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Hemoglobin M-Saskatoon clarified at molecular level by DNA sequencing of the β-globin gene

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ABSTRACTS

Purnomo Suryantoro - ***Hemoglobin M. Saskatoon clarified at molecular level by DNA sequencing of the β-globin gene***

DNA sequencing of the β-globin gene was done and clarify the Hemoglobin M-Saskatoon at molecular level. A boy was detected to suffer from β-thalassemia since three year old. At four years of age, he underwent splenectomy due to severe splenomegaly. At 10 years of age blood sample was withdrawn and enzymatic sequencing of blood lympocyte DNA showed a mutation at Codon 63 (CAT→TAT). Therefore, hemoglobin M-Saskatoon was diagnosed. This mutation was also found in his mother detected by using NLA III restriction enzyme which digests the wild type of DNA at the CATG/region. This is the first report to demonstrate sequencing technique identifying hemoglobin M instead of using the biophysical examination of the blood oxygen binding affinity.

Key words: β-thalassemia - hemoglobin M - Saskatoon - DNA sequencing - endonuclease restriction enzyme

ABSTRAK

Purnomo Suryantoro - ***Hemoglobin M-Saskatoon yang dipastikan pada tingkat molekular dengan sekuensing DNA gena globin β***

Telah dilakukan sekuensing DNA pada gena globin β dan menemukan adanya hemoglobin M Saskatoon pada tingkat molekular. Seorang anak laki laki terdiagnosis thalassemia β sejak usia 3 tahun. Pada usia 4 tahun dikerjakan splenektomi karena splenomegali berat. Pada usia 10 tahun sekuensing DNA ensimatik menunjukkan mutasi pada kodon 63 (CAT→TAT) ini membuktikan hemoglobin M-Saskatoon. Mutasi tersebut juga dibuktikan terdapat pada ibu dengan mempergunakan ensim restriksi NLA III yang dapat memotong sekuen CATG/. Ini adalah laporan pertama yang menunjukkan teknik sekuensing untuk mengidentifikasi hemoglobin-M tanpa menggunakan pemeriksaan biofisik dan afinitas oksigen eritrosit.

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INTRODUCTION

Thalassemia is not uncommon in Indonesia. It is estimated that not less than 6 million cases are thalassemia or HbE carriers among 154 million people in 1986[1]. The β-thalassemia disease is caused by mutation in the β-globin gene or its immediate flanking regions[2], resulting in abnormal expression of the β-globin gene, i.e. imbalanced ratio of α to β-globin chain synthesis. The heterozygous state are characterized by hypochromic microcytic red blood cells, an increase in proportion of the minor adult hemoglobin (HbA2).

Hemoglobin M is a rare hemoglobin disease in which hemoglobin is partially oxidized to methemoglobin thus reducing oxygen affinity. Cyanosis can be detected since birth without any evidence of respiratory and/or circulatory problems. In certain circumstances, such as when γ-chain was replaced by the β-chain synthesis, cyanosis can be relieved at the age of five weeks.

Measuring oxygen affinity, which is not a simple technique, is always needed to reach the diagnosis of the Hb-M. In this report we showed that this disease could be diagnosed at molecular level using DNA sequencing technique. We have an opportunity to screen 35 β-thalassemia cases and described a case of Hb-M disease from Yogyakarta, Indonesia.

The aim of this report is to present a case of Hemoglobin M (Hb M) Saskatoon disease disclosed by sequencing technique and restriction endonuclease digestion.

CASE

In studying 35 blood samples of β-thalassemia patients from Yogyakarta by DNA sequencing technique, the author found mutation at codon 63 (CAT-TAT) or HbM-Saskatoon in the blood sample of a 10 year old boy. DNA were extracted from the fresh blood sample. The exon 1, 2 and 3 of the β-globin gene were amplified by PCR technique as described elsewhere[3].

Three forward primers were as follows:

ThA : 5'> ACC TCA CCC TGT GGA GCC AC< 3'
ThH : 5'> AGA AAC TGC GCA TGT GGA GA<3
and ThI: 5'> ATT CTG AGT CCA AGC TAG GC<3'

combined with three respective reverse primers :

ThG : 5' >TGA TAG GCA CTG ACT CTC<3'
ThB : 5' >CCC CTT CCT ATG ACA TGA ATT TC<3'
and ThK: 5' >TGC ACT GAC CTC CCA CAT TC <3'

These combined primer will amplify the first region 336 bps (Exon 1), the second region 385 bps (Exon 2) and the third region 384 bps (Exon 3) respectively (FIGURE 1)

After amplification, the PCR products were ligated to pT7 blue vector plasmid and transformed to the competent cells of *E. coli* XL-1 blue. Plasmid DNA extraction was done by alkaline lysis. Plasmid DNA purification was done by using gene cleaned (Boehringer M..?) and DNA sequencing of exon 1, 2 and 3 were carried out by using ABI-Prisms sequencing system. The result shown in FIGURE 2 indicates single mutation on Codon 63 Exon 2 (CAT to TAT).

