III. RESULTS

III.1 Isolation and purification of the pEMBL α_2 and pEMBL β plasmids.

From the alkali lysis procedure, the plasmid DNA was extracted including some chromosomal DNA and the low molecular weight contaminates such as RNA, oligonucleotides and proteins. This crude preparation was purified by using the Sephacryl S-1000 gel filtration chromatography. The elution profile corresponding to plasmid preparation appeared in two peaks as shown in Figure III.1. Each eluted fraction from the column was identified by agarose gel electrophoresis. The electrophoretic analysis corresponding to the absorption peaks showed that the first peak was the plasmid DNA and the second peak was very large amounts of RNA (47,48). In addition, it was possible to find some chromosomal DNA contaminations appeared in the front part of the plasmid peak. They could be eliminated from the plasmid peak by discarding the contaminated fractions after electrophoresis examination. Thus it was obtained a plasmid preparation free from measurable amounts of chromosomal DNA.

III.2 Preparation of α_2 - and β -specific fragments

The purified plasmids were digested with specific restriction endonuclease enzymes, pEMBL α_2 was digested with PstI, giving the 1.5

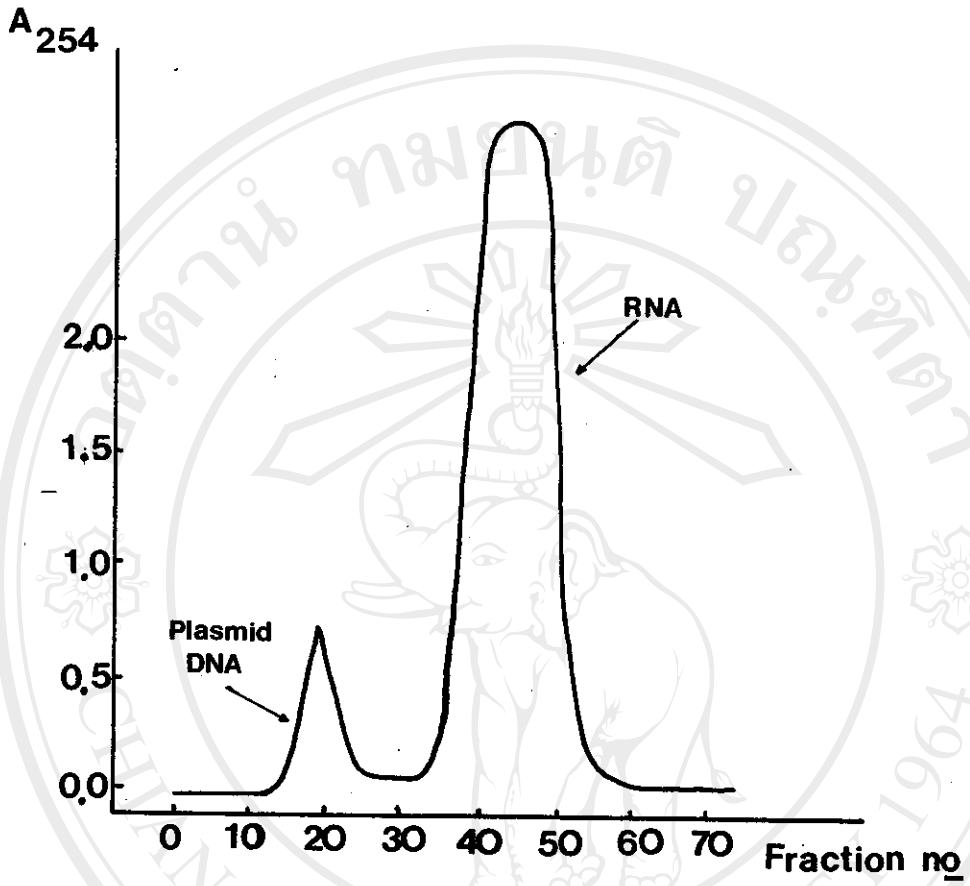

Figure III.1 Elution profile of plasmid purification on Sephacryl S-1000 column chromatography.

kb fragment seperated from the pEMBL vector. pEMBLζ was digested with Pst I and Hind III restriction enzymes, giving the 400 bp fragment.

