HAEMATOLOGY

HbA2 levels in β-thalassaemia carriers with the Filipino β0-deletion: are the levels higher than what is found with non-deletional forms of β0-thalassaemia?

E. GEORGE*, LAI KUAN TEH*, JAMA TAN†, MEI I. LAI* AND LILY WONG‡

*Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, †Department of Molecular Medicine, Faculty of Medicine, University of Malaya, and ‡Department of Medicine, Queen Elizabeth Hospital, Kota Kinabalu, Sabah, Malaysia

Summary

Aims: Classical carriers of β0-thalassaemia are identified by a raised HbA2 level. Earlier studies indicated that the Filipino β0-deletion has high raised HbA2 levels. The introduction of automated high performance liquid chromatography (HPLC) for thalassaemia screening is an important advancement in technology for haematology laboratories. The BioRad Variant II Hb analyser is a common instrument used to quantify HbA2 levels in thalassaemia screening. This study aimed to determine HbA2 levels in carriers of Filipino β0-mutation using the BioRad Variant II Hb analyser.

Methods: The Filipino β0-deletion was identified using gap-polymerase chain reaction (PCR) in the parents of transfusion dependent β0-thalassaemia patients who were homozygous for the Filipino β0-deletion in the indigenous population of Sabah, Malaysia. Hb subtypes were quantified on the BioRad Variant II Hb analyser. Concurrent α-thalassaemia was identified by multiplex gap-PCR for deletions and amplification refractory mutation system (ARMS)-PCR for non-deletional mutations.

Results: The mean HbA2 level for Filipino β0-thalassaemia trait was 5.9 ± 0.47 and with coinheritance of α-thalassaemia was 6.3 ± 0.44 (α–3.7 heterozygous) and 6.7 ± 0.36 (α–3.7 homozygous). The HbA2 levels were all >4% in keeping with the findings of classical β-thalassaemia trait and significantly higher than levels seen in non-deletional forms of β-thalassaemia.

Conclusion: The HbA2 level measured on the BioRad Variant II Hb analyser was lower than the level in the first description of the Filipino β0-thalassaemia. β-thalassaemia trait with coinheritance of α-thalassaemia (α–3.7) is associated with significantly higher HbA2 level.

Key words: Filipino β0-deletion, HbA2 levels, indigenous population of Sabah, β-thalassaemia.

Received 30 May, revised 30 July, accepted 31 July 2012

INTRODUCTION

The thalassaemias are an autosomal recessively inherited group of disorders of haemoglobin (Hb) synthesis characterised by the absence or reduction of one or more of the globin chains of human Hb. The most severe form of β-thalassaemia, β-thalassaemia major results in transfusion dependent anaemia where cure is possible with human leukocyte antigen (HLA) matched stem-cell transplantation and perhaps gene therapy.

The molecular basis of β-thalassaemia is a consequence of inheritance of mutations in the β-globin gene complex. The common β-thalassaemia alleles are point mutations and extensive deletions are rare. In the Kadazan/Dusun, an indigenous population of Sabah, a state of Malaysia in the north east of Borneo, homozygosity of Filipino β0-deletion is the common mutation seen in transfusion dependent thalassaemia patients.1–3 The β-thalassaemia Filipino β0-deletion was first reported by Motum et al.4 and later by other investigators.5–9 Carriers of β-thalassaemia have normal Hb level or mild anaemia with hypochromic and microcytic cells. In a screening program on complete blood counts (CBC) for thalassaemia, the red cell indices MCV of <80 fl and MCH of <27 pg are used as cut-off points for further studies.10 The diagnosis of a β-thalassaemia carrier requires accurate quantitation of HbA2. The methods used for quantitation include cellulose acetate electrophoresis at alkaline pH with elution and spectrophotometry, densitometric scanning, isoelectric focusing, high performance liquid chromatography (HPLC) and more recently capillary electrophoresis. The HbA2 level is raised in classical β-thalassaemia carriers. Earlier studies on β-thalassaemia Filipino β0-deletion indicated that carriers had high raised HbA2 levels.8 Introduction of automated HPLC for thalassaemia screening is an important advance in technology for haematology laboratories. Cation-exchange high performance liquid chromatography (CE-HPLC) has become the method of choice for quantitation of HbA2 in laboratories.11–14 The BioRad Variant II, an automated Hb analyser, is the instrument that is used in Malaysia.15,16 This study reports on the HbA2 level in carriers of β-thalassaemia with the Filipino β0-deletion and its implication in screening of thalassaemia.

