CHAPTER IV

RESULTS

1. Laboratory studies

1.1 Amplification of the β-globin gene exon by PCR

The β-globin gene amplification by PCR. The PCR components and PCR conditions had been titrated to bring the best result, which provide a clear single band at the expected size of 340 bp for exon 1, 412 bp for exon 2 and 357 bp for exon 3. Optimization of exon 1, 2 and 3 of some parameter such as the titration of glycerol (Figure 15,16 and 17). In exon 3, the bests result because it a clear single band . The specificity of the PCR products of three exons is necessary for using as the template by cycle sequencing.

Figure 15. The effect of glycerol concentrations in the amplification of exon 1. The percentages of glycerol were 0, 5, 10 and15 in lane 1, 2, 3 and 4 respectively. The concentration of 10% glycerol was suitable for exon 1 amplification.

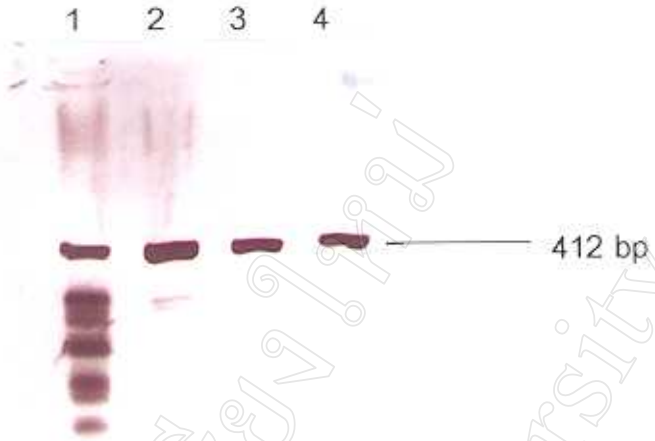

Figure 16. The effect of glycerol concentrations in the amplification of exon 2. The percentages of glycerol were 0, 5, 10 and15 in lane 1, 2, 3 and 4 respectively. The concentration of 10% glycerol was suitable for exon 2 amplification.

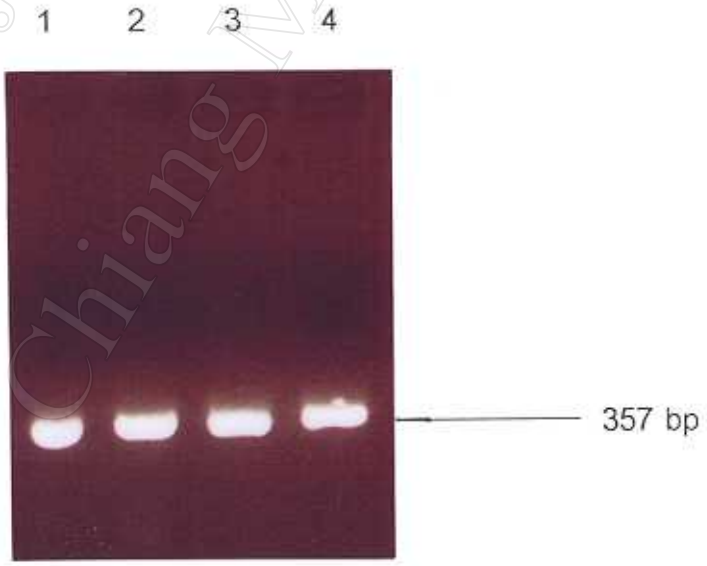

Figure 17. The constant PCR product as obtained in all different concentrations of glycerol indicates the best exon and the glycerol adding was not necessary.

1.2 Chain-termination cycle sequencing

The sequencing data of exon 1 of the β-globin gene PCR products showed a single base substitution at the nucleotide number 1631(position is listed according to the sequence from GeneBank, HUMHBB221) was observed as equally high peak of A and T nearly at the same position (Figure 18). This single base substitution caused the amino acid substitution of glutamic acid to valine (Glu→Val) at codon sixth or from the acidic residue to the neutral one.