The α_2- and ζ-specific fragments were obtained by low melting agarose gel electrophoresis and phenol extraction. The electrophoresis pattern of these two fragments is shown in Figure III.2.

III.3 Preparation of human DNA

The DNA samples were isolated from β-thalassemic blood, yielding about 40-80 ug/ml blood. The quality of the whole DNA samples was examined by electrophoresis. The good qualitative sample will give a big band of high molecular weight DNA without smear pattern of the shearing fragments. The ratio of absorbance A260/A280 were also examined. The values range from 1.70-1.92.

The DNA was completely digested with either EcORI or BglII. The complete digestion will give the sharp smear pattern.

III.4 Preparation of ^{32}P labelled DNA probes.

α_2 - and ζ - fragments were labelled with α-^{32}P dCTP by hexaprimer DNA labelling method. The percentage of incorporation was determined by using Sephadex G-75 microcolumn and blue dextran/phenol red mixed dye as a marker. The blue dextran fraction contains the labelled DNA probes and the red fraction contains the free radioactives. Activity of each fraction was detected by hand-held

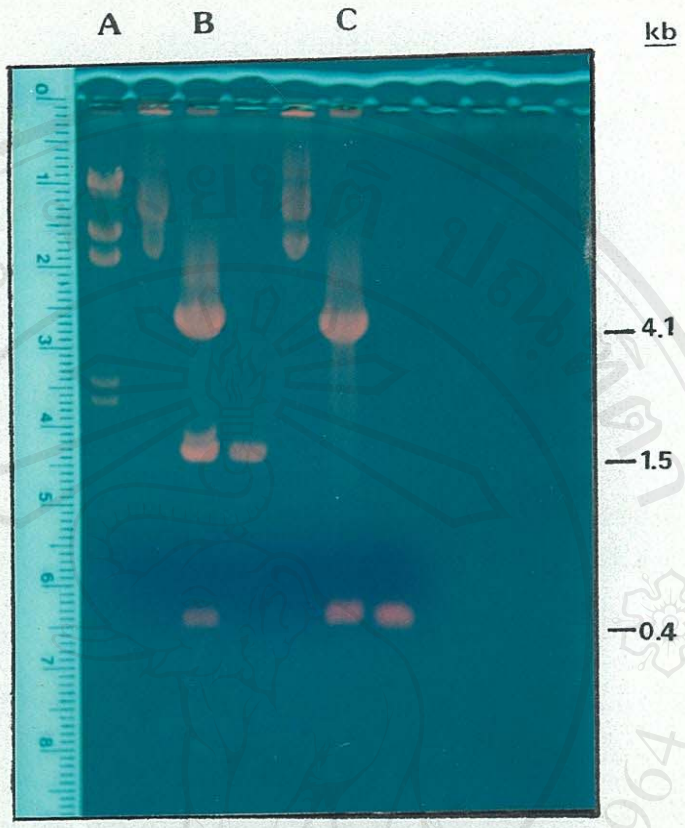

Figure III.2 Agarose gel electrophoresis for demonstration of α_2- and ζ-globin specific fragments.

A) λ Hind III markers

B) pEMBLα_2 plasmid : showing the digested pattern and isolated α_2 -globin specific fragment.

C) pEMBLζ plasmid : showing the digested pattern and isolated ζ -globin specific fragment.

Geiger counter and the percentage of incorporation was calculated as below :

Percentage of incorporation =

$$\frac{\text{activity of incorporated DNA probe (blue fraction), cps} \times 100}{\text{total activity (blue + red fractions), cps}}$$

The acceptable incorporation percentage should be at least 70%.

III.5 DNA mapping in the subjects

The α-globin gene deletions were determined by the EcORI enzyme digestion and the digest was hybridized with α_2- or ζ-specific probes. The restriction map and the restriction fragments which were obtained from hybridization with these probes are shown in Figure III.3 and Table III.1.