MATERIALS AND METHODS

Studies on thalassaemia in the indigenous population of Sabah were approved by the Medical Ethics Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, in accordance with the declaration of Helsinki. Written informed consent was obtained from the study participants prior to blood sample collection.

Samples

Parents of patients with homozygous Filipino β0-thalassaemia deletion from the indigenous population of Sabah formed the study group. DNA amplification techniques were performed to identify the Filipino β0-thalassaemia deletion.
PCR for agarose gel in 1x tris-acetic-EDTA (TAE) buffer at 10 volts/cm for 20 min. Scientific, USA) and gel electrophoresis using 0.8% ethidium bromide-stained agarose gels. The deletion was confirmed by DNA application across the deleted region of the -thalassaemia deletion.17–19 DNA amplification was carried out using primer: F31 (5'-CTTACAGGCATATCCC-3') and F33 (5'-CATTTAGCTCCCACACTCCT-3'), within the L1 repetitive element, 188 to 208 bp downstream of the Filipino -globin gene cap site), F32 (5'-TCAGAAGCAGAGCT-3'), and F32 primers, 0.6 M of F33 primers and 1x PCR-master solution with a mixture of 10 mM KCl, 10 mM Tris (pH 8.3), 200 μM of each dNTP, 0.02 U/μL Taq polymerase and 2% glycerol. Cycling conditions were carried out using a thermalcycler (Takara PCR Thermalcycler Dice, TP600 gradient; Takara Bio, Japan), with an initial denaturation for 5 min at 95°C, followed by 35 cycles of 95°C denaturation for 1 min, 60°C annealing for 1 min, 72°C extension for 1 min and a final extension for 10 min at 72°C. Each amplified product (10 μL) was analysed using 1.5% ethidium bromide stained-agarose gel in 1x TAE buffer at 10 volts/cm for 1 h. The gel was then visualised on an ultraviolet transilluminator (G:box Bioimaging Systems; Synoptics, UK). The deletion was amplified as a 376 bp fragment and the undeleted -globin gene sequence (wild type allele) amplified as a 482 bp fragment.

DNA amplification for α-thalassaemia
α-thalassaemia deletion mutation
The four most common deletions in α-thalassaemia, α-3.7-, α-4.2-, α-4.2L, and α-20.1-, were identified using a multiplex-PCR protocol to amplify across the breakpoints of each α-thalassaemia deletion.17–20 DNA amplification was carried out in 20μL reactions containing 100 ng genomic DNA, 0.5x Q-solution, primers and 1x Qiangen multiplex PCR-master solution with 3 mM MgCl2, HotStar Taq DNA polymerase and dNTP mix. Cycling conditions included initial denaturation for 15 min at 96°C, followed by 30 cycles of 98°C denaturation for 45 s, 66°C annealing for 90 s, 72°C extension for 1 min and a final extension for 5 min at 72°C using a thermocycler (Veriti Thermalcycler; Applied Biosystems, USA). All PCR products were electrophoresed on ethidium bromide stained agarose gels.

α-thalassaemia non-deletion mutation
Six α-thalassaemia non-deletion mutations, initiation codon (ATG→AGG), codon 30 (GAG), codon 35 (TCC→CCC), codon 59 (GGC→GAC; Hb Adana), codon 125 (CTG→CCG; Hb Quong Sze) and termination codon (TAA→CAA; Hb Constant Spring), were identified using a modified multiplex-PCR assay with the published primer sequences.20 DNA amplification was carried out in 20μL reactions containing 100 ng genomic DNA, 0.5x Q-solution, primers and 1x Qiangen multiplex PCR-master solution with 3 mM MgCl2, HotStar Taq DNA polymerase and dNTP mix. Cycling conditions included an initial denaturation for 12 min at 94°C, followed by 32 cycles of 94°C denaturation for 40s, 64°C annealing for 20s, 72°C extension for 3 min and a final extension for 5 min at 72°C. All PCR products were electrophoresed on ethidium bromide stained agarose gels.

