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Identification of α^o-thalassaemia ($-^{SEA}$) Using an Enzyme-Linked Immunosorbent Assay (ELISA) for Embryonic Zeta-globin Chain Detection – A Preliminary Study

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ABSTRACT

Objectives: This study aimed to evaluate the UBI MAGIWEL™ ζ-GLOBIN ELISA Kit for the presumptive diagnosis of α^o-thalassaemia. The ELISA results obtained were confirmed by molecular characterisation of α^o-thalassaemia using a Duplex-PCR. **Methods:** Routine peripheral blood counts and red cell indices were determined in 94 blood samples sent for Hb analysis. Hb subtypes were quantified by high performance liquid chromatography (HPLC) and Hb electrophoresis conducted on agarose gel at pH 8.5. Zeta-globin chain levels were determined using the UBI MAGIWEL™ ζ-GLOBIN ELISA Kit. Molecular analysis was performed using a duplex-PCR which simultaneously amplifies a normal 136 bp sequence between the ψα–α2-globin genes and a 730 bp Southeast Asian deletion-specific sequence ($-^{SEA}$) between the ψα2–θ1-globin genes. **Results:** Using the ELISA assay kit, 20 blood samples were presumptively identified as α-thalassaemia carriers from elevated ζ-globin chains (OD>0.3) while the remaining 74 blood samples showed OD<0.3. Molecular characterisation confirmed amplification of the 136 bp normal fragment in all the blood samples. Seventeen of the 20 DNA samples from the α-thalassaemia carriers amplified the SEA-deletion specific fragment. The remaining three DNA samples did not amplify the SEA-deletion specific fragment but amplified the normal α-globin gene sequence, indicating the presence of amplifiable DNA in these samples. Further molecular characterisation of the $\alpha^{-3.7}$ and $-\alpha^{4.2}$ deletions and Hb Constant Spring (CS) mutation showed the absence of these defects in these 3 DNA samples. **Conclusion:** This preliminary investigation showed the sensitivity and specificity of the UBI MAGIWEL ζ-globin ELISA kit as 100 % and 96.1 % respectively in the detection of α-thalassaemia-1 carriers ($-^{SEA}$).

Keywords: ELISA, ζ-globin chains, α-thalassaemia-1, Duplex-PCR, Southeast Asian deletion

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INTRODUCTION

Thalassaemia is a genetic blood disorder of haemoglobin synthesis and is the most common inherited haematological disorder in Malaysia. Thalassaemia is a public health problem in Malaysia as the incidence of α-thalassaemia carriers among the Malays and Chinese is approximately 3-4 %.[1] However, the majority of these α-thalassaemia carriers are unaware of their thalassaemia status as α-thalassaemia-1 or α^0 (–/aa) and α-thalassaemia-2 or α^+ (-α/αα) are asymptomatic and difficult to detect haematologically.

Alpha-thalassaemia is caused by deletions or mutations within the α-globin gene complex and this leads to a decrease or an absence of α-globin chain production. Alpha-thalassaemia can result from a deletion of either one (α^+-thalassaemia) or both (α^0-thalassaemia) α-globin genes.[2] The most severe form of α-thalassaemia is Hb Bart's hydrops foetalis, a condition that is not compatible with life. Hb Bart's hydrops foetalis is common in Southeast Asia [3] and is responsible for 60-90 % of Bart's hydrops foetalis cases in this region.[4] The estimated frequency of α-thalassaemia carriers in Southeast Asia is approximately 3-5 % [5] and the Southeast Asian deletion ($-^{SEA}$) is the most common type of deletion involved.[6] The $-^{SEA}$ deletion of 17.5 kb along the α-globin gene involves the deletion of the ψα2-, ψα1-, α2-, α1-and θ1-genes leaving only the ζ-genes intact (*Fig.1*).[7, 8]