DNA from subjects who were carriers for α-thalassemia 2 was further investigated by digestion with the Bgl II enzyme and hybridized with α_2- specific probe. This was used to determine the type of α-thalassemia 2, rightward type ($--\alpha^{3.7}/$) or leftward type ($/--\alpha^{4.2}$). The Bgl II restriction map and the fragment size in various genotypes are shown in Figure III.4 and Table III.2.

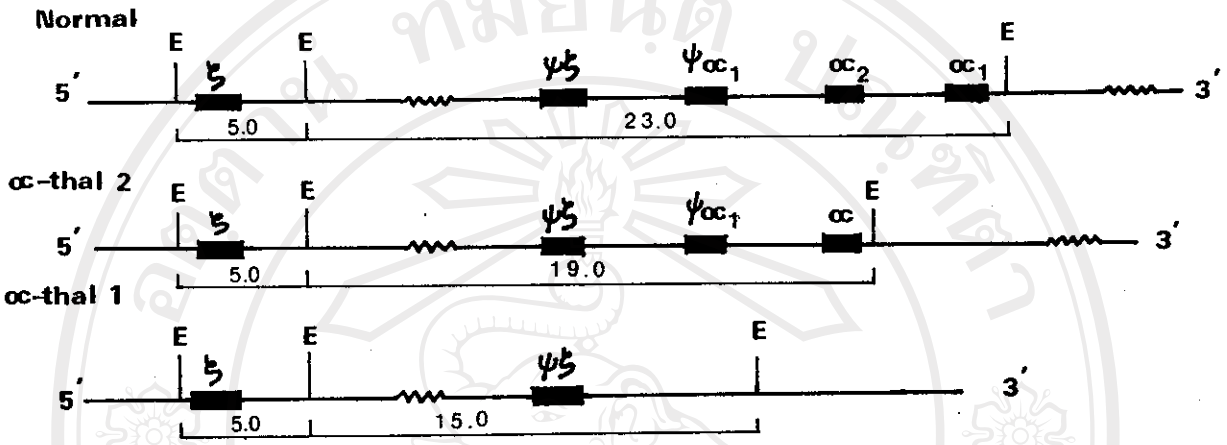

Figure III.3 EcoRI restriction endonuclease maps of the area around the α-globin gene cluster in normal, α-thalassemia 2 and α-thalassemia 1.

Table III.1 The EcoRI restriction fragment size after hybridization with α_2 - or ζ -globin specific probe

Genotypes	EcoRI α_2-gene restriction fragment (kb)	EcoRI ζ-gene restriction fragment (kb)
αα/αα	23	23,5
_α/αα	23,19	23,19,5
_α/_α	19	19,5
__/αα	23	23,15,5
__/_α	19	19,15,5