Hb S

	5	6	7
	Pro	Glu	Glu
Normal	CCT	GAG	GAG
		↓	
Patient	CCT	GTG	GAG
	Pro	Val	Glu

This agreed with the Hb typing data which detected that abnormal hemoglobin of patient was slower moving to the anode in cellulose acetate gel electrophoresis at pH 8.5 comparing with normal Hb A (see Figure 2 and 3).

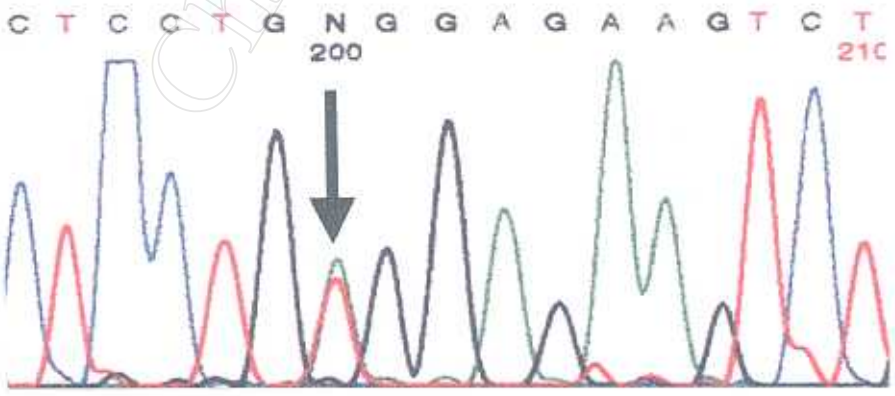

Figure 18. Exon1 sequencing analysis shows mutation at codon 6 [GAG (Glu)→GTG (Val)].

Moreover, according to the sequencing data of exon 1 of β-globin gene PCR products showed a single base substitution at the nucleotide number 1704 (position is listed according to the sequence from GeneBank, HUMHBB221) was observed as equally high peak of G and T nearly at the same position (IVS-1nt1) (Figure 18). This single base substitution caused to change this site of specific nucleotide sequence at the splice junction.

IVS-1nt1

	29	30	
	Gly	Arg	
Normal	GGC	AG	gtt ggtat
Patient	GGA	AG	ttt ggtat

This agreed with the Hb typing data that detected abnormal hemoglobin by using HPLC. The data derived from Miss Thasaneeya Chamrasratanakorn who detected Hb typing by using HPLC. The chromatographic elution time of patient was 4.44 minutes when performed by HPLC using the Bio-Rad Variant ™ (To see Figure 1). This elution time is the same as that for Hb S.

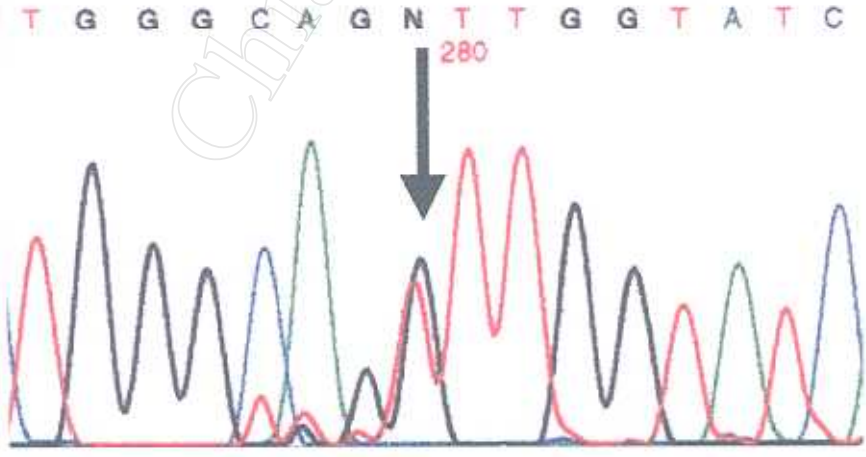

Figure 19. Exon1 sequencing analysis shows mutation at splice junction (G→T).