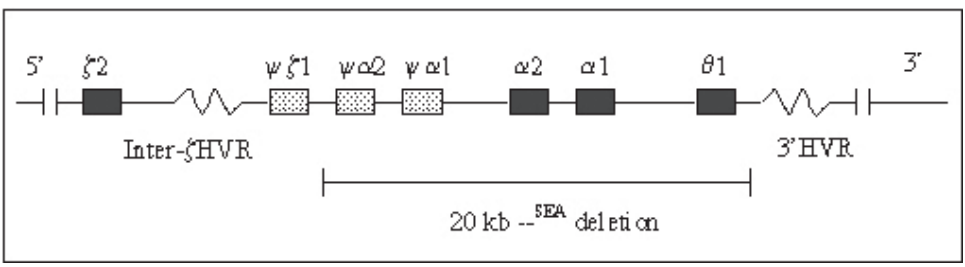

Figure 1. Southeast Asian deletion in the α-globin gene cluster

Note: Genes are represented as solid boxes, pseudogenes as dotted boxes and hypervariable regions as zigzag lines. The α-globin gene cluster on chromosome 16 is arranged in order telomere - ζ2, ψζ1, ψα2, ψα1, α2, α1, and θ1 – centromere. The Southeast Asian defect ($-^{SEA}$) of 17.5 kb deletes the ψα2, ψα1, α2, α1, and θ1 – globin genes and spares the ζ2, ψζ1 – globin genes.

There is an urgent need to identify carriers of α^o-thalassaemia in couples with the SEA-deletion as they are at risk of producing a gestation with Hb Bart's hydrops foetalis. Molecular characterisation and prenatal diagnosis for α-thalassaemia in Malaysia are currently carried out using a Duplex-PCR.[9] The duplex-PCR simultaneously amplifies both the normal sequence between the ψα–α2-globin genes and the SEA-specific deletion in a single PCR reaction, and thus offers a rapid and specific prenatal diagnosis method for α-thalassaemia in the Malaysian population.

Adult carriers of α^o-thalassaemia with the Southeast Asian deletion ($-^{SEA}$) have been reported to possess increased levels (0.01-1%) of embryonic ζ-globin chains in their erythrocytes.[10,11] The elevated embryonic ζ-globin chains can be detected using

fluorescence microscopy after immunocytological staining of erythrocytes with anti-human ζ-globin chain antibodies.[12, 13] A simpler ζ-globin chain immunosorbent assay (ELISA) was subsequently developed to detect α^o-thalassaemia carriers with the $-^{SEA}$ deletion [14, 15] and sensitivity of the ζ-globin ELISA assay was reported at 89.3% and specificity at 100% in a study carried out using peripheral blood.[16] The ζ-globin ELISA assay offers a simple and cost-effective diagnostic method for the identification of carriers of ($-^{SEA}$) deletional α^o-thalassaemia. In addition, this method can be used in diagnostic laboratories where equipment and trained staff for molecular diagnosis are not available. The UBI MAGIWEL ™ Zeta Globin (ZAM) Qualitative Test kit (United Biotech, Mountain View, California, USA) detects α^o-thalassaemia carriers with the ($-^{SEA}$) deletion and α-thalassaemia-1 carriers from other α-thalassaemia mutations that spare the embryonic ζ-globin genes and causes traces of ζ-peptides to persist throughout life.

This project is a preliminary study to evaluate the UBI MAGIWEL ™ ζ-globin ELISA kit for the presumptive diagnosis of α^o-thalassaemia carriers with the ($-^{SEA}$) deletion. The results obtained from the ELISA assay were compared with molecular studies using the Duplex-PCR to confirm the SEA deletion in α^o-thalassaemia carriers.

METHODS

Patient Samples

Three laboratories in the Klang Valley entered 94 (30, 30, 34 per site respectively) blood samples. Entrance criteria were Hb analysis testing ordered. The EDTA anticoagulated whole blood samples were used for automated blood counts, to quantify Hb subtypes by high performance liquid chromatography (HPLC) for UBI MAGIWEL ™ ζ-globin ELISA kit analysis and DNA studies. Routine peripheral blood counts and red cell indices were determined according to standard laboratory procedures. Hb analysis was performed on all blood samples with a MCV <80 fl and MCH <27 pg. Hb subtypes were quantified by HPLC using the Variant Hb Analyser (Bio-Rad Laboratories, Hercules, California, USA). Automated Hb electrophoresis was conducted on agarose gel at pH 8.5 (Sebia Hydrasys).

