

Spontaneous mutation of hemoglobin Leiden ($\beta 6$ or $7 \text{ Glu} \rightarrow 0$) in a Thai Girl

We report a case of 12-year old Thai girl suffering from mild non-transfusion-dependent thalassemia intermedia. She is the single child in her family. On examination she looked pale; there was no hepatosplenomegaly. The Hb concentration was 9 g/dL. Hb typing and molecular mutation study revealed compound heterozygosity for HbE and Hb Leiden ($\alpha_2\beta_2^{6/7\text{-Glu}}$, codon 6/7-GAG). The proportion of HbE was 47% whereas that of Hb Leiden was 39%. The patient had no HbA. Hb typing of her father and mother revealed HbE trait, and no Hb Leiden was demonstrated. As the paternity test confirmed the parenthood, we assume that Hb Leiden has arisen by spontaneous mutation. A study of the β -globin gene framework by molecular cloning and subsequent DNA sequencing of the β -globin gene in the members of the family indicated that the Hb Leiden mutation occurred on a maternal inherited chromosome. The deletion of codon 6 or 7 (-GAG) of the β -globin gene in the patient may be due to an unequal crossing over during the mother's oogenesis.

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Introduction. Hemoglobin Leiden ($\beta 6$ or $7 \text{ Glu} \rightarrow 0$) has been described in several populations (1-5). The abnormal Hb can be detected by HPLC⁶ and the mutation can be confirmed by DNA sequencing.⁷ Severe hemolytic anemia has been described in combination with β -thalassemia.² In one of the previous observations, Hb Leiden had probably arisen from a new mutation.⁵

According to Orkin et al.⁸ and Antonarakis et al.⁹ the micro-haplotype polymorphism of the β -globin gene designated as *framework* (FW) is defined by five single nucleotide polymorphisms (SNPs). Four main frameworks have been identified as shown in Table 1.

Patient. A twelve-year-old girl with moderate hemolytic anemia was referred to Chiang Mai University Hospital with her parents for diagnostic study. She had not received any blood transfusions since her first visit when she was a nine years old. Folate supplements had been given on several occasions when she was pale.

Methods. EDTA blood samples were collected after receiving informed consent. The samples were tested for abnormal hemoglobin using automated high perform-

ance liquid chromatography (HPLC) β -thalassemia short program (BIORAD, Variant).

For molecular analysis, DNA was extracted from whole blood using a modified version of the Chelex-100 extraction method.¹⁰ The target DNA was amplified using PCR. The sense primer was paired to the complementary area at -147 to -125 (S-x1,2) from the cap site whereas the antisense primer was paired to IVS-II-99 to IVS-II-130 (A-x1,2) of the gene. The PCR mixture and condition were previously reported.¹¹ The samples were electrophoresed on 2% TBE-agarose gel to check size and quality of the PCR product and purified using the QIAquick[®] PCR purification kit (QIAGEN GmbH) and electrophoresed to estimate the quantity of the PCR product. The purified PCR products were used as templates for the cycle sequencing reaction using BigDye[®] Terminator Ready Reaction Mix (Applied BioSystems). The sense primer (S-x1,2) was used for mutation detection of abnormal hemoglobin and the first polymorphic site at codon 2 nt 3. For IVS-II-16, IVS-II-74, and IVS-II-81, the sequencing primer was 5'-TCA CCT GGA CAA CCT CAA G-3' (S-ivs2), which paired to the complementary area at codon 76 nt 3 to codon 82 nt 3. The reaction mixture was then precipitated using ethanol-sodium acetate and capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer (Applied BioSystems) as described by the manufacturer.

To identify the β -globin gene FW linked to the Hb Leiden mutation in the patient, the PCR products of the β -globin gene were cloned into *E. coli* strain JM109 using pGEM[®]-T Easy Vector (Promega). The plasmids were extracted using QIAprep[®] Spin Miniprep Kit (QIAGEN) and were used as the template for DNA sequencing as described above.

A paternity test was carried out using six STR loci—D13S317, D16S539, D5S818, D8S1179, TH01, and vWA. The combined power of discrimination is 99.5755% in northern Thai (unpublished data).

Results. Physical examination of the patient revealed a pale-looking girl without thalassaemic facial changes and no jaundice. There was no hepato-splenomegaly. Her hematological data were Hb: 9.2 g/dL ; Hct: 29.2%; MCV: 77.6 fL; MCH: 24.8 pg; MCHC: 30.7 g/dL; the reticulocyte count was 10.6%. Microscopic examination of a blood smear showed moderate hypochromia, anisopoikilocytosis and few target red blood cells. The HPLC of the patient showed 47% HbE and 39% of another abnormal Hb in the position of S window. There was no HbA in the chromatogram (Figure 1). The hematological data of her parents were within normal limits. HPLC and direct DNA sequencing results of both parents showed heterozygosity of HbE and no other abnormal Hb. Direct DNA sequencing of the patient indicated compound heterozygosity of HbE ($\beta 26 \text{ nt } 1 \text{ G} \rightarrow \text{A}$) and Hb Leiden ($\beta 6$ or 7 -GAG). Four polymorphic sites on β -globin gene (codon 2 nt 3, IVS-II-16, IVS-II-74, and IVS-II-81) indicated homozygosity of FW2 in her father

Table 1. Classification of β -globin gene framework according to Orkin et al.⁸ and Antonarakis et al.⁹

