

## An Indonesian Case of Compound Heterozygote for Hemoglobin E and Deletion C at Nucleotide 391 of the $\beta$ -Globin Gene

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### Abstrak

Hemoglobin E (HbE), yang mengalami pergantian asam amino ke-26 dari asam glutamat menjadi lisin pada  $\beta$ -globin, adalah mutasi  $\beta$ -globin yang paling sering ditemukan di Asia Tenggara. Mutasi heterozigot ganda, yaitu yang menyebabkan pergantian asam amino ini bersama dengan mutasi lain pada gen  $\beta$ -globin, menyebabkan penyakit hemolitik berat yang disebut penyakit hemoglobin E/ $\beta$ -thalasemia, dan memerlukan transfusi darah berulang. Seorang anak perempuan Indonesia, penderita anemia berat dengan kadar HbF dan HbA<sub>2</sub>/HbE tinggi, sekuens gen  $\beta$ -globinnya dianalisis menggunakan sekuenser DNA otomatis. Hasilnya menunjukkan bahwa satu alel mengalami mutasi G  $\rightarrow$  A pada nukleotida (nt) 232, yang menyebabkan penggantian asam glutamat menjadi lisin pada codon 26, dan berakibat HbE; pada alel lainnya terjadi delesi C pada nt 391. Laporan kasus ini melaporkan satu kasus heterozigot ganda HbE dan delesi C pada nt. 391 pada gen  $\beta$ -thalasemia, yang berakibat anemia berat.

### Abstract

Hemoglobin E (HbE), having the substitution of glutamic acid with lysine at the 26th amino acid residue on  $\beta$ -globin, is the most common mutation of the  $\beta$ -globin gene in South East Asia. A compound heterozygote of this mutation with another mutation in the  $\beta$ -globin gene leads to a severe hemolytic disease known as hemoglobin E/ $\beta$ -thalassemia disease, where repeated blood transfusions are needed. In an Indonesian girl showing severe anemia and high levels of HbF and HbA<sub>2</sub>/HbE, the sequences of the  $\beta$ -globin gene were analyzed using an automatic DNA sequencer. The results showed one allele had G to A mutation at nucleotide (nt) 232 which resulted in the substitution of glutamic acid with lysine at codon 26 resulting in HbE; in the other allele deletion C at nt 391 was identified. This is a case report of a compound heterozygote of HbE and a deletion of C at nt 391 in the  $\beta$ -thalassemia region which showed severe anemia.

**Keywords:** Codon 26, nucleotide 232, codon 35,  $\beta$ -thalassemia.

The mutation substituting glutamic acid with lysine at the 26th residue of  $\beta$ -globin is called as hemoglobinopathy E (HbE) and is the result of a G to A mutation at the nucleotide 232 of the  $\beta$ -globin gene. HbE is the most prevalent hemoglobin variant in South East Asia, where the frequency is about 28 million people in a population of 338 millions.<sup>1</sup>

HbE homozygotes show microcytic hemolytic anemia, while heterozygotes for this mutation show only microcytic red blood cells but no clinical anemia. In contrast, a Hb E heterozygote can complicate  $\beta$ -thalassemia trait called Hb E/ $\beta$ -thalassemia, a disease with

variable clinical picture ranging from a mild anemia to a severe anemia nearly as severe as the  $\beta$ -thalassemia major.

In  $\beta$ -thalassemia more than four hundred mutations on the  $\beta$ -globin gene have been reported from all over the world and the major types of mutations have been clarified in each specific region. A heterocompound case of HbE and deletion of C at nucleotide 391 (391 del C) was first reported in Indonesia by Lie Injo et al,<sup>2</sup> without any clinical features of the case, while Yang et al<sup>3</sup> had a more clear case report of a heterocompound case of HbE and 391 del C. The second case exhibited a Hb of 7 g/dl, and consisted of HbF 48%, HbE 46%, and HbA<sub>2</sub> 4.4%. This deletion mutations is rare, and since then, no more report have been published about this compound heterozygote for both HbE and 391 del C.

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This paper will present such a case.