The restriction endonuclease NLA III, which cuts CATG sequence, was used to detect the mutation on his parent. The enzyme will cut the PCR product of the region 2 of normal sequence into two fragments (191 bps and 194 bps). The digested samples were electrophorized in 3% Nu Sieve Agarose. The result can be seen in FIGURE 3.

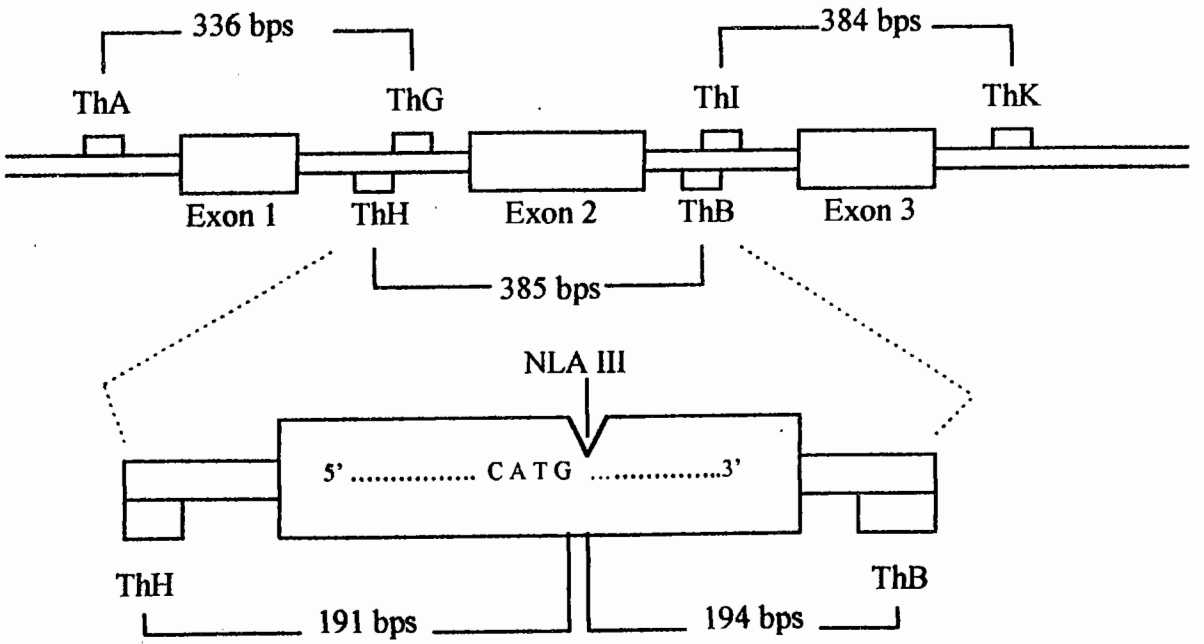

FIGURE 1. - Primer setting and NLA III digesting site

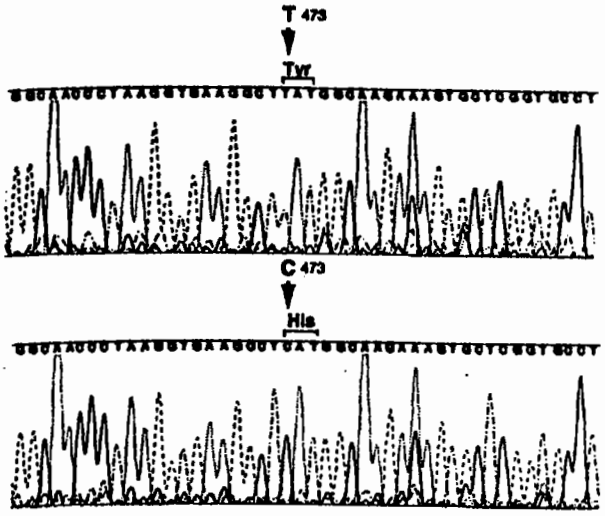

FIGURE 2. - DNA sequencing showed the wild type Co63(CAT) at the lower panel and mutated type Co63(TAT) at the upper panel.

