains near the origin in gel electrophoresis and is therefore difficult to detect, especially if other slow moving hemoglobins, e.g. Hb E,Hb A_2 are present (30,31).

Because of these methodological difficulties, previously published data on the prevalence of the alpha-thal traits are unreliable. The genetic defects of alpha-thalassemia were first studied at the DNAlevel by Orkin in 1979 (18). The Southern blotting hybridization technique was used to study the molecular basis of Hb H disease. Alpha-thal-1 and alpha-thal-2 were shown to have different patterns of RFLP markers, and two different molecular defects causing alpha-thal-2 were discovered: the leftward and the rightward type of deletion. These findings were confirmed by several investigations (17,32,33), and this standard DNA mapping technique was first applied for diagnostic purposes in 1982 (34,35).

Nondeletional alpha-thalassemia defects were discovered employing the DNA sequencing method of Maxam and Gilbert (36), and the methodology of oligonucleotide hybridization was developed and successfully used in the diagnosis of single base exchanges in 1983 (37).

At present, the DNA hybridization technique is widely used for the study of the molecular basis in many genetic diseases,and the study of hemoglobinopathies is the field where these methods have been most

successfully employed. This is particularly true for the clarification of the molecular basis of the alpha-thalassemias:

Alpha-thal-2, the most common and universally distributed form is caused by non homologous crossing over: the misalignment between two strands of DNA from two separate chromosomes during meiosis leads to deletion of one of the two alpha globin genes. One type of deletion involves the homologous regions 3' to the pseudoalpha gene on one chromosome and 3' to the $alpha_2$globin gene on the other and leads to deletion of 4.2 kb of DNA, whereas a second type involves the homologous segments containing the $alpha_2$ and $alpha_1$globin genes and leads to the deletion of 3.7 kb of DNA. These two forms are referred to as the leftward and rightward deletions. The outcome of these unequal crossovers is the generation of new chromosomes containing either a single alpha globin gene or triplicated alpha globin genes (38,39). All four possible chromosome types have been identified in various population where alpha-thalassemia occurs in high frequency. The leftward type of deletion is prevalent in Oriental individuals (40) and also occurs in low frequency in Saudi Arabia (39), but was not encountered in Mediteraneans or American blacks (17). The rightward deletion has a worldwide distribution among all racial groups (Orientals, Mediteraneans, and blacks) (26,41,42,43). The single alpha globin gene chromosomes are much more common than the triple alpha globin gene chromosomes (44,45). Greek Cypriots exhibit the highest frequency of alpha-thalassemia among white people and also have a relatively high frequency of triple alpha globin gene chromosomes (28).

It is well documented that the common forms of alpha-thal-2 have reached very high frequencies through a process of natural selection by malaria. The protection exerted against plasmodium falciparum has been assumed to explain the altitude dependence and frequency heterogeneity in the regions of high malaria endemicity. Provisional surveys in several population have indicated that the common rightward deletion has been produced by at least three different crossover events. The crossover typeI is observed in all populations,typeII in Jamaicans, black Africans, Saudi Arabians, Indians, Southeast Asians and Mediteraneans, typeIII in Melanesians and Polynesians (23,46,47)

The alpha-thal-2 may be caused by a number of nondeletional effects, most of them point mutations that reduce the expression of alpha globin genes. The most frequent type in Southeast Asia is Hb Constant Spring.The inheritance of such a mutation in combination with alpha-thal-1 or alpha-thal-2 deletion leads to a variable clinical pattern.

Several other types of deletion involve both alpha globin genes of a single chromosome ; these are the alpha-thal-1 mutations. The extent of these deletions has been defined by restriction endonuclease mapping, but the mechanism of their formation is not yet obvious. The superscript SEA or MED refers to the common alpha-thal-1 defects in Southeast Asian and Mediteranean subjects.The severe alpha-thalassemia syndromes (Hb H disease and Hb Bart's hydrops fetalis) which are prevalent in Southeast Asia are due to the alpha-thal-1 haplotype and the

rightward $-\alpha^{3.7}$deletion (Hb H disease $--^{SEA}/-\alpha^{3.7}$and Hb Bart's hydrops fetalis $--^{SEA}/--^{SEA}$).