## Extracorporeal Membrane Oxygenation (ECMO) : New Technology or Just A New Tool for Developing Countries ?

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### Abstrak

*Manajemen pasien dengan ventilasi mekanik pada gagal napas akut dan / atau adult respiratory distress syndrome di negara-negara berkembang biasanya dilakukan oleh ahli anesthesiologi. Di negara maju pun, gagal napas akut, terutama adult respiratory distress syndrome mempunyai mortalitas yang masih tinggi. Extracorporeal membrane oxygenator (ECMO) merupakan inovasi teknologi tinggi dalam bidang intensive care medicine yang dimulai sejak 20 tahun lampau. Pada beberapa unit perawatan intensif di negara maju, ECMO digunakan pada gagal napas akut sebagai rescue therapy atau sebagai terapi alternatif pada prediksi mortalitas tertentu. Di USA, ECMO telah merupakan terapi standar pada gagal napas neonatus. Hasil terapi ECMO berbeda-beda pada kelompok umur yang berbeda. Hasil terbaik didapat pada neonatus, yaitu dengan 70-90% berhasil dengan selamat, sedangkan pada anak dan dewasa, didapati mortalitas 45-55% untuk pasien yang diprediksi mempunyai mortalitas sekitar 80% dengan ventilasi mekanik. Apakah mungkin dilakukan ECMO di negara berkembang? ECMO tidak dapat disangkal sangat efektif untuk terapi pada neonatus dengan gagal napas, tetapi ECMO sangat membutuhkan tenaga. Biaya ECMO juga sangat tinggi, kira-kira 2 kali terapi perawatan intensif standar. Dengan mempertimbangkan cost benefit analysis, ECMO tampaknya lebih baik dilakukan di negara-negara berkembang hanya pada rumah sakit tertentu yang mempunyai cukup pengalaman operasi jantung terbuka, dan hanya dilakukan pada gagal napas neonatus.*

### Abstract

*The management of patient's mechanical ventilation, in acute respiratory failure and / or adult respiratory distress syndrome in developing countries is generally done by anesthesiologist. Even in developed countries, patients with acute respiratory failure and particularly adult respiratory distress syndrome have a very high mortality rate. Extracorporeal membrane oxygenation (ECMO) is an innovation of high technology in the intensive care medicine which emerged two decades ago. In certain centers in several developed countries, ECMO for acute respiratory failure is used as a rescue therapy or as an alternative therapy at a certain predicted mortality rate. In fact, in neonatal respiratory failure in the United States, ECMO is considered as a standard therapy. Unfortunately, the result of ECMO is different at different age groups. The best results is in neonates, i.e. 70-90% survival rate, while for older children and adults the mortality rate is 45-55% for patients with predicted mortality rate around 80% with mechanical ventilation. Would it be possible to start ECMO therapy in developing countries? ECMO has been unquestionably successful in treating a large number of term infants with respiratory failure, but ECMO is very labor intensive. The cost for ECMO is very high, it is about twice as high as standard intensive care treatment. Taking into considerations the cost benefit analysis and cost effective analysis ECMO would be better carried out in developing countries only at certain hospitals with enough bypass or open heart surgery experience (1-2 selected centers), and is best done only in neonatal respiratory failure.*

**Keywords:** ECMO, Developing countries, Cost, Neonates

### HISTORY

Extracorporeal membrane oxygenation (ECMO) is a form of invasive cardiopulmonary support that can provide temporary physiologic stabilization in reversible circulatory and/or respiratory failure. The history of ECMO application in clinical situation has been controversial. In essence, ECMO is an innovative

intensive care unit application of operating room cardiac technology. The use of an artificial lung for extended applications was not considered a serious possibility until Kolf and Clowes, demonstrated that the interposition of a gas permeable membrane between the blood and gas greatly reduced both blood trauma and embolic accidents due to direct gas blood exposure in the heart lung machines then in use.<sup>1</sup> Work with new fabrication and membrane materials and improved design concepts contributed to the steady evolution of membrane lungs through the 1950s and early 1960s.<sup>2,3</sup> Before the evolution of membrane

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## METHOD

### Case

An 11 year old Indonesian girl was admitted to Dr. Sardjito General Hospital Yogyakarta, Indonesia, because of anemia and pallor. For 2 years before admission she had apparently been weak compared with her friends of the same age. During the last two months she could not go to school because of intermittent fever. Therefore, the doctor from the primary health center referred her to the hospital.

