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ORIGINAL ARTICLE

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AS A SCREENING TOOL FOR CLASSICAL BETA-THALASSAEMIA TRAIT IN MALAYSIA

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Beta-thalassaemia is characterized by a decrease (β^+) or absence (β^0) in the synthesis of β-globin chains of human haemoglobin. The heterozygous state for β^+ or β^0 result in β-thalassaemia trait in which the hallmark is the presence of an elevated level of Haemoglobin *(Hb)* A_2 ($\alpha_2\delta_2$). In the past, the traditional methods such as cellulose acetate electrophoresis with elution and microcolumn chromatrography have been the techniques used by the majority of the laboratories in Malaysia for the estimation of *(Hb)* A_2 levels. The recommended method currently is high performance liquid chromatography which has only been introduced in a few laboratories in the country.

Aim of the study **-To determine the cut-off level for *(Hb)* A_2 when estimated by high performance liquid chromatography (HPLC) in the diagnosis of classical beta-thalassaemia trait, a condition in the homozygous state that results in beta-thalassaemia major and red blood cell transfusion dependency.**

Results **-High performance liquid chromatography (HPLC) as a method for the measurement of *(Hb)* A_2 was rapid, and technically easy. A cut-off level of *(Hb)* A_2 >4.0 % predict the majority of carriers of classical beta-thalassaemia .**

Conclusions **-A full blood count (FBC), together with red blood cell indices generated on an automated blood counter in conjunction with the measurement of Hb A_2 on the VARIANT-BioRad, an automated HPLC machine and the beta-thal short program is an appropriate approach for the screening and presumptive identification of carriers of classical beta-thalassaemia prior to DNA studies for definitive diagnosis. In carriers for classical beta-thalassaemia, the MCV and MCH are <75 fl and <27 pg respectively with a Hb A_2 cut-off level > 4.0% [range 5.9 (4.5-8.1)] on the VARIANT-BioRad.**

Key words : ***Beta thalassaemia trait. screen. Hb A_2. HPLC***

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Introduction

The thalassaemias are inherited haemoglobin disorders with profound implications for individuals, families, and health services. In Malaysia, they occur mainly in the Malays and Chinese-Malaysians. About 4.5 % of the Malays and Chinese-Malaysians are carriers for beta-thalassaemia. Beta-thalassaemia is characterized by a decrease (β^+) or absence (β^0) in the synthesis of β-globin chains of human haemoglobin. The heterozygous state for β^+ or β^0 result in β-thalassaemia trait. The homozygous state for β^0, (β^0/β^0) causes a severe transfusion dependent anaemia termed thalassaemia major. Each ethnic

group in Southeast Asia has its own set of mutations that cause β-thalassaemia. In Asia, the common mutations that result in β^0 are IVS 1-1(G to T), CD 17 (A to T), CD 35 (-C), FSC 41-42 (-TCTT), FSC 71-72 (+A), -619 bp, $-\beta^{FIL}$, and FSC 8-9 (+G). IVS 1-5 (G to C) and IVS 2-654 (C to T) are β^+-thalassaemia mutations which are clinically severe as only minimally amounts of Hb A are synthesized. The common β-thalassaemia haemoglobinopathies, Hb E and Hb Malay (CD 19 A to G) have a β^+ phenotype.

In the screening for classical beta-thalassaemia trait, the hallmark is the presence of an elevated level of Hb A_2 (1). This necessitates the accurate estimation of Hb A_2 ($\alpha_2\delta_2$) . A number of techniques are available for the measurement of Hb A_2. These techniques include haemoglobin separation on cellulose acetate electrophoresis pH 8.9, microcolumn chromatography followed by analysis of elution with spectrometry and high performance liquid chromatography (HPLC)(2,3). On cellulose acetate electrophoresis and microcolumn chromatography a level of Hb A_2 >3.4% is found in carriers of beta-thalassaemia Unstable haemoglobins can be associated with an increase in Hb A_2 and in these conditions the RBC indices may not fit in with classical beta-thalassaemia trait. Currently a number of instruments