A novel and potentially important deletion in the alpha globin gene cluster was characterized in two patients of North European origin with an unusual combination of acquired Hb H disease and mental retardation. This phenotype was apparently caused by the genetic combination of a rightward deletion chromosome and a deletion of the whole alpha globin gene cluster on the other chromosome. Another patient with mental retardation inherited a mutation from his father that inactivated both alpha globin genes ; this mutation was apparently of the nondeletional type (48).The combination of these unusual mutations and mental retardation might be coincidental or could imply the presence of DNA sequences that influence mental development near the alpha globin gene cluster (46). But there was no mental retardation in Hb H disease patients found in Thailand and the Philipines carrying $--^{THAI}$ and $--^{FIL}$ deletions both of which removed the entired alphaglobin gene cluster.

As in many other populations, alpha-thal-2 is the most common defect in Thailand, and the rightward type ($-\alpha^{3.7}$) is more prevalent than the leftward type ($-\alpha^{4.2}$). The crossover typeI is predominant and there are only few examples of typeII. The common alpha-thal-1 in northern Thailand is the $--^{SEA}$ type and the new type $--^{THAI}$ seems to be very rare. The common haplotypeI of $--^{SEA}$ is present in six

variants. The other variants (II-VII) differ only at the 5' breakpoint (19).

In the present study, standard DNA mapping techniques were used to study the molecular basis and the prevalence of alpha-thalassemia in Northern Thailand. Out of 112 samples, 33 (approximately 30%) had a single alpha globin gene deletion (alpha-thal-2). There were 25 heterozygotes and 8 homozygotes (Table III.1). Digestion with Bgl II identified four heterozygotes with the leftward type (Table III.2). All 29 mutations of the rightward type were subtypeI, according to Higgs et al (23) (Table III.2). All 13 cases of alpha-thal-1 were due to deletions of the common Southeast Asian type, and two of these showed triplicated zeta globin genes on one chromosome (J17,S18). Subtyping according to Winichagoon et al (19), revealed subtypeII in two cases, the others were subtypeI(Table III.3). These results agree with a recent study which showed that the subtypeI is the predominant type of alpha-thal-1 and alpha-thal-2 deletion in northern Thailand. One of the alpha-thal-1 mutations occurred in mixed heterozygosity with the rightward deletion, a combination expected to result in Hb H disease (P22 in Table III.1). The deletion of the entire zeta alpha globin gene cluster which was found in two cases of Hb H disease in central Thailand (25) was not found in this study. The Rsa I polymorphic site was found on 54 of 116 nonthalassemic chromosomes and in 3 of 34 alpha-thalassemic ones (Table III.8).

In the present study,an unusual mutant was detected in sample MT1. The sample was characterized by the normal state of alpha globin gene loci in the Bam HI and Bgl II digestion (Table IV.1) (Figure III.12), but with an anomalous 7.5 kb band representing the pseudozeta gene fragment. In study of the Rsa I polymorphic site, this sample showed a 1.5 kb fragment as expected in the case of a R+ rightward alpha-thal-2. These results suggested that this sample had two defects in the zeta alphaglobin gene cluster.One of these was located in the zeta or pseudozeta gene fragment. The 7.5 kb fragment may have been caused by a point mutation that generated a new cleavage site for Bam HI or by a partial deletion. The second defect was a point mutation in the alpha$_2$globin gene which generated a new Rsa I cleavage site and resulted in an anomalous 1.5 kb fragment. This MT1 sample was designated as $\alpha\alpha/\alpha\alpha^m$ where m stands for "mutant".

Table IV.1 : The RFLP of the MT1 sample in comparison with the normal and the rightward alpha-thal-2 (R+) haplotype.

