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

Purnomo Suryantoro
Child Health Department, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia.

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 to 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 At ten years of age blood sample was withdrawn and enzymatic sequencing of blood lymphocyte DNA showed a mutation at Codon 63 (CAT→TAT).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 gene globin β


INTRODUCTION

Thalassemia is not uncommon in Indonesia. It is estimated that not less than 6 million cases are thalassemia or HbE carrier among 154 million people in 1986. The β-thalassemia disease is caused by mutation in the β-globin gene or its immediate flanking region, 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 (fHbA2). 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.

Three forward primers were as follows:
- ThA: 5' - ACC TCA CCC TGT GGA GCA AC - 3'
- ThG: 5' - AGA AAC TGC GCA TGT GGA GA - 3'
- ThH: 5' - ATG CTC AGT CCA AGC TAG GC - 3'

combined with three respective reverse primers:
- ThG: 5' - TG A TAG GCA CTG ACT CTC - 3'
- ThH: 5' - CCC CTT ATG ATA GCA GAG ATT TC - 3'
- ThK: 5' - TGC ACT GAC CTG 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-Prism 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 Steve Agarose. The result can be seen in FIGURE 3.

![FIGURE 1. Primer setting and NLA III digesting site](image)
Iwate northeast corner of Honshu. Their blood was as black as Japanese soy sauce. This disease occurred since birth until five weeks of age when chain synthesis was replaced by \( \beta \)-chain.

The first biochemical analysis of M-hemoglobin (HbM) shows three variants: M-Boston (c. 55 His->Tyr.), M Saskatoon (c. 63 His->Tyr.) and M-Milwaukee-1 (c. 67 Val->Glu) whilst the Japanese kochiurou is the fourth variant HbM iwaue (c. 87 His->Tyr.). The fifth and the sixth were found by Heller et al.\(^{10}\) as HbM Hyde Park (c. 92 His->Tyr.) and Hayashi et al.\(^{11}\) as Hb FM-Osaka (c. 63 His->Tyr) respectively. Nagai et al., \(^{12}\) clearly reported the different spectrum of M-Saskatoon, M-Hyde Park, M-Boston and M-Iwaue by resonance Raman Spectra of the hemolysate. Hb M-iwaue could be directly identified upon RASAI digestion at the molecular levels\(^{13}\).

In the HBM-Saskatoon disease (codon 63), the amino acid of \( \beta \)-globin of distal to heme was changed from histidine to tyrosine, resulting only \( \alpha \) chain on this site will carry oxygen, \( \beta \)-chain does not. Therefore only two molecule instead of four molecules of oxygen are bound on this site because the absence of Bohr effect\(^{14}\).

The MetHb Saskatoon has a weak Fe-tetrasionate interaction. The heme contains six-coordinate heme like normal methb A, whereas methb Boston has only five-coordinate heme\(^{15}\). 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\(^{16}\). The role of carbon monoxide in reducing oxygen content in the HB was further discussed by Nagai et al., 1991\(^{17}\) and Lian et al., 1993\(^{18}\).

HbM Saskatoon has spread along Germany, Canada, Britain, USA, France, Denmark, Norway, Poland, Italy, South Africa, Japan and Russia\(^{19}\). It is rare in blacks\(^{20}\) 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\textsuperscript{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\textsuperscript{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.\textsuperscript{17} identified a family whose first and second babies with fetal methH F-M-Fort Ripley and at least\textsuperscript{1} additional members of that family were known to have a similar neonatal cyanosis. Kumagai et al.\textsuperscript{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 trait, but de novo mutation we not uncommon. Rotoli et al.\textsuperscript{19} described a 7 year old girl with 16% methemoglobin and their further investigation identified Hb M-Hide-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 NLAII 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 bp to 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 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 Sasketoon in an Indonesian boy of 10 years old diagnosed suffering 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.

REFERENCES