HPLC
Quantification of Hb subtypes was performed on the BioRad Variant II cation-exchange HPLC. HB analyser using the -thalassaemia short program and adhering to manufacturer’s instructions (BioRad Laboratories, USA). The Hb analyser, a fully automated HPLC system, uses double wave length detection (416 and 690 nm), measures HbA2 and HbF accurately, and presumptively identifies Hb variants by specific retention times. Each run takes 6.5 min and linearity for measurements of HbA2 and HbF are 12% and 40%, respectively. Normal adults have HbA2 levels between 2.1 and 3.3%. In classical β-thalassaemia trait the HbA2 levels are 4–9%.14,15 In our laboratory, the HbA2 levels for carriers of non-deletional β-thalassaemia are 5.2±0.55 (Table 1, Group 4).

Statistical analysis
Data analysis was performed using SPSS (Version 19; SPSS, USA). All data are represented as mean ± SD. Independent Student’s t-test was used to compare two means. A p value of less than 0.5 was regarded as significant.

RESULTS
A total of 139 samples from parents who were carriers of Filipino β-thalassaemia were studied. They belonged to the Kadazan/Dusun indigenous group that comprises the largest indigenous population in Sabah, East Malaysia. All the children of these carriers are β-thalassaemia major patients and are homozygous for the Filipino β-deletion. Molecular characterisation for deletional α-thalassaemia showed 98 (70.5%) did not possess α-thalassaemia. The alpha rightward deletion −α(7) (α7) was seen in 37 (26.6%) individuals and four (2.8%) were homozygous for the −α(7) deletion (Table 1). The Southeast Asian (−SEA) and −α(2) deletions that have been reported in Malaysia were absent in all the 139 individuals studied. In addition, the non-deletion α-thalassaemia mutations: initiation codon (ATG→AGG), codon 30 (GAG), codon 35 (TCC→CCC), codon 59 (GGC→GAC; Hb Adana), codon 125 (CTG→CCG; Hb Quong Sze) and termination codon (TAA→CAA; Hb Constant Spring) were also absent in the β-thalassaemia carriers studied. The mean HbA2 level in Filipino β-thalassaemia trait was 5.9%. The HbA2 level was significantly higher in those

### Table 1

Heterozygous Filipino β-thalassaemia deletion (n = 139)

<table>
<thead>
<tr>
<th>Group</th>
<th>Fil del heterozygous, n = 139</th>
<th>HBF (Mean ± SD)</th>
<th>p value</th>
<th>HBA2 (Mean ± SD)</th>
<th>p value</th>
<th>HBA (Mean ± SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Fil del het only</td>
<td>98</td>
<td>2.92 ± 1.48</td>
<td>5.93 ± 0.47</td>
<td>81.87 ± 1.67</td>
<td>1.72 ± 0.55</td>
<td>0.36</td>
</tr>
<tr>
<td>Group 2</td>
<td>Fil del het + α-3.7 het</td>
<td>37</td>
<td>2.50 ± 1.59</td>
<td>6.27 ± 0.44</td>
<td>0.001</td>
<td>81.85 ± 1.54</td>
<td>0.95</td>
</tr>
<tr>
<td>Group 3</td>
<td>Fil del het + α-3.7 hom</td>
<td>4</td>
<td>1.48 ± 1.05</td>
<td>6.73 ± 0.36</td>
<td>0.001</td>
<td>82.83 ± 2.05</td>
<td>0.267</td>
</tr>
<tr>
<td>Group 4</td>
<td>Non-deletion β-thal het</td>
<td>180</td>
<td>2.84 ± 1.26</td>
<td>5.20 ± 0.55</td>
<td>0.000</td>
<td>81.94 ± 1.72</td>
<td>0.554</td>
</tr>
</tbody>
</table>

1. p < 0.05 was taken to be statistically significant.
2. Group 4: Non-deletional forms of β-thalassaemia (data extracted from laboratory records).
with cointheritance of $-\alpha^{3.7}$ (mean HbA2 level was 6.3 for heterozygous $-\alpha^{3.7}$ and 6.7 for homozygous $-\alpha^{3.7}$).