Table 1: Haemoglobin (Hb), red blood cell count (RBC), red cell indices (MCV, MCH, MCHC), red cell distribution width (RDW), Hb A_2 in carriers for classical beta-thalassaemia

	(n=25)		
	Beta-thalassaemia Carrier (female) n=16 mean ± 2s.d	**Beta-thalassaemia Carrier (male) n=9 mean ± 2s.d**	
Hb	11.1±2.0	12.9.1±2.8	gms/dl
RBC	5.2±1.3*	6.2±1.8*	$X10^{12}$/L
HCT	33.1±.6.1	40.1±9.6	L
MCV	64.9±7.0*	64.0±8.0*	fl
MCH	21.7±3.5*	20.6±2.0*	pg
MCHC	33.4±2.1	32.2±2.6	gms/dl
RDW	15.2±0.8	15.4±1.4	%
Hb A_2	5.9±1.4*	6.3±1.8*	%
Hb F	1.4±0.6	2.1±3.9	%

*significant differences between beta-thalassaemia carriers and normal persons ($p<0.001$)

are available for Hb A_2 quantitation by HPLC. HPLC is a sensitive and precise method for the identification of Hb A_2, Hb F and abnormal haemoglobins. It has become the method of choice for thalassaemia screening because of its speed and reliability. An automatic HPLC system, the VARIANT (Bio-Rad, 2000 Alfred Nobel Dr., Hercules, CA 94547, United States) (4,5) is currently available primarily for the detection of β-thalassaemia carriers and the common abnormal haemoglobins (Hb S, Hb C, HbE). In this study, we used the Variant, HPLC system with the beta-thalassaemia short program (BTS) to determine the cut-off level for Hb A_2 in carriers of classical beta-thalassaemia.

Materials and Methods

Subjects

Twenty-six parents of patients with transfusion dependent beta-thalassaemia from the Paediatric Unit, Hospital Universiti Kebangsaan Malaysia formed the study group. Following informed consent, blood samples were collected by venepuncture in E.D.T.A from the parents of children with transfusion dependent beta-thalassaemia when

Table 2. Screening for beta-thalassaemia trait in Malaysia : MCV, MCH and Hb A_2 levels

	RBC indices	**Hb A2 %**
(a) Classical β-thal trait	MCV < 75 fl MCH < 27 pg	>4.4 (4.5-8.1)
(b) Variant β-thal trait		
Type 1 silent	MCV > 80 fl MCH > 27 pg	<4.0
Type 2 `α-thal phenotype'		
β^+ allele (mild)	MCV 75-<77 fl MCH 24-<27 pg	<4.0
β^+ allele (mild) with δ-thal	MCV 75-<77 fl MCH 24-27 pg	<4.0

they accompanied the children to the Day-Care centre for blood transfusions.

Haematological

Haemoglobin, red blood cell counts and red cell indices were collected on an automated blood counter (Coulter STKS, Coulter Corporation, 11800 SW, 147, Miami, Florida 33196-2500, United States of America). Classical red cell indices for beta-thalassaemia trait are indicated by a MCV <75 fl and MCH <27 pg (6).