Confirmation of single base substitution at splice junction in exon 1 of β-globin gene by using chain-termination cycle sequencing of patient's mother

To confirm that single base substitution at splice junction in exon 1 of β-globin gene by using chain-termination cycle sequencing of patient's mother. The sequencing data of exon 1 of β-globin gene PCR products showed a single base substitution at the nucleotide number 1704 (position is listed according to the sequence from GeneBank, HUMHBB221) was observed as equally high peak of G and T nearly at the same position (IVS-1nt1)(Figure 20). This single base substitution caused to change these sites of specific nucleotide sequence at the splice junction. It's the same as patient.

IVS-1nt1

	29	30	
	Gly	Arg	
Normal	GGC	AG ¦	gtt ggtat
Patient	GGA	AG ¦	ttt ggtat

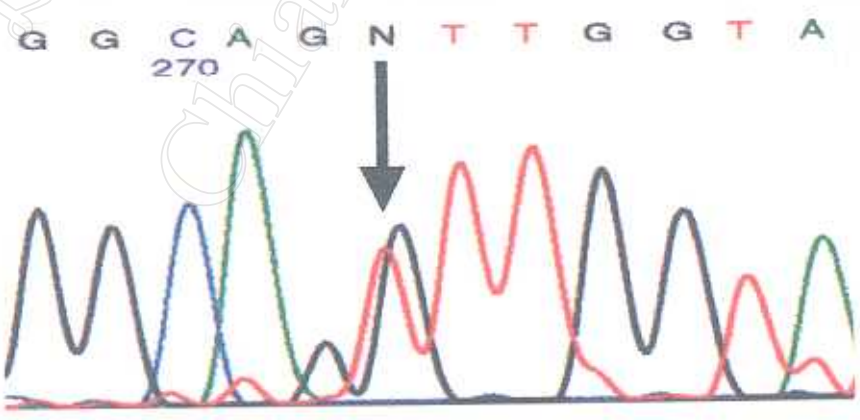

Figure 20. Exon1 sequencing analysis shows mutation at splice junction (G→T).

From the results it can be concluded that the patient's father is Hb S trait and the mother is β^{O}-IVS-1nt1. The inheritance of mutated hemoglobin genes from parents caused the patient to be Hb S/β^{O}- IVS-1nt1 (Figure 20).

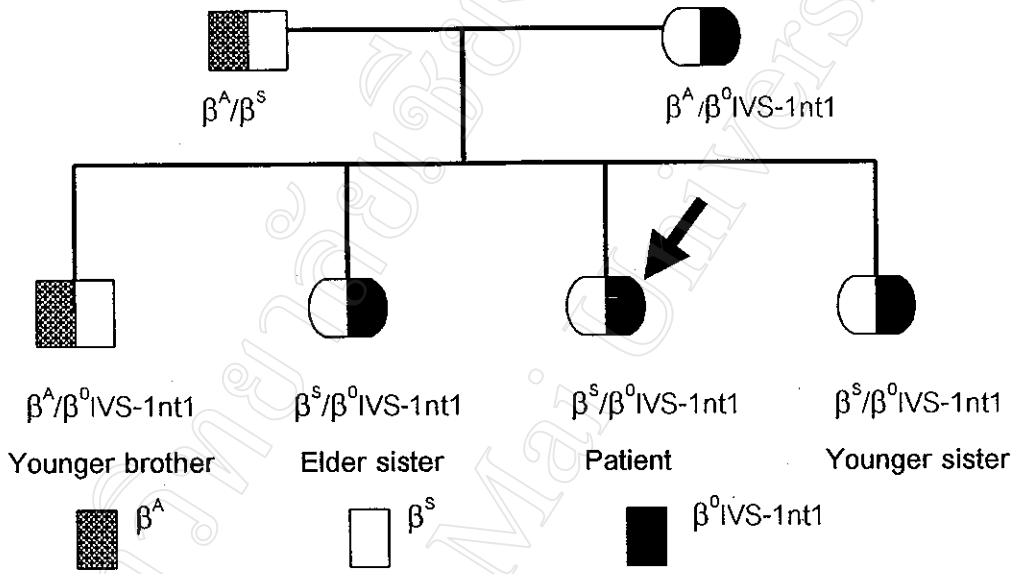

Figure 21. Diagram of Pedigree of the patient and family members.