DISCUSSION

Recombinant plasmid first hit the scientific headlines when it was constructed by Cohen S.N. et al. in 1973 (50). The important properties of plasmids are as follow: they are capable of extrachromosomal replication in bacterial cells, they contain a selectable marker (an antibiotic resistance gene), and they present suitable sites for cloning. These properties gave a great adventage in development of recombinant DNA technique. Complementary DNA (cDNA) was inserted to the plasmid vector to construct the recombinant plasmid and introduced into bacterial host cells for the purpose of the clone-DNA amplification.

In the present study, pEMBLα_2 and pEMBLζ recombinant plasmid were used as the source of complementary globin specific DNA. They were constructed by Wilson and Maniatis by ligation of the α_2- and ζ -globin specific fragments to the pEMBL vectors and transformed into E.Coli LK111. The α_2- and ζ -specific fragments can be used to study the structure of human α-globin gene cluster by the hybridization technique. To obtain the α_2- and ζ -fragments, the pEMBLα_2 and pEMBLζ plasmids were isolated from E. Coli by alkali lysis procedure. It was found that an important chore in laboratories working with recombinant DNA is the purity of plasmid; it is essential to eliminate chromosomal DNA and low molecular weight contaminants such as RNA, oligonucleotides, and proteins. For this reason, the cesium chloride gradient procedure was commonly used for purification in which the

close-circular plasmid DNA can be seperated from the linear DNA (chromosomal DNA and nicked circular DNA) and the RNA in the equilibrium gradient centrifugation. This procedure is highly efficient but yields a low recovery and is time-consuming (36-48 hours). Hence, in this study, it was used a more simple and rapid procedure for purification of plasmid by gel filtration chromatography (Sephacryl S-1000, Pharmacia). Plasmid DNA which is purified by this procedure, can be digested by restriction enzymes and produce the pure DNA probe for analysis of human genomic DNA by hybridization technique.

The specific DNA fragments which can be isolated in a pure form by recombinant DNA technology has made possible the detailed analysis of gene structure, and when this knowledge is applied to the study of genetic diseases, it has provided a wealth of information about their molecular basis at the DNA level. There has been much progress in understanding the molecular defects reponsible for the thalassemia disorders and other types of hemoglobinopathies (51).

The fundamental principle of most methods of DNA analysis is the molecular hybridization of a small amount of pure labelled DNA probe to a much larger amount of genomic DNA. The probe and target DNA are first denatured to be single-stranded and then, under the appropriate experimental conditions, they are allowed to reassociate with one another to form double-stranded labelled molecules. In this way, the probe only hybridizes to a perfectly-matching genomic DNA sequence (complementary sequence) and the unhybridized and mismatched probe is removed by washing. The remaining probe-genomic DNA hybrids

are identified by autoradiography.

In the present study, the DNA mapping technique was used to determine the coinheritance of α-thalassemia with α-globin gene deletion in the homozygous β-thalassemia subjects and for studying a correlation between α-globin gene status and the phenotypic manifestation of the subjects.

The homozygous state for β-thalassemia usually results in thalassemia major, a severe disorder necessitating regular blood transfusion from early infancy. However, some homozygotes are less severely affected with a milder non-transfusion-dependent disorder referred to as thalassemia intermedia (6). It was interesting to determine what is the reason for their milder phenotypes. The coinheritance of α-thalassemia has been found to be one obvious method in balancing the globin chains which leads to less severe phenotypic manifestations.

There are two common types of deletional α-thalassemia: α-thalalassemia 1 and α-thalassemia 2. The former is due to deletion of both of the duplicated α-globin genes and results in no production of α-globin chain. The latter is due to deletion of one α-globin gene which leads to decreased synthesis of α-globin chain.

Out of 36 samples, 24(67%) had normal α-globin gene, 8 (22%) had a single α-globin gene deletion (α-thalassemia 2). There were 7 samples of heterozygotes and only one sample with homozygote. Identification of the deletion types revealed that all samples with α-thalassemia 2 were due to the rightward type ($-\alpha^{3.7}$/and

$-\alpha^{3.7}/-\alpha^{3.7}$) of deletion. The remaining 4 samples (11%) had two α-globin gene deletions (α-thalassemia 1).

Mean ($\bar{X}$) and standard deviation (S.D.) of the hematologic and clinical presentation of subjects were compared according to the α-globin genotypes (Table III.4). The variable range in Hb concentrations among three groups of genotypes failed to show significant difference between a group of subjects with normal and deleted α-globin genes. Factors that may be responsible for the Hb concentration, besides the anemia status, are the living standard of subject's parents (possibly being the cause of nutritional deficiency) and infections. Hb A_2 levels were found significantly higher ($p<0.05$ and $p<0.01$, respectively) in both groups of either one or two α-globin gene deletion than a group of normal α-globin gene. These results agree with many previous reports in which the high levels of Hb A_2 always occur in patients who are homozygous β-thalassemia and also carry one or more α-thalassemia genes(16). It can be explained that in the heterogeneous red cell population in β-thalassemia homozygotes, they consist either of cells with high Hb F content and low δ chain synthesis or of cells with low HbF and higher δ chain synthesis. The latter group of cells is usually destroyed in the bone marrow, because they lack sufficient chain to bind excess α chains. In the presence of one or more α-thalassemia genes, globin chain imbalance should be reduced. Therefore it was possible for this cell population to appear in the peripheral blood and cause an increase in absolute amount of HbA_2.