UBI MAGIWEL ™ ζ-globin ELISA Kit

The UBI MAGIWEL™ Zeta Globullin (ZAM) Qualitative Test (United Biotech, Mountain View, California, USA) is a solid phase enzyme-linked immunosorbent assay (ELISA) that provides screening for elevated ζ-globin levels in whole blood. The ELISA was performed following manufacturer's instructions. Patient's blood samples (100 μl) were mixed with 200 sample diluent (200 μl). 100 μl of the treated blood sample were dispensed into microplate wells coated with immobilised antibodies. After incubation at 37°C for 60 minutes, the wells were washed five times with washing buffer. Enzyme conjugate was then added and the wells incubated for another 30 minutes at 37°C before another 5 washes with washing buffer. Two solutions (A and B) were mixed and then added to the wells for 15 minutes at room temperature. The reaction was stopped with the addition of 50 ml of 1N H_2SO_4. Optical density was read at Absorbance 450 using a microplate reader. A positive colour (yellow) reaction (OD>0.3) indicates the presence of ζ-globin chain peptides in the blood sample. A

sample is negative when the OD reading is less than 0.2. Positive (blood from α-thalassaemia-1 patients with the (–SEA) deletion) and negative (blood from normal individuals) control blood and blank wells were included in every ELISA assay.

DNA Extraction

DNA was extracted using proteinase K and sodium dodecyl sulphate. Extracted DNA was purified by phenol-chloroform and precipitated with ethanol. Precipitated DNA was washed in alcohol, air-dried and then solubilised in double distilled water. DNA concentration and purity were measured spectrophotometrically.

Duplex-PCR

The duplex-PCR was performed using primers SEA1: 5'-CTCTGTGTTCTCAGTATTGGAGG-3' and SEA2: 5'-ATATATGGGTCTGGAAGTGTATC-3' to amplify the 730 bp SEA deletion-specific sequence. Primers α1: 5'-TACTGTAGATACCCGTGTACAA-3' and α2: 5'-ATCATGATGGAAACATAGTAAT-3'[17] were used to amplify the 136 bp normal sequence between the $\psi\alpha$–α2-globin genes. The PCR cycling conditions were initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 93°C for 1 minute, annealing at 50°C for 1 minute, synthesis at 72°C for 3 minutes and a final extension at 72°C for 6 minutes. DNA from a known heterozygote for α-thalassaemia ($-^{SEA}/\alpha\alpha$), a homozygous α^o-thalassaemia ($-^{SEA}/-^{SEA}$) and a normal individual ($\alpha\alpha/\alpha\alpha$) were amplified concurrently in every PCR to act as positive and negative controls.

Gel Electrophoresis of PCR Products

Amplified PCR products were resolved by electrophoresis on 1.5% agarose gels. Electrophoresis was carried out at 90 V for 45 minutes. DNA bands were observed using ultraviolet light irradiation after ethidium bromide staining and were recorded using a gel documentation system.

RESULTS

Out of the 94 blood samples sent for Hb analysis, 20 were presumptively identified as α^o-thalassaemia carriers by peripheral blood counts, red cell indices, HPLC analysis and Hb electrophoresis. The 20 samples consisted of 14 Chinese and 6 Malay patients. Using the UBI MAGIWEL ™ ζ-globin ELISA kit, all 20 samples showed elevated ζ-globin chains (OD>0.3). The OD reading of the remaining blood samples were <0.3.

Using the duplex PCR, the 136 bp normal $\psi\alpha$–α2-globin gene sequence was confirmed in all blood samples. The SEA-deletion specific sequence was not amplified from DNA in those with an OD reading <0.3. Seventeen out of the 20 DNA samples from α^o-thalassaemia carriers with elevated ζ-globin chains amplified the SEA-deletion specific sequence. Three DNA samples did not amplify the SEA-deletion but amplified the normal 136 bp $\psi\alpha$–α2-globin gene sequence, indicating the presence of amplifiable DNA in these samples .