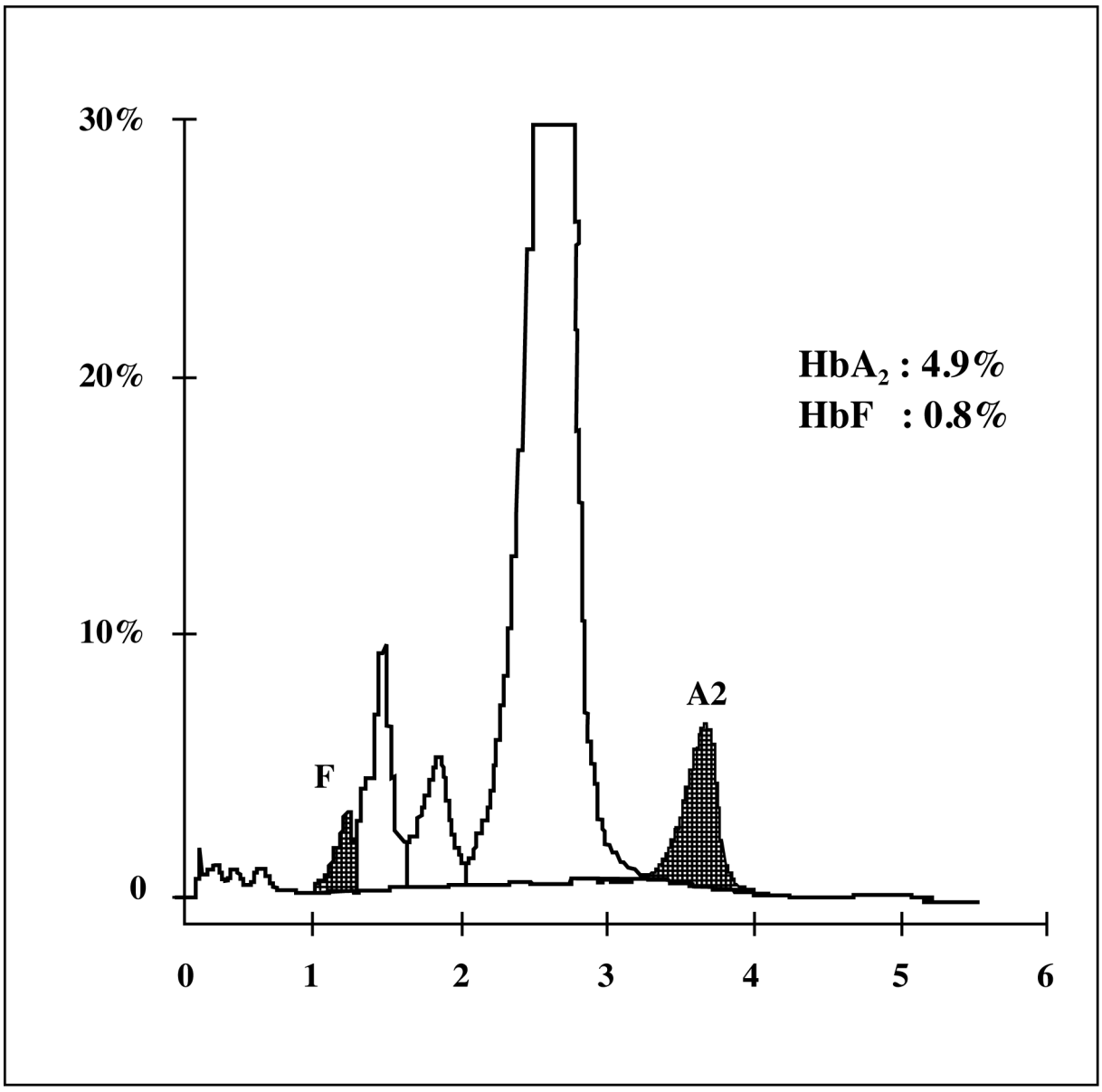
HPLC

The VARIANT (Bio-Rad, 2000 Alfred Nobel Dr., Hercules, CA 94547, United States of America), a fully automated high performance liquid chromatography (HPLC) system uses double-wave length detection (416 and 690 nM). This cationic-exchange column chromatography enables qualitative determinations of Hb A_2, Hb F and abnormal haemoglobins in 6.5 minutes on a haemolysate prepared from 5 ml of venous blood.. In the β-thal short program (BTS), a 3 X 4.6 cm non-porous cationic exchange column is eluted at a flow rate of 2 ml / min by a gradient created by two phosphate buffers that differ in pH and ionic strength. The reference for normal persons by the VARIANT is 2.1-3.7% in Malaysia. A chromatrogram of a case with beta-thalassaemia trait detected by the BTS program on the BioRad Variant is seen in figure 1.

