C TO T TRANSITION AT THE FIRST NUCLEOTIDE OF CODON 63 OF THE β-GLOBIN GENE CORRESPONDING TO HEMOGLOBIN M-SASKATOON IN AN INDONESIAN BOY

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Summary   Hemoglobin (Hb) M-Saskatoon, a β variant of methemo-globin, is characterized by mild hemolysis.   It is caused by the substitution of a histidine by a tyrosine at the 63rd amino acid residue of the β-globin chain.   Amplification and sequence analysis of genomic β-globin DNA from an Indonesian boy diagnosed as having the more severe disease thalas-semia demonstrated the presence of a C to T transition at nucleotide 473 in one of the two β-blogin genes resulting in a histidine to tyrosine sub-stitution at 63rd residue.   This amino acid change matched with that reported in Hb M-Saskatoon.   This nucleotide change abolished a recogni-tion site for the restriction endonuclease *Nla*III.   *Nla*III digestion of the corresponding β-globin DNA amplified from the patient's parents indicated that the mutation was inherited through from his mother.   This result shows that the world-wide distribution of Hb M-Saskatoon extends to Indonesia, where it was not previously identified.   Possible causes of the unusually severe symptoms observed in the case are discussed.
Key Words   hemoglobin M, hemoglobin M-Saskatoon, β-globin, thalassemia

INTRODUCTION

Methemoglobins (Hb Ms) are a group of mutant hemoglobins (Hbs) which have amino acid substitutions in the heme pocket that directly affects the heme-globin bond.   Amino acid analysis of Hb Ms disclosed substitutions of histidine

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residues in either the α or β chains of globin. The abnormal chains are maintained in the ferric (methemoglobin) form and cannot undergo reversible oxygenation. Patients with α-chain hemoglobin M show severe clinical symptoms such as cyanosis from birth, but β-chain hemoglobin M induces only mild clinical symptoms. Hb M Saskatoon is one of the β-chain Hb Ms. It shows a dominant inheritance pattern and is characterized by the substitution of a histidine by a tyrosine at the 63rd residue (Bunn and Forget, 1986).

Several lines of evidence indicate that β-globin of Hb M Saskatoon is slightly unstable. Hb M-Saskatoon produces only mild compensated hemolysis (Baine *et al.*, 1980), which is sometimes markedly accelerated by exposure to heat or the administration of an oxidant drug (Bunn and Forget, 1986). There has been no report of a Hb M-Saskatoon case showing severe anemia such as β-thalassemia.

Hb M-Saskatoon has been found in Germany, Canada, Britain, the United States, France, Denmark, Norway, Poland, Italy, South Africa, Japan, the Soviet Union (Bunn and Forget, 1986), and Algeria (Arbane-Dahmane *et al.*, 1985). The various exotic names that have been applied to Hb M-Saskatoon provide further testimony to the world-wide distribution of these variants. However, no case has been reported from South East Asia where many other kinds of mutations of the β-globin gene have been reported (Fucharoen *et al.*, 1989; Lie-Injo *et al.*, 1989).

In the past, Hb M-Saskatoon has been identified by analyzing the amino acid sequence of β-globin (Bunn and Forget, 1986). Recent advances in molecular biology make it possible to determine mutations at DNA sequence level. Until now there has been no report describing the nucleotide changes in Hb M-Saskatoon, although a C to T transition must have occurred at nt. 473 in order to cause the observed amino acid substitution at the 63rd residue. In this report we demonstrated that codon 63 was changed from CAT to TAT in an Indonesian patient showing signs of thalassemia, and that *Nla*III digestion of PCR amplified exon 2 region from genomic β-globin DNA can be used as a simple method to detect this mutation.

CASE AND METHODS

Case. A 10-year-old Indonesian male was admitted to Dr. Sardjito Hospital, Yogyakarta, Indonesia, because of paleness. He was born to Indonesian parents and had no siblings. Although his father and mother had been in healthy condition, they showed mild anemia (11.8 and 10.2 g/dl, respectively) and high fetal hemoglobin (6.8 and 7.8%, respectively: control, <0.4%). No other abnormal peak was pointed out on their electrophoretic pattern of hemoglobin. No definitive clinical diagnosis has been established in his parents.