Enzyme and Probe	Fragment length (kb)		
	MT1($\alpha\alpha/\alpha\alpha^m$)	($\alpha\alpha/-\alpha$ R+)	($-\alpha/-\alpha$ R-)
Bam HI /$\alpha_2$probe	14.1	14.1	14.1/10.5
Bam HI /ζ probe	6/7.5/8-11	6/8-11	6/8-11
Bgl II /$\alpha_2$probe	12.5/7.3	12.5/7.3	16.0
Rsa I /$\alpha_2$probe	2.4/1.75/1.5	2.4/1.75	1.5

The results of the present study confirm a remarkably high frequency of alpha-thalassemias in the northern part of Thailand, with a combined frequency of the rightward and leftward deletion of 33/224 = 0.147. Alpha-thal-1 ($--^{SEA}$) was found on 13 of 224 chromosomes, giving a frequency of 0.058. The expectations for the clinically important alpha-thalassemia syndromes were calculated from the observed haplotype frequencies (Table III.6): Hb Bart's hydrops fetalis (genotype --/--) 0.0034 or approximately 1 : 290, Hb H disease (genotype--/-α) 0.021 or approximately 1 : 48. This means in four of one thousand fetuses are at risk to die of Hb Bart's hydrops fetalis, and 20 newborn infants are expected to have Hb H disease.

In comparison with the recent study of alpha-thalassemia in northern Thailand (53), the prevalence of alpha-thal-2 in the rural areas of Chiang Mai province is twice as high in the present study (Table IV.2). Both studies indicate a much higher frequency of alpha-thal-1 in northern Thailand in comparison with Bangkok and central Thailand. These findings are in agreement with the observed high prevalence of Hb H disease and Hb Bart's hydrops fetalis in northern Thailand.

Table IV.2 The prevalence of deletional alpha-thal-2 and alpha-thal-1 in different areas of Thailand

Population	Haplotype		Reference
	Total	α-thal-2 α-thal-1	
Central Thailand (Bangkok)	812	0.0825 0.0185	(30)
Northern Thailand			
(central area)	212	0.0991 0.0236	(49)
(rural area)	224	0.1830 0.0580	present study

The present study confirms that alpha-thal-2 deletion is the common deletional type in Thailand, and that the rightward subtypeI is predominant (Table III.2).

Most of the alpha-thal-1 chromosomes are subtypeI (11/13) with few of subtypeII (2/13). This supports the proposition of Winichagoon (19) that the common of alpha-thal-1 deletion in Thailand is $--^{SEA(I)}$ (Southeast Asian deletion, subtypeI).

The RsaI polymorphic site which is present at the 5'end of the Z-box region is found more frequently on nonthalassemic chromosomes

than on $-\alpha^{3.7}$ thalassemic ones. A similar distribution has been observed in Laos, Cambodia and Vietnam (47).

As mentioned above,the misalignment of a crossing-over between two homologous chromosomes may result in an alpha-thal-2 deletion chromosome and a chromosome with triplicated alpha globin genes. Accordingly, alpha globin gene triplications should be present in population where alpha-thal-2 is found. In the present study, however, no alpha globin gene triplications were detected. If the hypothesis of malaria protection in favor of alpha-thal-2 is correct, this may explain the discrepancy.Whereas the low production of alpha globin chains in alpha-thal-2 is expected to create an intraerythrocytic environment which is unfavorable for the propagation of Plasmodium falciparum,this effect is probably absent in the case of a triplicated alpha globin gene. Therefore, it is not surprising to find many more alpha-thal-2 chromosomes with single alpha globin genes than chromosomes with triplicated alpha-globin genes in population under strong malaria pressure.In contrast, a low, but similar frequency of these two anomalies is expected in areas where malaria is absent. This expectation was confirmed in a recent study in Germany,where alpha globin deletions and triplication do not seem to be extremely rare (54).