**DISCUSSION**

$\beta$-thalassaemia is characterised by decrease ($\beta^+$) or absence ($\beta^0$) in the synthesis of $\beta$-globin chains of human haemoglobin (Hb). The heterozygous state of $\beta^+$ or $\beta^0$ results in $\beta$-thalassaemia trait where the hallmark is the presence of elevated HbA2 ($\alpha_2\delta_2$). The homozygous state of $\beta^0$ ($\beta^0/\beta^0$) causes severe transfusion dependent thalassaemia major. The $\beta$-thalassaemia mutations in the major ethnic groups in West Malaysia have been systematically characterised since 1994. Each ethnic group has its own set of specific mutations. Studies from East Malaysia are limited. In 1993, a novel large deletion ($\beta^0$) in the $\beta$-globin gene was described in two separate papers. In both papers, the deletion was described to start near the 5' end and extend beyond the 3' end of the $\beta$-globin gene and it was also stated that the mutation came from families of Filipino descent. Using pulse field gel electrophoresis, the deletion was reported to be approximately 45 kb long. In the following year, a PCR method incorporating gap-PCR was developed to identify this deletion. In this study, gap-PCR was adapted to identify the Filipino $\beta^0$-thalassaemia deletion. The deletion breakpoint has been further characterised and is described as 118 kb in length as it extends to the downstream olfactory receptor region where it deletes a single olfactory receptor gene that contains a $\gamma$-globin enhancer.

Sabah is a Malaysian state located on the northern portion of the island of Borneo. It has a population estimated at 3.2 million (www.statistics.gov.my). The population is heterogeneous and culturally diverse with more than 30 different ethnic races. There are 32 indigenous groups that make up 60% of the total population of Sabah where the Kadazan/Dusun ethnicity comprises almost 30%. The native population of Borneo appears to be derived from several migrations to the island. The Indo-Malayan migration occurred around 2500 to 2000 BC from South China, through Formosa and the Philippines to northern Borneo. The Filipino $\beta^0$-deletion has been described in Filipinos in Taiwan and Indonesians from the eastern part of Indonesia. In Sabah, it is the most the most common $\beta^0$-thalassaemia mutation resulting in transfusion dependent thalassaemia. There are over 1000 transfusion dependent $\beta$-thalassaemia patients in Sabah. In contrast, in keeping with the historical origins of the population of Peninsular Malaysia, the Filipino $\beta^0$-deletion is not a cause for transfusion dependent $\beta$-thalassaemia major in Orang Asli, the indigenous population and in the main Malay, Chinese and Indian races.

In the Filipino $\beta^0$-thalassaemia deletion, the 118 kb deletion of the $\beta$-globin gene also encompasses the olfactory receptor genes (OR51V1, OR52Z1, OR51A1P, OR52A1, OR52A5, OR52A4). OR52A1 contains a $\gamma$-globin enhancer. In this study, the HbF level was also simultaneously measured by the BioRad Variant II Hb analyser. The HbF level in an adult is normally less than 1–2%. HbF levels may be mildly raised in $\beta$-thalassaemia trait, usually to <6%. The mean HbF level in Filipino $\beta^0$-thalassaemia trait was 2.9 ± 1.48 and in those with cointheritance of $\alpha$-thalassaemia were 2.2 ± 1.29 (heterozygous $-\alpha^{3.7}$) and 1.5 ± 1.05 (homozygous $-\alpha^{3.7}$), respectively. However, the expected reduced HbF level is seen only in homozygous Filipino $\beta^0$-deletion state and not in carriers.