Stastical analysis

All statistical analyses were conducted using

Figure 1. Beta-thalassaemia trait chromatogram by the BTS program on the BioRad Variant



the statistical package for social sciences (SPSS). Results were expressed as mean ± 2 s.d. Only those with a classical pattern for beta-thalassaemia trait in their RBC indices (MCV < 75 fl, MCH < 27 pg) were analysed.

Results

Twenty six parents were studied. Twenty five (96.2%) had an MCV <75 fl and MCH <27pg (Table 1). One (3.8%) case had to be excluded as the red blood cell indices did not meet the criteria for inclusion as a carrier of classical beta-thalassaemia . The RBC, MCV, MCH, and Hb A_2, showed significant differences between the female beta-thalassaemia carriers and normal females ($p<0.001$). In 15 (93.7%) of the females, MCV was <70fl and MCH <24pg. In the male beta-thalassaemia carriers, MCV, MCH, and Hb A_2 showed significant differences between male beta-thalassaemia carriers and normal males. The red blood cell count in the males was significantly different from normal males ($p < 0.05$). In the male beta-thalassaemia carriers, MCV was <70 fl in 8 (88.9%) and in all the MCH was <24 pg. An MCV <75 fl, MCH <27 pg and Hb A2 > 4.0 will identify all cases of classical beta-thalassaemia. There was no significant difference in the Hb A2 levels between the females and males, $p<0.001$. The levels of Hb A2 in the females were 5.9 (4.5-7.3 %), and in the males 6.3 (4.5-8.1%) respectively.

Discussion

The thalassaemias are the commonest inherited single gene disorder in Malaysia. About 4.5 % of the people are heterozygous (or carriers) for beta-thalassaemia. Each year, approximately 121 newborn infants are afflicted with transfusion dependent beta-thalassaemia. There are an estimated 5,600 patients with transfusion dependent beta-thalassaemia in Malaysia. The management of patients with transfusion dependent thalasssaemia constitutes a heavy burden to health authorities, both clinically and financially. One vial (500 mg) of Desferal (deferrioxamine) currently costs RM 15.30. Patients require 1-2 vials a days as chelation therapy at a dose of 35 mg/kg, for 5-7 days a week for life once regular blood transfusions are commenced. Malaysia needs a community-based screening program for thalassaemia. The aim of screening is to offer carrier testing to every member of the population, ideally before they have children (prospective carrier diagnosis). The purpose being to identify thalassaemia carrier couples and inform them of their risk and the options to avoid the birth of a transfusion dependent beta-thalassaemia child.

The measurement of elevated levels of Hb A_2 is recognized as the most practical way to identify classical beta-thalassaemia trait. Accurate measurements of Hb A_2 is a prerequisite in any screening program. Traditional techniques are the estimation of Hb A_2 by elution after cellulose acetate electrophoresis or by microcolumn chromatography. By cellulose acetate electrohoresis, care needs to be taken for cutting the strips, and methaemoglobin formation in stored samples may cause less clear separation between bands making estimations less reliable. The duplicates should agree to within 0.2% (s.d.<0.05), which is very much dependent on the operator's skill. With microcolumn chromatrography, separation of haemoglobin is dependent on pH, ionic strength and the DEAE cellulose (Whatman DE-52). The developers are stored at 4^0C and they need to be allowed to come to room temperature before use. In addition the amount of haemoglobin that needs to be applied must be carefully controlled. Overloading with more than 7-8 mg Hb will cause a contamination of Hb A_2, and when the Hb is less than 2 mg it will result in an eluate with an absorbance too low for accurate estimation. The flow-rate must also be adjusted for standardization of the run. These traditional methods for Hb A2 estimations are lengthy, delicate and manually laborious procedures and influenced by methodological factors. Accuracy is much dependent upon stringent quality assurance programs. Automated chromatography as in high performance liquid chromatography (HPLC) enables Hb A_2 estimations to be done rapidly and accurately. This is the method recommended for Hb A2 estimations in the screening of classical beta-thalassaemia (6-7).