Physical examination revealed thalassemic facies<sup>4</sup> moderate splenomegaly and mild hepatomegaly. Conjunctivae were anemic and sclera slightly icteric. Hemoglobin concentration was only 5.2 g/dl, HbF detected by the alkali-denaturation method was 5.2%, and HbE+HbA<sub>2</sub> by cellulose acetate membrane diffusion method was 12.8%. Her father and mother had mild anemia (Hb 11.2 and 9.04 g/dl). Her father's and mother's HbF were 6.5 and 7.2 % (normal 0.4 %), and total HbE+HbA<sub>2</sub> were 5.7 and 27.1% respectively (Table 1).

Table 1. Blood examination of the case and her family members.

		Hb g/dl	PCV (%)	HbF (%)	HbE and HbA <sub>2</sub> * %
I.1	Father	11.20	38	6.5	5.7
I.2	Mother	9.04	32	7.2	27.1
II.1	Brother	10.89	36	5.9	20.9
II.2	Sister	9.82	34	4.2	24.2
II.3	Case	5.18	21	5.2	12.8

\* by Cellulose Acetate Membrane diffusion the HbE comigrated with HbA<sub>2</sub>.

### Screening for $\beta$ -globin mutation

A blood sample was taken before transfusion, and the DNA was extracted by the standard phenol/chloroform method for further  $\beta$ -globin investigation and sequencing.

Screening for  $\beta$ -globin mutation was conducted by PCR using amplification refractory mutation system (ARMS) primers as described elsewhere.<sup>5</sup> This method was applied to analyse the 5 most prevalent single mutations in South East Asia (codon 26 GAG  $\rightarrow$  AAG) and Asian-Indians: IVS-1 position 5 (G  $\rightarrow$  C), codon 15 (G  $\rightarrow$  A), IVS-2 position 654 (G  $\rightarrow$  T) and codon 30 (G  $\rightarrow$  C).

### Sequencing

For the sequencing, the  $\beta$ -globin gene was amplified as three separate fragments (fragment I, II, and III) by using three sets of primers (Table 2). Each of the three fragments cover an entire exon and a few nucleotides of flanking introns. The PCR mixture of 20  $\mu$ l contained approximately 0.3-0.5  $\mu$ g of the case's DNA, 5 pmols of each primer, 0.1 unit of Taq polymerase, 30  $\mu$ Mol of each dNTP in 10mM Tris HCl (pH 8.4), 50 mM KCl and 1.5 mM MgCl<sub>2</sub>. The thermal cycle consisted of primary denaturation temperature at 94°C for 6 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, combined with annealing temperature at 67°C for 1 minute, and extension at 72°C for 90 seconds. The last extension temperature was prolonged for 4 minutes (modified from Varrawalla et al, 1991).<sup>6</sup> The amplified DNA fragments were separated by electrophoresis on a 3% agarose gel and photographed after ethidium bromide staining.

The amplified DNA fragments were directly subcloned into pT7Blue T-vector (Novagen, Madison, WI). The sequences of each inserted DNA fragments (from 9-10 clones) were determined using an automatic DNA sequencer (model 373A; Applied Biosystem, Foster City, CA) with Taq dye primer cycle sequencing kit (Applied Biosystem, Foster City, CA).

Table 2. Primer sequences used to amplify the three fragments of the  $\beta$ -globin gene

Fragment	Sequence 5' $\rightarrow$ 3'	Complementary site*
I	ACC TCA CCC TGT GGA GCC AC	- 5 to 14
	GAG AGA GTC AGT GCC TAT CA	346 to 327
II	AGA AAC TGG GCA TGT GGA GA	286 to 305
	CCC CTT CCT ATG ACA TGA ACT TAA	670 to 647
III	ATT CTG AGT CCA AGC TAG GC	1386 to 1405
	TGC ACT GAC CTC CCA CAT TC	1771 to 1752

\*Numbering of the  $\beta$ -globin sequence is according to Lawn et al (1980).