Unusually high levels of HbA2 in excess of 6% have been described to occur with deletion forms of $\beta$-thalassaemia. This was attributed to extensive deletions that remove the $\beta$-globin gene promoter and the release of rate limiting transcription factors which interact with the locus control region (LCR) to increase the rates of transcription of the $\beta$-globin gene.

It is the proportion of HbA2 relative to other haemoglobins present that is used in the diagnosis of $\beta$-thalassaemia trait where HbA2 is expressed as a percentage of total Hb. The relative amount of HbA2 in adult healthy subjects is usually between 2.1 and 3.3%. Thus, correct quantification of HbA2 is of paramount importance for routine diagnosis of $\beta$-thalassaemia trait as $\beta$-thalassaemia major has major implications for affected individuals and their families. Classical $\beta$-thalassaemia trait has HbA2 levels >4%. However, a number of heterozygotes for $\beta$-thalassaemia may have normal or borderline HbA2 levels. Reporting these results requires a disclaimer and necessitates more extensive molecular work-up including DNA sequencing. The emergence of new methods for quantification of HbA2 requires laboratories involved in screening for thalassaemia to establish reference intervals and levels that have diagnostic significance for $\beta$-thalassaemia trait. Despite the vast heterogeneity of mutations causing $\beta$-thalassaemia, the magnitude of increased HbA2 is in the order of 5% and rarely exceeds 6%. In this study, the HbA2 level for Filipino $\beta^0$-thalassaemia trait was 5.9 ± 0.47 and 6.3 ± 0.44 with cointheritance of $\alpha$-thalassaemia ($-\alpha^{3.7}$ heterozygous) and 6.7 ± 0.36 ($-\alpha^{3.7}$ homozygous). In non-deletional forms of $\beta$-thalassaemia, HbA2 levels were significantly lower at 5.2 ± 0.55. In all 139 with Filipino $\beta^0$-thalassaemia trait, HbA2 levels were >4%, in keeping with the findings of classical $\beta$-thalassaemia trait: none were in the borderline range. However, the HbA2 levels were lower than that reported in its first description in a small number of patients. The estimation of HbA2 level then was carried out by cellulose acetate electrophoresis followed by elution, while in our study it was by automated high performance liquid chromatography on the BioRad Variant II Hb analyser, an instrument dedicated for HbA2 measurement. There was a significant difference in the HbA2 levels between the Filipino $\beta^0$-thalassaemia trait and those with cointheritance of $-\alpha^{3.7}$. Higher HbA2 levels were observed in those who have cointheritance of $\alpha$-thalassaemia. There was no significant difference in the HbA2 levels of those who were heterozygous or homozygous for the $-\alpha^{3.7}$ deletion. In HbH disease, $\alpha$-thalassaemia intermedia ($-\alpha$), the HbA2 level is less than normal. The higher HbA2 level seen in thalassaemia trait with Filipino $\beta^0$-deletion and cointheritance of $-\alpha^{3.7}$ deletion could be due to increased affinity of $\alpha$ chains for $\beta$-globin chains or activation of transcriptional factors that occur in the presence of $\alpha$-thalassaemia. Further studies are necessary to elucidate the cause of these findings. In conclusion, the mean HbA2 level in $\beta$-thalassaemia carriers with the Filipino $\beta^0$-deletion was 5.9 ± 0.47, 6.3 ± 0.44 in individuals with cointheritance of $-\alpha^{3.7}$ (heterozygous) and 6.7 ± 0.36 in individuals with co-inheritance of $-\alpha^{3.7}$ (homozygous) when measured on an automated dedicated HPLC system for HbA2 measurements. The HbA2 level in Filipino $\beta^0$-deletion was also significantly higher than that seen non-deletional forms of $\beta$-thalassaemia. It is important to have HbA2 assays that are both accurate and precise to diagnose a trait of Filipino $\beta^0$-deletion as the homozygous state results in $\beta$-thalassaemia major, a condition with major clinical implications for affected individuals and families.

Conflicts of interest and sources of funding: This study was supported by research grant E Science (MOSTI) 02-01-04-
References