RESEARCH NOTE

A 65 BP DELETION IN BAND 3 GENE OF β-THALASSEMIA PATIENTS IN INDONESIA

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Abstract. We investigated whether in addition to the well known genetic alteration in red blood cell membrane band 3 protein, a deletion of 9 amino acids leading to ovalocytosis, other mutations to band 3 also exist. In 12 % of our thalassemia major patients investigated, we found two bands in the agarose gel-electrophoresis of PCR products from band 3 gene with a difference of 65 ± 10 bp, equivalent to a deletion of 20 to 25 amino acids in band 3 protein. Thus, a co-existing band 3-mutant allele in addition to the thalassemic globin gene defects, could also contribute to erythrocyte membrane defects and to the spectrum of clinical symptoms of these thalassemia major patients.

Keywords: thalassemia, band-3 gene, deletion, PCR, electrophoresis, Indonesia

INTRODUCTION

The red blood cell (RBC) membrane is roughly composed of 50% phospholipid and 50% membrane proteins. In previous studies, we investigated membrane order parameters with spin-labeled fatty acids and N-ethylmaleimide (NEM) derivatives to obtain information about membrane structure (Udyaningsih-Freisleben *et al*, 2003), both in lipid and protein domains, especially under the influence of oxidative stress in thalassemic patients (Laksmitawati *et al*, 2003).

The stability of RBCs depends primarily on its membrane and is strongly influenced by the properties of the cytoskeleton proteins (spectrin, ankyrin) and their interactions with components of integral membrane proteins (band 3, gylcophorins). Band 3 has cytoplasma domains interacting with spectrin, mediated via ankyrin and contributing to the structural stabilization of RBC. On the other hand, in the membrane, band 3 functions as an anion exchanger. Hence, band-3 protein is essential to structure as well as function of erythrocytes (Bruce *et al*, 2000).

In Southeast Asia, a vast variety of mutations and deletions in anion trans-

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porters has been reported including band 3 protein in erythrocyte membrane (Bruce *et al*, 2000). Band 3 gene alterations may lead to spherocytosis with mutations in the cytoplasmatic domain (Jarolim *et al*, 1995), acanthocytosis with mutation of 868 proline to leucine (P868L), or ovalocytosis with a deletion of 27 bp at codon 400-408 of exon 11 (a loss of 9 amino acids) and a substitution of adenine-56 by guanine (Jarolim *et al*, 1991; Mohandas *et al*, 1992).

In this report, we investigated whether β-thalassemia patients in Jakarta, Indonesia, have deletions in band 3 gene as determined by analysis of PCR products using agarose gel-electrophoresis.

MATERIALS AND METHODS

Blood samples were collected from healthy controls and β-thalassemia patients, intermedia (not yet transfused) and major (transfused) from the Thalassemia ward of RSCM Jakarta, Indonesia. Samples from 50 transfused patients were taken immediately before their transfusion. Healthy controls did not exhibit signs of anemia, *ie*, they had normal Hb levels and did not have a family history of thalassemia or ovalocytosis. All subjects signed their consent to participate in the study which was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia.

Isolation of DNA from white blood cells

For DNA isolation, the buffy coat was carefully removed from sedimented RBCs (Eppendorf Centrifuge, Uppsala, Sweden), a 200 µl aliquot was added to 200 µl of binding buffer (High Pure Template Preparation Kit; Roche, Indianapolis, IN) and 40 µl of proteinase K and incubated for 10 minutes at 72ºC (Sambrook and Russel, 2001). Then, 100 µl of isopropanol (Boehringer 1119915, RNase-DNase-free) were added and thoroughly mixed. The mixture was transferred into a High Pure Purification Filter connected to a collecting tube and centrifuged for one minute at 5,500*g*. The solution in the collecting tube was discarded and the tube re-connected to the filter cup, which contained the DNA. A 500 µl aliquot of inhibitor removal buffer was added into the cup and centrifuged for one minute at 5,500*g*. The DNA was washed twice with wash buffer. A 200 µl aliquot of elution buffer pre-heated to 70ºC was added and centrifuged for one minute at 5,500*g*. The solution of purified DNA was stored at -4ºC. DNA concentration was measured in a Shimadzu double-beam spectrophotometer at 260 nm (A_{260}) and purity determined by A_{260}/A_{280} ratio (Holme and Peck, 1993).

Polymerase chain reaction (PCR)

For DNA amplification, a pair of primers was used: P1, 5'-GGGCCCA-GATGACCCTCTGC-3'; bases 1098-1117 and P2, 5'-GCCGAAGGTGATGGC-GGGTG-3'; bases 1272-1253 (Genset Biotech, Singapore). Primers, 60 µg of isolated DNA, PCR buffer and dNTP mix (PCR Core Kit Roche, Indianapolis, IN), and DNA AmpliTaq polymerase (1.25 U; Promega, Madison, WI) were mixed and total volume adjusted to 25 µl with sterile H_2O. The mixture was centrifuged for a few seconds to homogeneity at 18,000*g*. Thermocycling (PCR Mastercycler Personal Eppendorf) was carried out as follows: 95ºC for 5 minutes then 30 cycles of 95ºC for 30 seconds; 58 ºC for 30 seconds; 70ºC for 1 minute; followed by final extension step at 70 ºC for 5 minutes and holding temperature at 4ºC. The PCR products were stored at -20ºC until further analysis (Muladno, 2002). The resulting normal PCR product is 175 bp.