His mother noticed his paleness when he was 3 months old. At the age of 4, abdominal distention was noticed and an abdominal tumor was diagnosed by a doctor. At 4.5 year old, the abdominal tumor was identified as a distended spleen and splenectomy was performed. After the operation he was in good condition

and had no significant clinical abnormalities.   However, at the age of 8 he was found to be still anemic (10.3 g/dl); electrophoretic analysis of hemoglobin showed a high percentage of fetal hemoglobin (8.2%) but no other abnormality in electrophoretic pattern was pointed out.   He was clinically diagnosed as having thalassemia major.

After admission to hospital, he was examined for his paleness.   Physical examination showed no remarkable finding except paleness.   History taking disclosed no particular incident before his admission.   Only blood examination disclosed severe anemia (6.2 g/dl).   In spite of intensive care he died because of unexpected cardiac arrest.   Blood obtained before blood transfusion was subjected to a search for a mutation in the β-globin gene.

Methods.   DNA was extracted from blood cells by standard phenol/chloroform extraction methods and used as a template for polymerase chain reaction (PCR) amplification.   The β-globin gene was amplified as three separate fragments (fragment I, II, and III) by using three sets of primers (Table 1).   Each of three fragments covers one entire exon and its flanking introns.   PCR was carried out as described previously (Varawalla *et al.*, 1991).   The amplified DNA fragment was separated by electrophoresis on a 3% agarose gel and photographed after ethidium bromide staining.

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

In order to study the inheritance of the mutation and to determine its incidence among thalassemia patients, the amplified product containing exon 2 (fragment II in Table 1) was digested with *Nla*III according to the method suggested by the supplier (Toyobo, Osaka) and the digested fragments were electrophoresed in a 3% agarose gel.

RESULTS

Since the patient was clinically diagnosed as thalassemia major, we tried to identify mutations in the β-globin gene.   The β-globin gene were amplified as three

Table 1.   Primer sequences used to amplify three fragments of the β-globin gene.

Fragment	Sequence 5′——→3′	Complementary site [a]
I	A C C T C A C C C T G T G G A G C C A C	−5 to 14
	G A G A G A G T C A G T G C C T A T C A	346 to 327
II	A G A A A C T G G G C A T G T G G A G A	286 to 305
	C C C C T T C C T A T G A C A T G A A C T T A A	670 to 647
III	A T T C T G A G T C C A A G C T A G G C	1386 to 1405
	T G C A C T G A C C T C C C A C A T T C	1771 to 1752

[a] Numbering of the β-globin sequence is according to Lawn *et al.* (1980).

separate regions. Electrophoresis on agarose gels revealed the three amplified prod-
ucts as single DNA bands which comigrated with the corresponding products
amplified from the genomic DNA of a normal individual. To determine whether
a point mutation was present in the patient's β-globin genes we sequenced all three
amplified fragments as described in the methods section. The nucleotide sequences
of amplified DNAs covering exon 1 and its flanking introns (fragment I) or exon 3
and its flanking introns (fragment III) were identical to those of the wild-type gene
in all clones studied. In particular, two types of DNA clone were obtained by
subcloning the amplified exon 2 encompassing DNA (fragment II). The sequence
of one of them was identical to that of wild type exon 2 region (Fig. 1 lower panel).
The other type of clone had a single nucleotide change, from C to T, at position
473 (Fig. 1 upper panel). The mutated nucleotide corresponded to the first base of
codon 63 of β-globin; the codon is changed from CAT to TAT, resulting in a his-
tidine to tyrosine substitution. This amino acid substitution has been reported
as Hb M-Saskatoon. Thus, we diagnosed the case as Hb M-Saskatoon at the
molecular level.