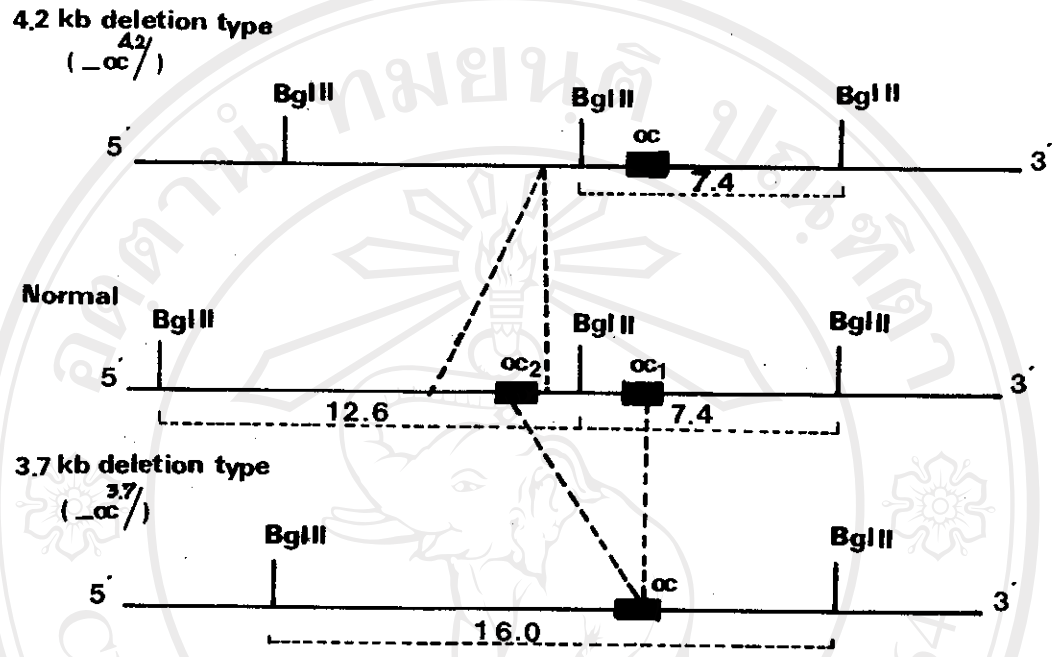

Figure III.4 BglII restriction endonuclease maps of the area around the α-globin gene cluster in normal and both α-thalassemia 2 genotypes.

Table III.2 The BglII restriction fragment size after hybridization with α_2- globin specific probe.

Genotypes	BglII α_2-gene restriction fragment (kb)
$\alpha\alpha/\alpha\alpha$	12.6, 7.4
$-\alpha^{4.2}/\alpha\alpha$	12.6, 7.4
$-\alpha^{4.2}/-\alpha^{4.2}$	7.4
$-\alpha^{3.7}/\alpha\alpha$	16, 12.6, 7.4
$-\alpha^{3.7}/-\alpha^{3.7}$	16
$-\alpha^{4.2}/-\alpha^{3.7}$	16. 7.4

III.6 α-Globin structural gene analysis

α-Globin structural genes of 36 subjects with different clinical severity were analysed. The distribution of α-globin genotypes among the subjects was as follow : 24/36 subjects had normal α-globin gene ($\alpha\alpha/\alpha\alpha$), 7/36 subjects had one α-globin gene deletion ($-\alpha/\alpha\alpha$), which are called α-thalassemia 2, 4/36 subjects had two α-globin gene deletion in a single chromosome ($--/\alpha\alpha$) which are called α-thalassemia 1 and only one subject showed the deletion of two α-globin genes from both chromosomes ($-\alpha/-\alpha$) which is called homozygous α-thalassemia 2.

The investigation of deletion types was performed in 8 subjects with α-thalassemia 2. The hybridized Bgl II fragments showed the rightward type ($-\alpha^{3.7}/\alpha\alpha$) of deletion in all cases of α-thalassemia 2, including a case who carries homozygous α-thalassemia 2 gene (subject No. 36); this case also shows the same type of deletion ($-\alpha^{3.7}/-\alpha^{3.7}$).

Autoradiographs of the hybridized EcoRI fragments with α_2- and ζ - specific probes are shown in Figure III.5 and III.6, respectively. The autoradiograph of Bgl II fragments, hybridizing with α_2- specific probe is shown in Figure III.7.

III.7. Hematologic and clinical studies.