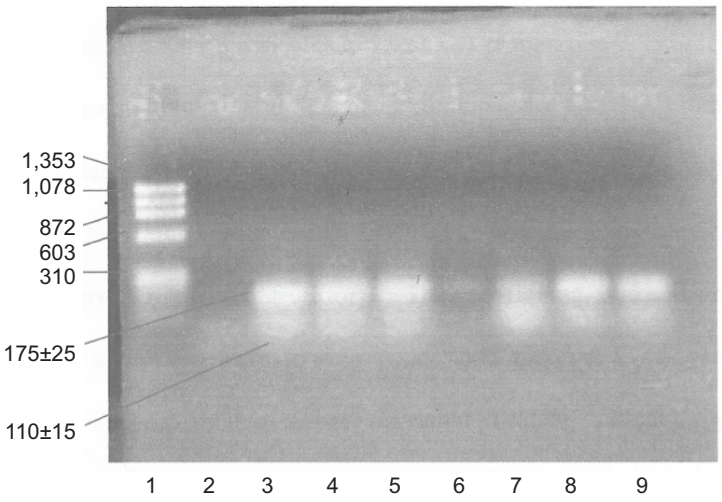

Fig 1–Agarose gel-electrophoresis of PCR products from band 3 gene: lane 1, DNA markers; lane 2, empty (no sample); lane 6, DNA sample from healthy control; lanes 3, 4, 5, 7, 8, 9, DNA samples from 6 individual thalassemia major patients.

Agarose gel electrophoresis

The 2% agarose gel was cast in TAE 1X buffer pH 8.4 and aliquots of PCR products and Roche DNA marker (X174 DNA cleaved with *Hae*III, with a size range from 72 to 1,353bp) were prepared with loading dye (bromphenol blue and xylene cyanol; Merck, Damstadt, Germany) and elecrophoresis at 95V for 30 minutes, stained with 0.02% ethidium bromide (Bio-Rad, Hercules, CA) for 2 minutes, washed twice with H_2O, then visualized and documented (Lewis, 2001) using an UV transluminator, Vilber Lourmat TCX-15.M and Polaroid Fuji 3000B.

RESULTS

Fig 1 shows agarose gel-electrophoresis of a DNA sample from a healthy control (lane 6) and on lanes 3, 4, 5, 7, 8, and 9 are samples from 6 thalassemia major patients, indicating two bands, one at normal 175 bp and a deleted band at 110 bp, the latter indicating a loss of about 20-25 amino acids. This was observed in 6/50 (12%) of the samples from thalassemia major patients.

DISCUSSION

The PCR product of the band 3 gene of healthy controls exhibited a length of 175 ± 25 bp. However, 12% of samples from thalassemia major patients demonstrated two PCR products, one of 175 bp and another one of 110 ± 15 bp. Patients with ovalocytosis also have two PCR products, one of 175 bp and another of 148 bp (Jarolim *et al*, 1991). The loss of 9 amino acids in the band 3 protein leads to perturbation to the protein components of the cytoskeleton interacting with band 3, resulting in membrane with the abnormal form of "ovalocytosis" and reduction in flexibility and deformability (Mohandas *et al*, 1992). Band 3 gene deletion in some thalassemia patients certainly contributes to pathologic alterations of the erythrocyte membrane similar to ovalocytosis. In other words, these patients have a more complex membrane pathology.

Future investigations are needed to clarify the sequence and location of the deletion in the band 3 gene and its immediate impact on structure and function of band 3 protein, and to correlate it to the clinical conditions of the patients. Although we have detected one novel deletion in band 3 gene in thalassemia patients this does not mean that it is the only such gene alteration (deletion or mutation) existing in thalassemic patients in Indonesia.

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REFERENCES

Bruce LJ, Wrong O, Toye AM, *et al*. Band 3 mutations, renal tubular acidosis and South-East Asian ovalocytosis in Malaysia and Papua New Guinea: loss of up to 95% band 3 transport in red cells. *Biochem J* 2000; 350: 41-51.

Holme DJ, Peck H. Analytical biochemistry. 2nd ed. New York: Longman Scientific & Technical Publishers, 1993.

Jarolim P, Hillard LR, Vaclay B, *et al*. Mutation of conserved arginines in the membrane domain of erythroid band 3 lead to a decrease in membrane associated band 3 and to the phenotype of hereditary spherocytosis. *Blood* 1995; 85: 634-40.

Jarolim P, Palek J, Amato D, *et al*. Deletion in erythrocyte band 3 gene in malaria-resistant Southeast Asian ovalocytosis. *Proc Natl Acad Sci USA* 1991; 88: 11022-6.

Laksmitawati DR, Handayani S, Udyaningsih-Freisleben SK, *et al*. Iron status and oxidative stress in β-thalassemia patients in Jakarta. *BioFactors* 2003; 19: 53-62.

Lewis M. Agarose gel electrophoresis (basic method). Biological protocols. Liverpool: University of Liverpool, 2001. [Cited 2013 Oct 14]. Avilable from: URL: http://www.methodbook.net/dna/agarogel.html

Mohandas N, Winardi R, Knowles D, *et al*. Molecular basis for membrane rigidity of hereditary ovalocytosis, a novel mechanism involving the cytoplasmic domain of band 3. *J Clin Invest* 1992; 89: 686-92.

Muladno. Around the Technology of genetic engineering. Bogor: Penerbit Pustaka Wirausaha Muda, 2002 (In Bahasa Indonesia).

Sambrook J, Russel DW. Molecular cloning. A laboratory manual. 3rd ed. New York: Cold Spring Harbor Laboratory Press, 2001.

Udyaningsih-Freisleben SK, Kurniati V, Prasetyo PB, *et al.* Isolated erythrocyte membranes of transfusion-dependent and non-transfused thalassemia patients in Jakarta – investigated by electron paramagnetic resonance spectroscopy, *BioFactors* 2003; 19: 87-100.