As the C to T transition was located in the sequence CATG recognized by the
restriction endonuclease *Nla*III, the DNA fragment harboring the mutation should
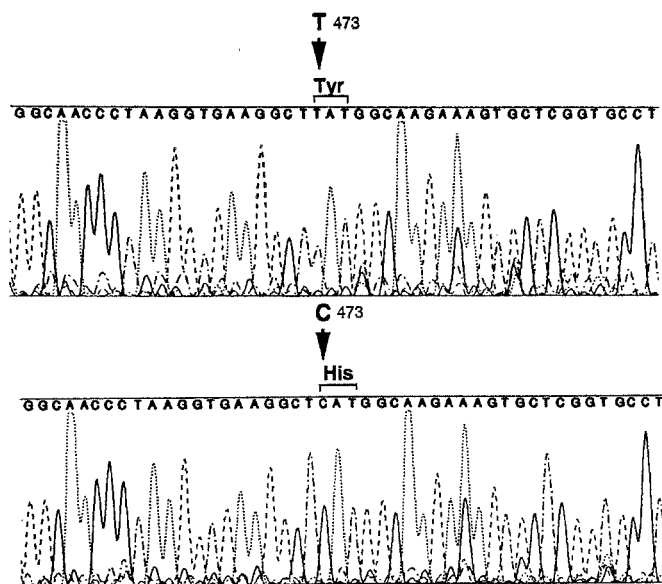

Fig. 1. Nucleotide sequence of part of the β-globin gene. Only the nucleotide sequences
around the site of point mutation of exon 2 were described. In the nucleotide
sequence of the patient's DNA nucleotide T (nt. 473) at the first nucleotide of
codon 63 (bottom) was replaced by C nucleotide (top). Bracket indicates 63rd
codon. The *Nla*III restriction enzyme recognition site (CATG) was absent from
the patient's sequence. The numbering is according to the β-globin sequence
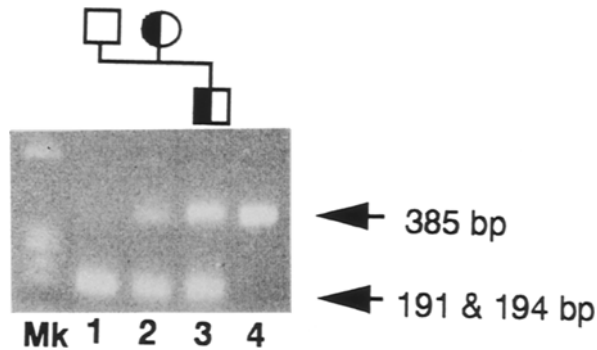(Lawn *et al.*, 1980).

Fig. 2. NlaIII digestion of PCR-amplified genomic DNA. A gemonic DNA fragment encompassing exon 2 of the β-globin gene (fragment II) was amplified, digested with NlaIII, and subjected to electrophoresis. The allele with a point mutation at T to C remained intact as a 385 bp fragment which corresponds to the un-digested product (lane 4), but digestion of the normal DNA yielded one visible fragment consisting of 191 and 194 bp fragments (lane 2). Top, pedigree with squares representing males and circles representing females. The affected individual is represented by a filled symbol and the carrier state is represented by half-filled symbol. The size marker is a size ladder HincII digested øX174 phage DNA (Toyobo, Osaka).

not be cleaved by this enzyme. The normal amplified product was indeed digested completely to produce two fragments (191 and 194 bp) which comigrated upon electrophoresis (Fig. 2 lane 1). In contrast, two bands were visualized on the aga-rose gel when the digested product of the patient was applied (Fig. 2 lane 3). The larger band was identified as uncleaved DNA (Fig. 2 lane 4), the other band re-presenting the mixed 191 and 194 bp bands found in the digested DNA from the normal individual (Fig. 2 lane 1). These results confirmed that the patient was heterozygous for the mutation.

To determine the inheritance pattern of the mutation, amplified products from the parents were also subjected to NlaIII restriction enzyme digestion. Although amplified exon 2 encompassing region (fragment II) from the father was completely digested by the enzyme, fragment II from his mother produced a mixture of two clearly resolved bands identical to those obtained with fragment II from the patient. This result showed his mother carries the same mutation and thus that the Hb M-Saskatoon allele was inherited from his mother.