Another question concerns the high frequency of homozygotes for alpha-thal-2 in the present study. In contrast to a recent study of alpha-thalassemia in northern Thailand (50),there was not only a much higher frequency of alpha-thal-2,but also an unusually high number of

alpha-thal-2 homozygotes in the results presented here: among the 112 probands,no less than 8 of the total of 33 subjects with alpha-thal-2 were homozygotes. The high frequency of homozygous alpha-thal-2 chromosome was particularly pronounced in three of the four districts: 5/18 in Smeang, 1/5 in Mae Tang and 2/7 in Prou (Table III.1). Table III.7 shows a disagreement between the observed distribution and the Hardy-Weinberg expectation. In the total sample, 3.8 alpha-thal-2 homozygotes were expected, and 8 were found, compared with 33.5 heterozygotes expected and only 25 found. One of the factors that causes an excess of homozygotes in a population is inbreeding. In comparison with an equilibrium population with the distribution p^2, 2pq, q^2 of the three genotypes at a dimorphic autosomal locus (AA, Aa, aa),an inbred population has the corresponding genotype frequencies of p^2+Fpq, $2pq(1-F)$, q^2+Fpq , where F is the coefficient of inbreeding. The calculation of F from the data in the present population sample shows that a coefficient of inbreeding of 0.254 is required to explain the deviation from equilibrium. This value for F is far higher than any coefficient of inbreeding ever observed in human population. Therefore, it is concluded that inbreeding alone cannot explain the high frequency of alpha-thal-2 homozygotes. It is more likely that chance effects, inbreeding and an unrecognized population stratification cause the extreme deviation from Hardy-Weinberg equilibrium.

The oligonucleotide hybridization technique was used to detect Hb Constant Spring, the common type of nondeletional alpha-thal-2 in Thailand. The mutant oligonucleotide probe was homologous to the 5'-3'

strand of the mutant gene in general, but the normal probe was homologous to the 3'- 5' strand to avoid a G-T mismatch. This is necessary because several studies have shown that G-T and A-G mismatches are more stable than A-C and T-C mismatches (51,52). To obtain the maximum destabilizing effect, the normal probe was synthesized homologous to the 3'-5'strand to produce an A-C mismatch instead.

Four of 112 samples showed heterozygosity for the Hb Constant Spring gene. Two were genotypically combined with an alpha-thal-2 haplotype, a rightward deletion and a leftward deletion (Table III.5). The frequency in the present sample is different from the recent study (Table IV.3).

Table IV.3 Distribution of the Hb Constant Spring (Hb CS) gene in different areas of Thailand.

Population	No. examined	Hb CS frequency
Northern Thailand :		
1. Chiang Mai, urban area	151	0.0331
2. Tak Province	32	0.0156
3. Rural area of Chiang Mai	224	0.0178
Northeastern Thailand:		
4. Khonkaen area	17	0.0588
5. Ubol Rachathani	45	0.0556

Population 1, 2, 4 and 5 from (53); Population 3: present study.

The frequency of the Hb Constant Spring gene in the rural area of Chiang Mai was only half of that in the central area, but similar to Tak.The high frequency in Northeastern Thailand corresponds with that of a beta-thalassemia mutation: Hb E. The recent study showed a correlation between the high frequency of Hb CS and Hb E; this indicates that these two mutations were selected for by the same environmental factor (53).

Hb CS may be classified as alpha-thal-2 because of the reduced availability of functional alpha globin chains. When combined with alpha-thal-1 the Hb CS gene causes Hb CS-Hb H disease, a syndrome similar to Hb H disease. The expected frequency of this syndrome in northern Thai was calculated from the haplotype frequencies as 0.002 or approximately 1:500.

Previous studies of the human alpha globin gene cluster showed that there was a considerable number of different genetic rearrangements in this DNA region. In the Thai population, there were at least 18 different types of chromosomes carrying independent alpha-thalassemia mutations (19). In the present study, 7 different types were detected:

1. alpha-thal-1, subtypeI.
2. alpha-thal-1, subtypeII.
3. alpha-thal-2, rightward subtypeI with Rsa I polymorphic site.
4. alpha-thal-2, rightward subtypeI without Rsa I polymorphic site.
5. alpha-thal-2, leftward type.
6. triplicated zeta globin gene type.
7. Hb Constant Spring type.