The hematologic data, clinical presentation, and α-globin

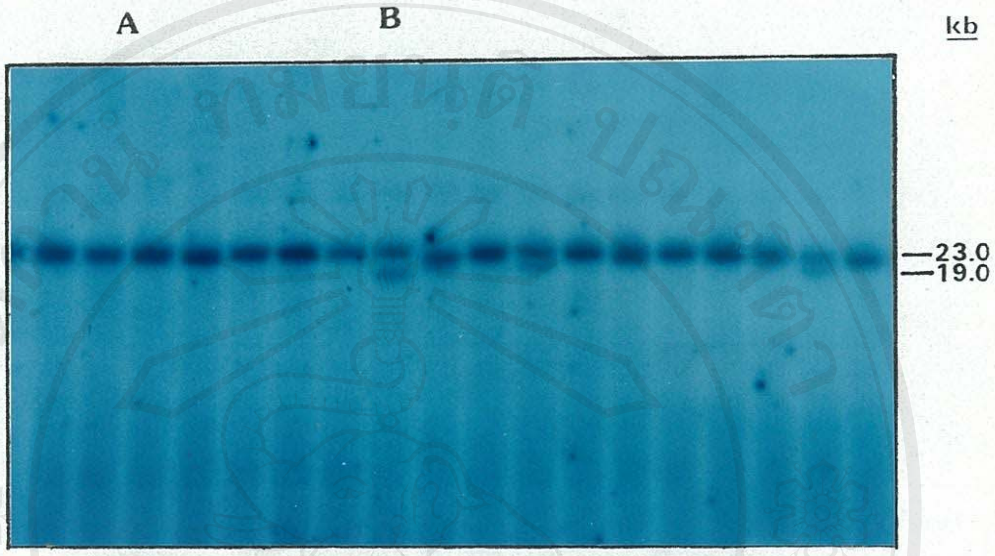

Figure III.5 Autoradiograph shows EcoRI digest DNA hybridized with α_2-specific probe.

A) normal sample

B) heterozygous α-thalassemia 2.

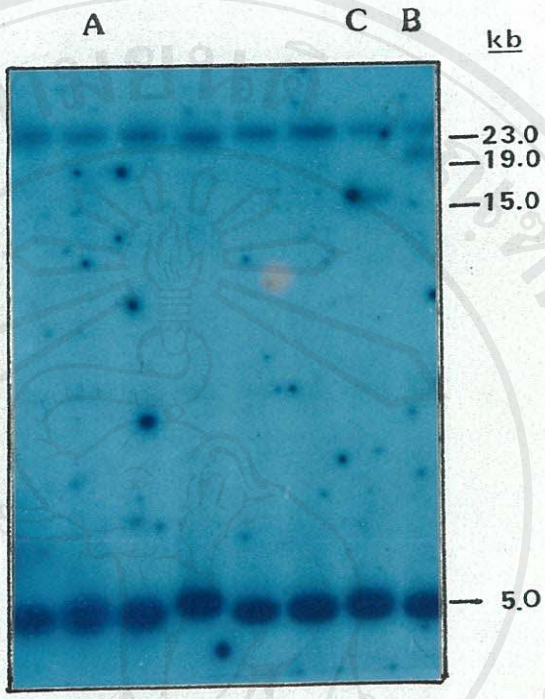

Figure III.6 Autoradiograph shows EcoRI digested DNA hybridized with ζ-specific probe

A) normal sample

B) heterozygous α-thalassemia 2

C) heterozygous α-thalassemia 1

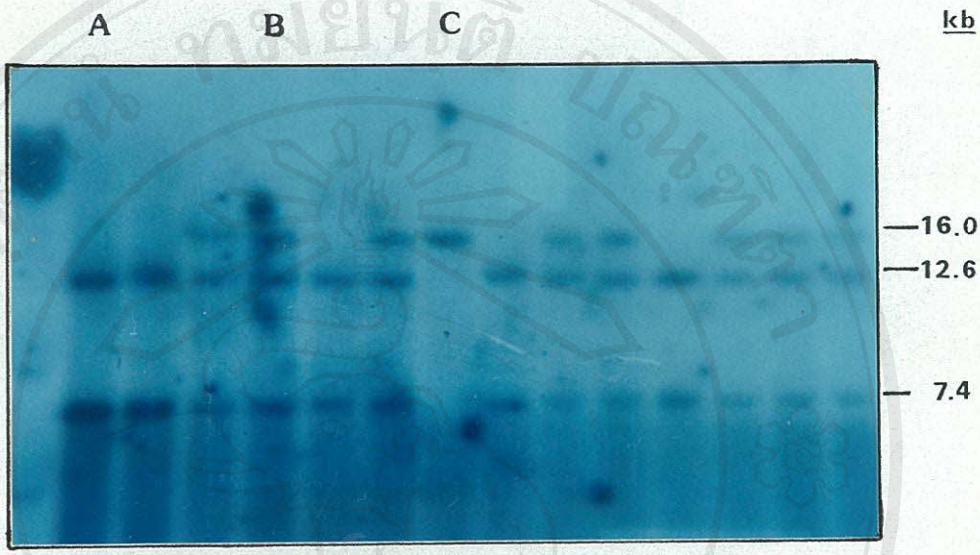

Figure III.7 Autoradiograph shows BglII digested DNA hybridized with α_2-specific probe (15).