To determine the incidence of the mutation among Indonesian thalassemia patients, amplified fragment II from 50 cases was digested with NlaIII. In all 50 cases, the amplified DNA fragment was correctly and completely cleaved by the restriction enzyme, indicating that the mutation is not common among thalassemia patients.

DISCUSSION

Hb M-Saskatoon results from the substitution of the 63rd residue of β-globin chain, histidine, by tyrosine. The only point mutation which can cause this amino acid change is a C to T transition at the first nucleotide of the codon, as shown to be the case here.

Our result confirmed that Hb M-Saskatoon is also present in Indonesia, where β-thalassemia is common. In malaria endemic areas as Indonesia, three hemoglobinopathies including α- and β-thalassemias and sickle cell anemia are very common genetic diseases since they confer resistance to malarial infection. In contrast to these hemoglobinopathies, the hemoglobinopathy of Hb M-Saskatoon has been reported to be rare in black people, although a case has been reported in Africa (Arbane-Dahmane *et al.*, 1985). In the present study we failed to identify the same mutation in group of 50 Indonesia patients with thalassemia. Thus, the mutation seems to be rare even though mutations in the β-globin gene are common among Indonesians.

If we accept that all Hb M-Saskatoon cases are due to a single ancestral mutation, then it must have spread throughout the world by population migration. Another possibility is that an unknown factor predisposing the same mutation may be present around the mutation site in the β-globin gene. C to T transitions such as that found in Hb M-Saskatoon are the commonest point mutations identified in human genes (Labuda and Striker, 1989).

In the present case, we could not identify any mutation other than Mb H-Saskatoon in the analyzed regions of the β-globin gene including both exon and intron sequences (Table 1). Thus the thalassemia diagnosis could not be confirmed at the molecular level. However, the patient's symptoms were clearly those of β-thalassemia rather than of Hb M-Saskatoon. We consider that the severe symptoms might be explained in three ways. First, both parents have high levels of blood fetal hemoglobin, and are therefore likely to be thalassemia carriers. Thus, the patient may have been a compound heterozygote for thalassemia, a situation which is not uncommon among South-East Asians. However, we have so-far failed to identify any nucleotide changes (other than the C to T transition in exon 2) in the sequenced segments of the child's β-globin gene, which would argue against this idea.

The second possibility is that the case inherited the Hb M-Saskatoon trait from his mother and the β-thalassemia trait from his father. The β chain bearing the tyrosine substitution is reportedly synthesized at the normal rate, but is more likely to be degraded than normal β chains (Baine *et al.*, 1980). This may induce an imbalance of the levels of α and β chains in red blood cells, as observed in thalassemia. Thus, a patient who is compound-heterozygous for both Hb M-Saskatoon and β-thalassemia may exhibit severe anemia like that shown by patients with β-thalassemia. Since, as mentioned above, we have not yet identified the mutation

responsible for the thalassemia heterozygosity in this case, this supposition must be tested by further studies.

The third proposed explanation for the severe clinical signs is that they are indeed due solely to Hb M-Saskatoon. In one case of Hb M-Saskatoon, signs of mild hemolysis, anemia, splenomegaly, reticulocytosis, and hyperbilirubinemia were reported (Shibata et al., 1967), although all of these signs are fully compatible with β-thalassemia. Thus, the index case be another unusual case of Hb M-Saskatoon, but why did he develop severe anemia at the last stage? The answer might be that he was exposed to an unknown oxidizing agent which triggered the severe hemolytic crisis as described in some Hb M cases (Bunn and Forget, 1986). However, this hypothesis seems incompatible with the fact that the mother carrying the same mutation did not show signs of anemia during the same period, even though she would probably have been exposed to the same conditions as her son. One way to determine the cause of the severe anemia and to discover its genetic basis will be to study the case histories of other patients with Hb M-Saskatoon. NlaIII digestion of amplified β-globin exon 2 region provides a simple method for the molecular diagnosis of this disease, and could prove particularly useful in such a study.

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