From the result of hematologic and clinical study (Table III.4), the effect of α-thalassemia on the severity of homozygous β-thalassemia did not seem to be consistent. The heterozygous α-thalassemia 2 (--α/αα) showed no consistent modification of the common severe clinical picture. The same phenomenon was also found in subjects with α-thalassemia 1 heterozygote except in the view of disease onset and transfusion dependence. The deletion of two α-globin genes seemed to slightly modify the phenotype expression of subjects by delay of clinical presentation and onset of transfusion dependence.

In consideration of each subject who carried the heterozygous state for α-thalassemia 1 gene (subject No.33-36), there were two subjects which recieved a regular blood transfusion (8-11 times/year); one had a moderate rate (6 times/year) and another one had a very low rate of blood transfusion. The finding suggested that the coexistence of α-thalassemia in these subjects might delay the clinical manifestation but could not modify the common severe clinical picture.

The incidence of α-thalassemia gene was observed in two groups of subjects with homozygous β-thalassemia, one with the clinical phenotype of thalassemia major and the other with the phenotype of the intermediate forms (Table III.5). The similar percentage (30 and 37.5%) in the incidence of α-thalassemia between two groups of subjects can be use as a basis for judging the influence of coinheritance of α-thalassemia . Distribution of the coinheritance of α-thalassemia in both two groups of subjects made it

possible to say that the coinheritance of α-thalassemia, which is due to α-globin gene deletion, did not show the ameliorating effect on the severity of homozygous β-thalassemia in the group used for this study.

The results seemed to be different from the previous studies in Cyprus and Sardinia. The investigation of α-globin gene in Cypriot population (5) reported a significant difference in the incidence of α-thalassemia between two groups of subjects with homozygous β-thalassemia; there were 4/30 (13%) of subjects with thalassemia major and 14/27 (52%) of subjects with thalassemia intermedia. The same studies in Sardinian population (3,25,52) showed a different result. The incidence of α-thalassemia in two groups of subjects were 44/109 (40%) of subjects with thalassemia major and 29/50 (58%) of subjects with thalassemia intermedia.

Comparison between these two observations in different ethnic groups. It had been found that β^+-thalassemia mutation predominated in Cyprus whereas in Sardinia, β°-thalassemia mutation was the most common form (53). It seemed that the molecular defect of β-gene was an influential factor in which the study in Cypriot population showed a positive effect whereas in Sardinia population, the effect was much less.

In Thailand, a recent study of β-globin gene clusters in Thai homozygous β-thalassemic patients revealed that there were four common mechanisms which cause β-thalassemia in Thailand, including Bangkok and Chiang Mai. The study can identify over 80% of

β -thalassemic mutations present in Thailand. The most common mutation is a four-nucleotide deletion between codons 41 and 42 of the β -globin gene. This deletion shifts the reading frame, resulting in an in-phase terminator at codon 59. The others are : 1). the nucleotide substitution (C ⟶ T) at position 654 of IVS-2, which generates a new GT dinucleotide that forms an apparent donor-like splice sequence; 2) the nonsense mutation at codon 17 of the β -gene; 3) the insertion of A between codons 71 and 72. All these mutations lead to β°-thalassemia in which no β -globin chain is synthesized (54).

The finding might be the reason for explaining the present observation. Because of the β°-thalassemia homozygotes, the clinical severity was strong and could not be modified by the less imbalance of chains caused by α-thalassemia.

However, further study of individual subjects, including the coinheritance of α-thalassemia associated with the β -gene mutation, will provide more information to confirm the evidence found in the present investigation.

The diagnosis of homozygous β -thalassemia or thalassemia major has in the past been associated with expectation of an early death. The enhanced understanding of the clinical pathophysiology of thalassemia, coupled with advances in molecular biology of this disease, have provided better approaches to treatment and management.

The prevalence of homozygous β -thalassemia in Thailand makes a major public health problem that consumes a large portion of the medical resources of our country. Identification and counselling of

the couples at risk of giving birth to children carrying two anomalous genes has been a first approach to stopping the transmission of this disease. Prevention of such couples from having a family causes this approach to have a low probability of success. The introduction of prenatal diagnosis is another approach to reducing this problem. The present study tries to define a factor which can modify the severity and prolong survival of homozygous β -thalassemia patients. Recognition of the existence of this type of disorder gives hope for carriers of these genes and provides evidence of a basis for such a program. Further studies in getting more evidence to confirm the present work will make it possible to predict the severity of the disorder which the child might have. If the fetus carries genes that would produce severe disorder, a decision could be made as to the termination of the pregnancy.