A) normal sample

B) heterozygous α-thalassemia 2 (rightward type)

C) homozygous α-thalassemia 2 (rightward type)

genotypes in subjects with homozygous β-thalassemia are summarized in Table III.3.

According to the different α-globin genotypes, mean ($\bar{X}$) and standard deviation (S.D.) of hematologic and clinical parameters were statistically compared to study the relation between the coinheritance of α-thalassemia and severity of subjects. Data are presented in Table III.4.

Hematologic findings

There was no difference in the Hb concentration between subjects with a normal α-globin genotype and those with the deletion of either one or two α-globin genes.

The Hb A_2 levels were significantly higher in subjects with either one or two α-globin gene deletions than in those with a normal α-globin gene ($p<0.05$ and $p<0.01$, respectively)

Clinical presentation

Disease onset and transfusion dependence were significantly later ($p<0.05$ and $p<0.01$, respectively) in subjects with the deletion of two α-globin genes (--/αα) than in those with a normal α-globin gene (αα/αα). In those subjects with one α-globin gene deleted (-α/αα), there was no difference of either the disease onset or transfusion dependence.

The variation of Hb concentration at the time of disease onset and transfusion dependence among subjects with different

Table III.3 Summary of the hematologic data, clinical presentation and α-globin genotypes in subjucts with homozygous β-thalassemia

Subject No.	Sex/Age (years)	Hb (g/dl)	HbA_2 (%)	Age at presentation (months)	Age at first transfusion (months)	Hb concentration at presentation (g/dl)	Hb concentration at first transfusion (g/dl)	Transfusion requirement (times/year)	α-Globin genotype
1	F/8	5.2	3.2	6	11	6.0	5.0	10	αα/αα
2	F/10	6.2	1.9	12	19	5.8	3.7	11	αα/αα
3	F/10	5.5	0.3	3	6	5.2	4.6	10	αα/αα
4	F/11	6.1	3.8	4	6	7.4	5.5	8	αα/αα
5	F/6	6.8	2.3	12	18	7.5	4.8	3	αα/αα
6	M/10	6.0	3.4	10	18	6.3	5.8	9	αα/αα
7	F/10	5.0	1.9	5	8	5.4	4.6	11	αα/αα
8	M/13	6.9	3.2	17	48	7.0	6.1	12	αα/αα
9	M/7	6.0	1.9	3	5	6.5	2.7	11	αα/αα
10	F/17	6.7	3.4	15	32	6.3	5.4	10	αα/αα
11	M/8	6.0	4.3	3	4	5.0	3.8	12	αα/αα
12	M/8	5.7	2.7	6	10	5.6	4.8	10	αα/αα

Table III.3 continued

Subject No.	Sex/Age (years)	Hb (g/dl)	HbA_2 (%)	Age at presentation (months)	Age at first transfusion (months)	Hb concentration at presentation (g/dl)	Hb concentration at first transfusion (g/dl)	Transfusion requirement (times/year)	α-Globin genotype
13	F/8	5.3	3.6	3	5	5.2	2.7	9	αα/αα
14	M/7	6.6	2.8	5	12	7.0	6.5	9	αα/αα
15	M/7	6.8	1.9	7	16	7.0	6.0	6	αα/αα
16	F/5	8.2	2.6	24	40	7.5	7.0	3	αα/αα
17	F/7	7.4	1.9	48	51	7.2	6.7	2	αα/αα
18	F/6	7.8	2.8	16	25	8.3	8.0	7	αα/αα
19	F/18	7.9	5.5	24	68	5.6	4.8	3	αα/αα
20	F/17	6.7	2.7	36	72	6.5	5.8	3	αα/αα
21	F/6	7.5	2.9	8	11	7.0	5.6	6	αα/αα
22	F/10	8.3	2.9	25	34	7.8	6.2	6	αα/αα
23	F/5	7.4	3.0	41	48	7.7	5.7	2	αα/αα
24	F/19	8.0	4.4	12	38	7.0	4.8	2	αα/αα