Table 1. Classification of β -globin gene framework according to Orkin et al. (8) and Antonarakis et al. (9)

| | codon 2 | | IVS-II | | |
|------|---------|-------|--------|-------|--------|
| | nt 2 | nt 16 | nt 74 | nt 81 | nt 666 |
| FW1 | C | C | G | C | F |
| FW2 | C | C | T | C | F |
| FW3A | T | G | T | C | C |
| FW3 | T | G | T | T | C |

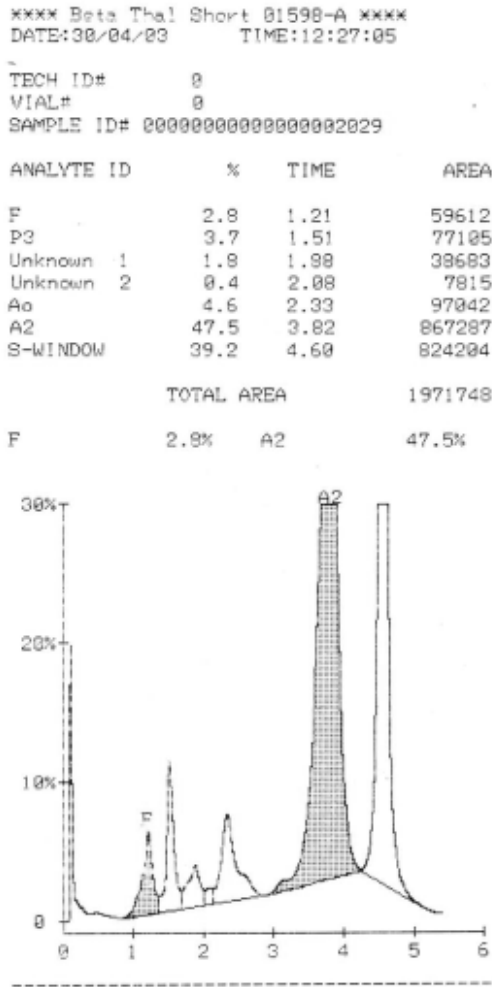


Figure 1. Hb HPLC of the patient indicated HbE (shown as A2 in gray) 47% and another abnormal Hb 39% in the position of the S-window.

while her mother and the patient were heterozygous for FW2 and FW3A. Subsequent cloning of PCR product followed by DNA sequencing of the patient showed Hb Leiden in FW3A (Figure 2). Paternity test of the six STR loci confirmed the parenthood.

Discussion. A 12-year old girl with a clinical picture of mild non-transfusion-dependent thalassemia intermedia was shown to be a compound heterozygote for HbE ($\alpha_2\beta_2^{26\text{Glu}\rightarrow\text{Lys}}$) and Hb Leiden ($\alpha_2\beta_2^{6/7\text{-Glu}}$). Both parents were HbE trait without Hb Leiden. The clinical expression was more severe than usually seen in HbE homozygotes. This may be caused by a higher instability of Hb Leiden as suggested by the reduced ration of this Hb in comparison with that of HbE. The patient's clinical picture was similar to the milder form of β -thalassemia/HbE with no transfusion dependent.

It is highly probable that the deletion of codon 6 or 7 of the β -globin gene in our patient is due to a spontaneous mutation on a maternal chromosome. Juricic *et al.*⁵

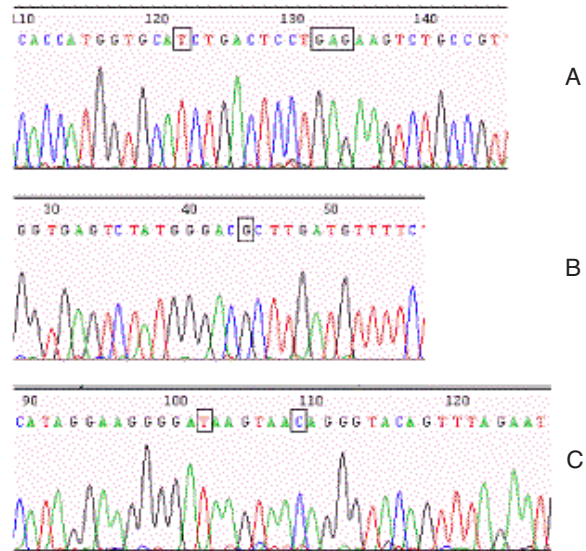


Figure 2: Electropherogram of plasmid containing PCR product of Hb Leiden. In figure A, the sequencing primer was a sense PCR primer (S-x1,2) while in figures B and C the sequencing primer was S-ivs2 which is complementary to codon 76 to 82. (A) The left box indicates the codon 2 nt 3 of FW3A while the 3 bases in the right rectangle box are the remaining codon 6 or 7. (B) The box shows the SNP at IVS-II-16. (C) The boxes show the SNPs at IVS-II-74 and IVS-II-81.

concluded the same for their Yugoslavian patient, although they did not perform a paternity test. In our case paternity was confirmed with a high probability. The finding of the Hb Leiden mutation on a β -globin gene FW3A chromosome was further proof that the mutation was not inherited from the father. The presence of a FW3A chromosome and the absence of Hb Leiden in the mother indicated that the deletion of codon 6 or 7 in the β -globin gene arose as a result of unequal crossing over during oogenesis.

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