## RESULTS

Using ARMS PCR method, mutant (M) and wild type (W) primer can bind to mutant or wild type specific sequence respectively (Figure 1). By detecting the amplified fragment of HbE gene from each reaction, normal, hetero or homozygotes can be identified. Results in Figure 2 disclosed that the mother (I.2) was a HbE homozygote consistent with her high HbE levels

(27.1%). The brother (II.1) and sister (II.2) were identified as heterozygotes. The case itself (II.3) has proven to be a HbE heterozygote but she has high HbF (5.2%) which is phenotypical of a  $\beta$ -thalassemia gene carrier, possibly inherited from her father.

Pr A : 5' ACC TCA CCC TGT GGA GCC AC  
 Pr 1 : 5' ACC AAC CTG CCC AGG GGC TT  
 Pr 2 : 5' ACC AAC CTG CCC AGG GGC TC

Figure 1. The sequences of the primers used for detecting HbE cases.

The Pr A, cited from Varawalla et al (1991) is used as forward primer for both the reversal primer Pr 1 and Pr 2. The Pr 1 primer, a mutant primer, will anneal only if mutant G to A at nt 232 exists and Pr 2, a wild type primer, will anneal if the mutation does not exist.

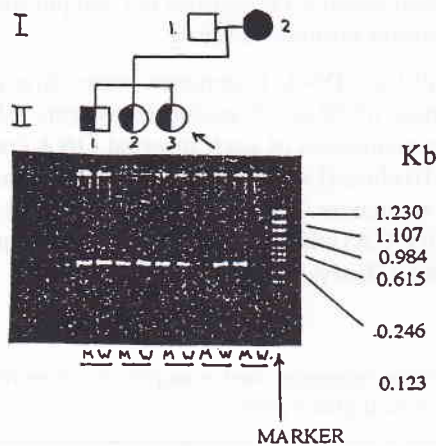


Figure 2. Gel electrophoresis of amplified products detecting the existence of HbE gene using ARMS method.

Wild type primer will amplify normal sequence (W) and mutant primer will amplify mutant sequence (M). This figure shows that all family members are heterozygote except the mother, where only the mutated type at Co26 (GAG  $\rightarrow$  AAG) band appears, therefore being a homozygote. The father shows only a wild type band therefore there is no mutation at Co26 on his side.

To clarify the HbE/ $\beta$ -thalassemia disorder state, her  $\beta$ -globin gene was subjected to sequencing using an automatic DNA sequencer.

Results of the nucleotide sequences of amplified DNAs encompassing exon 3 (fragment III) showed identical sequences to those of wild-type gene in all clones studied. In particular, two type of DNA clones were obtained by subcloning the amplified fragment I and fragment II encompassing exon 1 and 2 respective-

ly. In fragment I, one type of clone had a DNA sequence which was identical with the wild type of exon 1 (Figure 3 upper panel) and the other type had a single nucleotide mutation from G to A at position 232 (Figure 3 lower panel) which corresponds to the HbE mutation. Therefore, this confirmed the result of previous PCR as shown in Figure 2. In fragment II, one type of clone had a DNA sequence which was identical with the wild type of exon 2 (Figure 4 upper panel), however the other type had a single nucleotide (C) deletion at position 391 (Figure 4 lower panel). These results indicate that the patient was compound heterozygote at the molecular level.

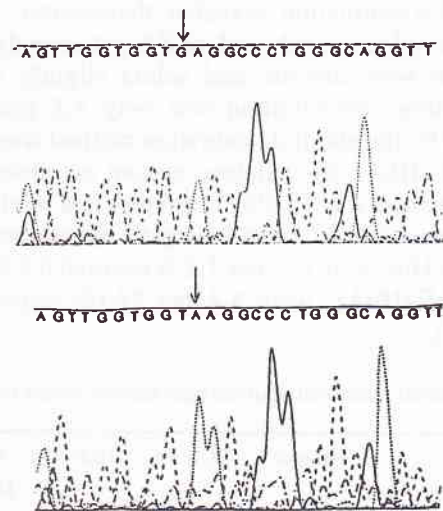


Figure 3. Nucleotide sequences of a part of the exon 1 of the  $\beta$ -globin gene.