Table III.3 continued

Subject No.	Sex/Age (years)	Hb (g/dl)	HbA_2 (%)	Age at presentation (months)	Age at first transfusion (months)	Hb concentration at presentation (g/dl)	Hb concentration at first transfusion (g/dl)	Transfusion requirement (times/year)	α-Globin genotype
25	M/13	5.7	4.9	27	39	6.3	4.9	11	$-\alpha/\alpha\alpha$
26	F/18	6.2	4.4	24	30	6.1	4.4	11	$-\alpha/\alpha\alpha$
27	F/14	5.2	3.1	12	18	6.7	3.1	10	$-\alpha/\alpha\alpha$
28	F/8	5.9	4.4	5	12	5.4	4.4	10	$-\alpha/\alpha\alpha$
29	F/18	7.9	3.4	12	15	6.0	3.6	4	$-\alpha/\alpha\alpha$
30	M/25	7.4	4.2	36	48	7.0	6.4	very rarely	$-\alpha/\alpha\alpha$
31	F/12	7.0	7.6	56	63	5.2	3.3	5	$-\alpha/\alpha\alpha$
32	M/8	9.3	4.1	-	-	-	-	never	$-\alpha/-\alpha$
33	F/13	7.3	5.5	65	72	6.8	5.4	8	$--/\alpha\alpha$
34	F/12	6.3	3.6	19	24	7.0	5.8	11	$--/\alpha\alpha$
35	M/9	7.2	4.8	54	61	7.5	5.5	6	$--/\alpha\alpha$
36	M/4	8.4	4.2	48	50	6.8	6.8	very rarely	$--/\alpha\alpha$

Table III.4 Mean($\bar{X}$) and standard deviation (S.D.) of hemotologic and clinical data in subjects with homozygous β-thalassemia according to α-globin genotype.

	α-Globin genotype		
	αα/αα	−α/αα	−−/αα
Number of subjects	24	7	4
Hb concentration (g/dl)	6.67±0.99	6.47±0.98	7.30±0.86
Hb A_2 level (%)	2.89±1.05	4.57±1.47	4.53±0.81
Age at presentation (months)	14.38±12.66	24.57±17.43	46.50±19.64
Age at first transfusion (months)	25.21±20.40	32.14±18.95	51.57±20.56
Hb concentration at presentation (g/dl)	6.58±0.93	6.10±0.65	7.03±0.33
Hb concentration at first transfusion (g/dl)	5.28±1.26	4.33±1.11	5.88±0.64
Transfusion requirement (times/year)	7.29±3.53	7.33±4.23	6.38±4.42

* This table does not show data of subject No. 32 who carries homozygous α-thalassemia 2 (−α/−α)

α-globin genotypes resulted in no significant difference between subjects of either one or two α-globin genes deletion and the normal α-globin gene.

Transfusion regimen

There was no difference in the transfusion requirement between subjects with a normal α-globin genotype and those with the deletion of either one or two α-globin genes.

The frequency of α-thalassemia in homozygous β-thalassemia with the clinical phenotype of thalassemia major and thalassemia intermedia was studied. Data are presented in Table III.5.

It was found that there was no significant difference in the incidence of the α-thalassemia with α-globin gene deletion between two groups of β-thalassemia homozygotes.

Table III.5 Frequency of α-thalassemia in subjects with thalassemia major and thalassemia intermedia.

α-Globin genotype	Thalassemia major		Thalassemia intermedia	
	No. case	percent	No. case	percent
αα/αα	14	70	10	62.5
_α/αα	4	20	3	18.75
__/αα	2	10	2	12.5
_α/_α	0	0	1	6.25
Total	20	100	16	100