The identification of beta-thalassaemia trait is often presumptive based on a characteristic red blood cell count, red cell indices, raised levels of Hb A_2 and unbalanced globin chain synthesis in an individual of an appropriate ethnic origin. Definite identification usually requires DNA analysis or amino acid sequencing. Family studies are of importance in elucidating this inherited disorder of Hb synthesis. Classical beta-thalassaemia trait is commonly first suspected by a specific pattern in the red blood cell counts and red blood cell indices generated by an automated blood counter. In this condition, the haemoglobin (Hb) level is normal or

minimally reduced, the red cell count raised, the mean corpuscular volume (MCV) and the mean corpuscular haemoglobin (MCH) being less than 75 fl and 27 pg respectively. Studies have indicated that the degree of microcytosis relates with the clinical severity of the β-thalassaemia mutation. In almost all cases, carriers of β^0 mutations had an MCV below 67 fl (8). In our studies 93.7 % of the female carriers and 88.9 % of the male carriers had an MCV < 70 fl. Couples who are both carriers for a β^0 mutation are at a risk of producing a child with homozygous beta-thalassaemia. Clinicians frequently review red cell indices when they are trying to diagnose a patient . The red cell indices also get a close scrutiny when full blood counts are done on automated blood counters with blood samples during a general medical examination. Clinicians assume that all three indices, MCV, MCH, and MCHC are equally reliable. Studies have indicated that the MCH is the only one that approaches accuracy achieved by routine hematology instruments. The Hbs and RBC have r^2 values on the order of 95%. On the following blood counters, Coulter, Technicon and Cell Dyn 3000, the MCH, r^2 values are at least 80% truthful, and with MCV 60-70% but MCHC 20-50% (9). The British Committee for Standardization in Haematology (BCSH) recommends the use of MCH as a screening tool for thalassaemia carrier identification (6-7). In our study, 93.7 % of the female carriers and all the male carriers had an MCH <24 pg.

In our study only a single parent had red blood cell indices MCV > 75 fl and MCH >27 pg. This latter parent also had a Hb A_2 level at 3.2%. Problems may be encountered when the person is a beta-thalassaemia carrier but the Hb A_2 levels are in the normal range (Table 2). Two phenotypes are seen : type 1 with normal red cell indices and type 2 where the red cell indices is typical of heterozygous beta-thalassaemia. DNA studies have indicated that type 1 may arise with the inheritance of a mild β^+ thalassaemia mutation . The mutations that have been implicated are –101 (C to T) and –92 (C to T). In the type 2 phenotype, the mutations CAP +1 (A to C), and IVS 1-6 (T to C) have been implicated. The mild β^+ mutation CAP +1 (A to C) has been described in Southeast Asian Indians. This type of 'β-thalassaemia' phenotype may also occur when co-inheritance of d-thalassaemia (cis or trans) with classical β-thalassaemia with high Hb A_2. The interaction of heterozygous β^0-thalassaemia with a-thalassaemia, due to the deletion of one or both a-globin genes may also lead to the production of red blood cells with normal red blood cell indices (10). If the co-inheritance is an interaction with a mild beta-thalassaemia allele such as CAP + 1 (A to C), the Hb A_2 levels, the red blood cell indices will be both normal and the diagnosis of β-thalassaemia trait will be missed. Iron deficiency can lower the Hb A_2 concentration (11). In most cases beta-thalassaemia trait can be diagnosed in the presence of iron deficiency. However, in the presence of iron deficiency, cases with a mild elevation of Hb A_2 will be missed.

Conclusion

The hallmark of classical beta-thalassaemia trait is the presence of an elevation of Hb A_2, where the recommended method of measurement is by automated HPLC. Our study, is the first to document the cut-off levels for Hb A_2 by HPLC in the identification of carriers for classical beta-thalassaemia in Malaysia. In Malaysia where 4.5% of the population carry the thalassaemia gene, all blood samples with an MCV <80 fl and an MCH < 27 pg should be investigated. For the screening of beta-thalassaemia, the measurement of Hb A_2 should be done by automated HPLC where a level of Hb A_2 > 4.0 would be indicative of classical beta-thalassaemia trait.

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