Table 1. Molecular characterisation of DNA samples

Sample number	Ethnic group	A_{450}	136 bp ψα–α2-globin gene sequence	—^{SEA}	$\alpha^{3.7}$	-$\alpha^{4.2}$	HbCS
1219	Chinese	1.648	Positive	Negative	Negative	Negative	Negative
1208	Malay	0.850	Positive	Negative	Negative	Negative	Negative
1209	Malay	0.987	Positive	Negative	Negative	Negative	Negative

Further molecular characterisation of the α-globin gene showed the absence of the -$\alpha^{3.7}$ and -$\alpha^{4.2}$ deletions in these three samples (Table 1). In addition, Hb Constant Spring due to a T-C bp substitution in the termination codon of the α-2 globin gene was also not present in the three DNA samples.

On comparing the ELISA method with molecular characterisation using Duplex-PCR, the ELISA method was found to have a sensitivity of 100 % and a specificity of 96.1 %.

DISCUSSION

Molecular characterisation using the duplex-PCR was used to evaluate the ELISA immunological technique for the detection of α^o-thalassaemia carriers with the Southeast Asian deletion. The ELISA kit used was the UBI MAGIWEL ™ ζ-globin ELISA kit that detects α^o-thalassaemia carriers resulting from the SEA deletion and α-thalassaemia-1 carriers from other α-thalassaemia mutations that spare the embryonic ζ-globin genes and causes traces of ζ-peptides to persist throughout life. Results from this study showed the sensitivity and specificity of the ELISA kit as 100% and 96.1 % respectively, when using an OD cut-off value of 0.3 as determined by the manufacturers. In a study of an ELISA method for ζ-globin chain detection for α^o-thalassaemia carriers with the SEA deletion where a cut-off value of 0.2 was used, the investigators reported the sensitivity and specificity of the ELISA to be 96.4 % and 98.2 % respectively.[16]

Three of the blood samples with elevated ζ-globin chains detected in this study did not amplify the SEA deletion-specific sequence (Table 1). In a study of 200 cord bloods for α-thalassaemia-1 using both ELISA and PCR techniques, one false positive was reported where the ζ-chains were elevated but PCR confirmed the patient as an α-thalassaemia-2 heterozygote.[18] The ELISA method was also reported to be unable to detect the SEA deletion in some cases of Hb H disease blood samples. [16] The investigators reported that incomplete lysis of hypochromic microcytic cells together with the low erythrocyte count in Hb H patients may have produced a smaller quantity of ζ-globin chains in the blood lysate and thus, produced the false-negative result.

Alpha-thalassaemia with two α-globin gene deletions (–^{MED}) with elevated ζ-globin chains and positive ELISA results have been reported.[11,16] However, molecular characterisation of the Mediterranean gene deletion was not carried out in the three DNA samples that were negative for the SEA deletion in this study. Molecular characterisation of the single α-gene deletions (-$\alpha^{3.7}$ and -$\alpha^{4.2}$) and the non-deletional α-thalassaemia (Hb Constant Spring) was carried out in the three DNA samples. The samples were found to be negative for these three gene defects.

Hb Bart's hydrops foetalis observed in the majority of hydropic pregnancies in the Malaysian Chinese results from the inheritance of the Southeast Asian deletion from both parents. Thus, a simple test for the confirmation of the double α-globin gene deletion in both chromosomes is sufficient for the antenatal diagnosis of Hb Bart's hydrops foetalis in Malaysia. The duplex-PCR allows the detection of the Southeast Asian deletion and the normal ψα-α2-globin gene sequence in a single PCR reaction. It is a rapid, sensitive and specific method. However, equipment for molecular analysis and trained staff is required. The UBI MAGIWEL ™ ζ-globin ELISA kit is a rapid assay and does not require sophisticated equipment or highly trained staff. This preliminary investigation showed sensitivity and specificity of the UBI MAGIWEL ™ ζ-globin ELISA kit as 100 % and 96.1 % respectively. The sample numbers involved in this study are small and a larger study is needed to document the specificity of the ELISA method in the detection of the SEA deletion in α^{o}-thalassemia carriers.

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