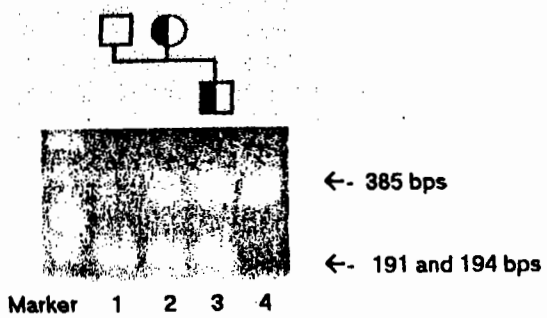

FIGURE 3. - NLA III digestion showed that Co63 (CAT→TAT) was inherited from his mother

DISCUSSION

Horlein and Weber in 1948 described a family in which certain members suffered from congenital cyanosis caused by abnormality in the globin and not in the heme. Their appearance were "lavender blue" and their blood appeared brown. This abnormality was transmitted as an autosomal dominant trait through four generation. In Japan this clinical symptoms have been recognized since 1800 ("*kochikuro*"/black mouth and "*chikuro*"/black blood) in the prefecture of Iwate northeast corner of Honshu. Their blood was as black as Japanese soy sauce. This disease occured since birth until five weeks of age when chain synthesis was replaced by β-chain.

The first biochemical analysis of M-hemoglobin (HbM) shows three variants: M-Boston (α 58 His→Tyr), M Saskatoon (β 63 His→Tyr) and M-Milwaukee-1 (β 67 Val→Glu) whilst the Japanese kochikuro is the fourth variant HbM Iwate (α 87 His→Tyr). The fifth and the sixth were found by Heller *et al*[4] as HbM Hyde Park (β 92 His→Tyr) and Hayashi *et al*[5] Hb FM-Osaka (γ 63 His→Tyr) respectively. Nagai *et al*, 1989[6] clearly reported the different spectrum of M-Saskatoon, M-Hyde Park, M-Boston and M Iwate by resonance Raman Spectra of the hemolysate. Hb M-Iwate could be directly identified upon RASAI digestion at the molecular levels[7].

In the HbM-Saskatoon disease (codon 63), the amino acid of β-globin of distal to heme was changed from histidine to tyrosine, resulting only α chain on this site will carry oxygen, β-chain does not. Therefore only two molecule instead of four molecules of oxygen are bound on this site because the absence of Bohr effect[8].

The MetHb Saskatoon has a weak Fe-tyrosinate interaction. The heme contains six-coordinate heme like normal metHb A, whereas metHb Boston has only five-coordinate heme[9]. It has been reported at p02 of 100 mmHg the oxygenation level of Hb M-Boston-chain was 44% only, while mutant chain was completely oxygenated[10]. The role of carbon monoxide in reducing oxygen content in the Hb was further discussed by Nagai *et al*, 1991[11] and Lian *et al*, 1993[12].

HbM Saskatoon has spread along Germany, Canada, Britain, USA, France, Denmark, Norway, Poland, Italy, South Africa, Japan and Russian[13]. It is rare in blacks[4] and never reported in the area of Southeast Asia. This disease is very rare, in which only 16 paper have been published during 10 years (1983-1993) compared with 293 papers for thalassemia in 1 year (1993) as summarized from Medline CD-Rom search.

Many methods have been used to reach the chemical diagnosis such as para magnetic resonance spectral characteristics, electrophoretic mobility of Hb in pH gradient, reaction with cyanide, thermal stability, and in vitro reduction

with methemoglobin reductase[14] to diagnose 17 cases in the USSR.

In our experiments we found one different nucleotide along the globin gene. So far the polymorphism is a normal genetic variation if it appeared in the region not encoding protein[15,16] .Therefore our finding Co63 (G→T) is a significant problem in which it will affect the individual because of the different amino acid translation. Molchanova TP *et al.*[17] identified a family whose first and second babies with fetal metHb F-M-Fort Ripley and at least[11] additional members of that family were known to have a similar neonatal cyanosis. Kumagai *et al.*[18] also reported a case of inherited Hb M-Iwate in a newborn and they described the similarity of the relative quantities of the fetal and adult form of Hb M-Iwate in their hemolysate.

This disease is inherited as an autosomal dominant traits, but de novo mutation are not uncommon. Rotoli *et al.*[19] discribed a 7 year old girl with 16% methemoglobin and their further investigation identified Hb M-Hyde-Park, neither the parents nor a sister of her showed any abnormality. So far in our case both parents do not seem to be clinically affected therefore it is thought that there is a possibility of de novo mutation.

The NLAIII restriction enzyme digests the wild type CATG/sequence whilst the mutated region (TATG/) is not digested. It is shown in figure 3 where the 385 bps of the region 2 flanking by ThH and ThB primers was completely digested into two fragments of 191 and 194 bps long in the wild type (lane 1),and the other case at lane 2 (the mother) and lane 3 (the case) which is heterozygous has two different alleles, the first is not digested (385 bps) and the other one was digested (191 and 194 bps) This evidence disclosed that the mother was also affected by the same mutation at Co63 (CAT→TAT).

CONCLUSION

Hemoglobin-M Saskatoon in an Indonesian boy of 10 years old diagnosed sufferring from β-thalassemia disease at three years of age is reported. As clinical and biochemical analysis could possibly mislead the diagnosis we conclude that the DNA analysis could give more accurate diagnosis.

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