The lower panel shows a mutation (G  $\rightarrow$  A mutation) on nucleotide 232 which confirms the ARMS PCR electrophoresis result of Figure 2. It proved the Co26 mutation GAG  $\rightarrow$  AAG.

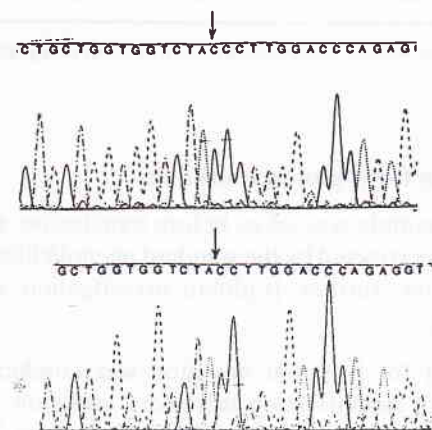


Figure 4. Nucleotide sequences of a part of the exon 2 of the  $\beta$ -globin gene.

The lower panel shows the deletion C at the nucleotide 391. The Co35 (TAC) remains unchanged but a frameshift mutation in the subsequent coding region will result.

## DISCUSSION

In dealing with single mutation screening, misincorporation of nucleotides by the Taq DNA polymerase is a problem to consider. According to Foecharoen et al<sup>7</sup> the misincorporation rate/nucleotide/cycle in the case of a 239-bp product in 30 cycles of PCR amplification was estimated to be  $2 \times 10^{-4}$ . In this report, at least three clones were shown to have the same mutation, and thereby the possibility of the misincorporation of the Taq polymerase was virtually eliminated.

We detected a single  $\beta$ -globin gene mutation from G to A at the position 232 which resulted in glutamic acid being substituted by lysine at codon 26. This mutation not only introduces a different amino acid (glu $\rightarrow$ lys) but also changes the splicing pattern of the mRNA because the codon 24-27 are actually a rather good fit for the consensus donor signal for splicing, therefore the mutation activates the "cryptic" splice site. The resultant 40% abnormal mRNA can not give any detectable  $\beta$ -globin protein, because a total of 16 nucleotides are removed from this exon.<sup>1,7</sup> Furthermore, instability of the mRNA in the erythroid cell and easy degradation in the cytoplasmic cell<sup>8,9</sup> will also reduce the amount of functional mRNA, therefore synthesis of normal  $\beta$ -globin is severely impaired.

Lie-Injo et al<sup>2</sup> reported that from 36 Indonesian thalassemia cases from Jakarta, a HbE gene was found in 13 chromosomes. Furthermore, from the analysis of 72 chromosomes (36 patients) they also described the most prevalent mutations as IVS-1 nt5 (G $\rightarrow$ C) (32 chromosomes), followed by IVS-2 nt5 (G $\rightarrow$ C) (7 chromosomes), IVS-1 nt1 (G $\rightarrow$ T) (6 chromosomes), codon 15 (TGG $\rightarrow$ TAG) (4 chromosomes) and others (7 chromosomes). They described 1 chromosome having deletion C at codon 35 without any information about the paired chromosomes. In the same year the same mutation was also published in the Malay ethnic group.<sup>3</sup>

In this report, we also clarified a single deletion C at nucleotide 391. This deletion introduces a translation reading frameshift providing a different amino acid sequence after the 35<sup>th</sup> the amino acid residue, before a premature nonsense termination codon at codon 60 (GTC  $\rightarrow$  TGA). Therefore people with the combination of mutation at codon 26 (GAG  $\rightarrow$  AAG) and codon 35 (del C) will suffer from severe impairment of  $\beta$ -globin production resulting in  $\beta^0$ -thalassemia.

In individual with a mutation at codon 26 (GAG $\rightarrow$ AAG) and 35 (del C), the impairment of  $\beta$ -globin gene expression might activate the  $\gamma$  and  $\beta$ -tan-

dem globin gene, introducing the HbF and HbA<sub>2</sub> production. The  $\delta$  chain is normally combined with a non  $\delta$  chain designated as  $\gamma$  chain resulting in increased HbF. In the erythrocyte this minor component composes about 2.5% (0.6-6%) of the total hemoglobin.

Compared with a similar case described by Yang et al<sup>3</sup> the HbF level of our case (5.2%) is far lower than that of the Malay case (48.7%). Subsequently, increased amount of HbA<sub>2</sub> was demonstrated to compensate for the low production of HbA.

Our case was a compound heterozygote for HbE and a mutation at codon 35 (del C) of the globin gene. Using the cellulose acetate membrane diffusion methods one cannot differentiate the HbA<sub>2</sub> from HbE, because this HbA<sub>2</sub> comigrated with HbE, even though from Table 1 we know that either HbE and/or HbA<sub>2</sub> level may not be abnormally increased. Our case showed a lower percentage of HbE and HbA<sub>2</sub> (12.8%) compared with those of the Malay case (46.9%). However, this results did not affect the clinical diagnosis. Also, the HbE levels can not identify the genotype (heterozygote, homozygote or heterocompounds) since there is no clear cut off between the three categories. Only by DNA analysis one can clarify the genotype.

In Malay patients the most prevalent  $\beta$ -thalassemias are mutations at IVS-1 nt5 (G $\rightarrow$ C), codons 41-42 (-TCCT), IVS-1 nt1 (G  $\rightarrow$  T), IVS-2 nt654 (C  $\rightarrow$  T) exhibited by Yang et al (1988)<sup>3</sup> on 20, 6, 4, 3 and 3 chromosomes respectively. This data shows close similarity with those described by Lie-Injo et al (1989)<sup>2</sup> and with the mutations described in the Indian subcontinent.<sup>6</sup> There is only distant similarity with those observed in the Chinese community where the common mutations are at promoter site e.g. -29 (A $\rightarrow$ G) and -28 (A $\rightarrow$ G), followed by codon 17 (A  $\rightarrow$  T), IVS-1 nt1 (G  $\rightarrow$  T) and IVS-1 nt5 (G  $\rightarrow$  C).

Lie-Injo et al<sup>2</sup> collected their samples from the surrounding area of the Jakarta metropolitan city while our case comes from an isolated family in a remote area 30 km from Yogyakarta, 600 km from Jakarta. The HbE gene in our case possibly originated from Thailand where the HbE carrier is prominent with a frequency of nearly 50-60%.<sup>10</sup> It is possibly true that 3000 years ago there were tribal movements from north SEA to the south. We can assume that the HbE mutation is inherited from the Thai, other  $\beta$ -thalassemia mutations from Asian-Indians, while the codon 35 (delC) is a specific mutation among the Malay-Indonesian (Figure 5). We also encourage to use the term Malay-Indonesian specific mutation instead of the

Muslim-Thai<sup>11</sup> since there is no correlation between religion and DNA mutation.

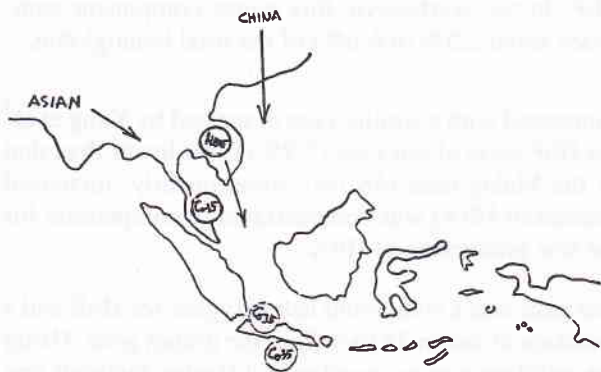


Figure 5. Geographic anthropology of the HbE and the  $\beta$ -thalassemia gene disease and the spread in the South East Asia (SEA).

HbE carrier rate is high in the North Eastern of Thailand and spread to the southern part of SEA nearly 3000 years ago. Other  $\beta$ -thalassemia mutations have spread from India to SEA including Indonesia nearly 5000-6000 years ago. Some new mutations may be specific to the Malay-Indonesian ethnic group.

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