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Monoclonal antibodies against hemoglobins for detecting thalassemia

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ABSTRACT

Background: Hemoglobin is composed of globin polypeptide chains, which serve as immunogens to induce the production of antibodies.

Objectives: This review article aims to describe the use of antibodies against human hemoglobins for the identification of thalassemia and hemoglobinopathies.

Materials and methods: Literature review to discusses the nature of normal human hemoglobin, hemoglobin switching, thalassemia and hemoglobinopathies, laboratory diagnosis, general properties of antibodies, production of antibodies against human hemoglobins, and clinical applications of these antibodies in identifying thalassemia and hemoglobinopathies.Antibody-based detection of hemoglobin is highly useful in diagnosing thalassemia and hemoglobinopathies.

Results: Polyclonal antibodies against HbF have been applied in sandwich ELISA to accurately detect HbF levels. Monoclonal antibodies against HbH and Hb Bart's have been produced and utilized in sandwich ELISA for detecting α-thalassemia. In addition, monoclonal antibodies against hemoglobin containing α-globin chains were developed and applied in sandwich ELISA to identify infants with Hb Bart's hydrops fetalis, a condition in which no α-globin chains are produced. For detecting β-thalassemia carriers, monoclonal antibodies against HbA_2 were produced, and sandwich ELISA was employed to measure HbA_2 levels, which are elevated in these individuals.

Conclusion: Antibody-based diagnosis of thalassemia and hemoglobinopathies enhances the quality of screening platforms and makes diagnosis of these disorders more reliable.

Introduction

In human, hemoglobin is the major protein in erythrocyte playing a major role in transporting oxygen from lung to tissue and carbon dioxide from tissue to lung. Hemoglobin naturally is composed to globin polypeptide chain, imbedded inside with heme molecule.[1] Two types of globin chains are produced along human developmental stage, including α-like globin chain (ζ and α-globin chains) and β-like globin chains (ε, γ, δ and β-globin chains). Functionally active hemoglobin molecule of human is of tetrameric structure composing of 2 α-like globin chains and 2 β-globin chains. Normal synthetic ratio of α-like globin chain and β-like globin chain is approximately equal, namely balanced globin chain synthesis. Normal hemoglobin composition in humans varies across developmental stages. During the embryonic stage (1–3 months of gestation), when hematopoiesis occurs in the yolk sac, the predominant hemoglobin is

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Hb Gower I ($\zeta_2\varepsilon_2$), followed by smaller amounts of Hb Gower II ($\alpha_2\varepsilon_2$), trace amounts of Hb Portland I ($\zeta_2\gamma_2$), and very limited amounts of Hb Portland II ($\zeta_2\beta_2$). In the fetal stage (4-6 months of gestation), with the liver and spleen serving as primary sites of hematopoiesis, approximately 90% of hemoglobin is HbF ($\alpha_2\gamma_2$), and about 10% is HbA ($\alpha_2\beta_2$). During the third trimester, HbA and HbF are produced in roughly equal proportions. At birth, a reciprocal shift occurs in the levels of HbF and HbA, and a small amount of HbA_2 ($\alpha_2\delta_2$) begins to appear. By around two years of age, the typical hemoglobin composition is approximately 95% HbA, 2.5% HbA_2, and less than 1% HbF.[1-3]

Thalassemia and hemoglobinopathies

Thalassemia is a group of genetic disorder caused by reduction or absence of globin chain production, leading to a condition termed imbalanced globin chain synthesis in which the normally synthesized globin chains become excessive. Hemoglobinopathy is also genetic disorder, but caused by production of abnormal globin chains, leading to production of abnormal hemoglobins. The abnormal hemoglobins may become either neutral or abnormal in physical and electrochemical properties.[3,5,6]

Several types of thalassemia have been identified, depending on globin chains that are reduced or absent. However, two main types of thalassemia have been found worldwide, including α-thalassemia and β-thalassemia. α-thalassemia is characterized by reduced or absent α-globin chain, while decreased or absent β-globin chain is unique in β-thalassemia. In α-thalassemia, α-globin chain containing hemoglobins, including Hb Gower II ($\alpha_2\varepsilon_2$), HbF ($\alpha_2\gamma_2$), HbA ($\alpha_2\beta_2$), and HbA_2 ($\alpha_2\delta_2$) are decreased or absent. In β-thalassemia, only HbA ($\alpha_2\beta_2$), the β-globin chain containing hemoglobin, is decreased. Two types of α-thalassemia are found; α^+ thalassemia or α-thalassemia 2 and α^0-thalassemia or α-thalassemia 1.[7] There are also 2 types of β-thalassemia; β^+-thalassemia and β^0-thalassemia.[8] Some α-globin chains are still produced in α^+-thalassemia, while no α-globin chains are produced in α^0-thalassemia. In the same way, some β-globin chains are still produced in β^+-thalassemia, while no β-globin chains are produced in β^0-thalassemia

Hemoglobinopathies, sometimes referred to as structural variants, are categorized into two main types: α-structural variants and β-structural variants.[9] α-hemoglobinopathies, or α-structural variants, involve abnormal hemoglobins formed by the assembly of abnormal α-globin chains with normal β-globin chains, such as Hb Constant Spring ($\alpha_2^{CS}\beta_2$) and Hb Pakse' ($\alpha_2^{PS}\beta_2$). As of now, approximately 841 structural variants have been identified worldwide.[10] Although some α-structural variants have a normal rate of synthesis, others exhibit reduced production, resulting in a phenotype resembling α^+-thalassemia. For example, Hb Constant Spring (CS) is an α-structural variant caused by a point mutation at codon 142 of the α2-globin gene (HBA2:c.427T>C). The mutated CS mRNA is unstable, leading to reduced synthesis of the CS α-globin chain. This makes Hb Constant Spring clinically like α^+-thalassemia. Clinical and hematological studies have shown that Hb Constant Spring presents a more severe clinical and hematological phenotype than deletional α^+-thalassemia. Patients with HbH disease due to Hb Constant Spring (HbH-CS disease) often experience more severe symptoms than those with deletional HbH disease and are particularly prone to severe hemolytic crises during infections. For this reason, Hb Constant Spring is classified as a severe α^+-thalassemia, and careful screening for thalassemia and hemoglobinopathy carriers is essential.[11-15] β-hemoglobinopathies, or β-structural variants, are abnormal hemoglobins formed by the combination of normal α-globin chains with abnormal β-globin chains, such as HbS (β^6Glu→Val) and HbE (β^26Glu→Lys). To date, approximately 958 β-structural variants have been identified worldwide.[10]

Abnormal globin genes that cause thalassemia and hemoglobinopathies are inherited in an autosomal recessive fashion. Those having these genes in heterozygous and doubly heterozygous forms are carriers or so-called traits of the disease. These carriers are clinically asymptomatic, requiring no medical care. Those homozygous or compound heterozygous for genes of severe types of thalassemia and hemoglobinopathies (- -/ - - for α-thalassemia and β^0/β^0 for β-thalassemia) are affected and need serious medical attention. Chance of having affected offspring of the heterozygous couples is definitely 25%. Therefore, screening for the carriers of thalassemia and hemoglobinopathies in the population is essential in controlling and preventing birth of the patients of this disease, especially in region rich of thalassemia and hemoglobinopathies.

Screening for carriers for thalassemia and hemoglobinopathies

Screening for carriers of α-thalassemia, β-thalassemia and abnormal hemoglobin such as HbE and Hb Constant Spring are crucial for preventing birth of babies with thalassemia diseases such as Hb Bart's hydrops fetalis (Homozygous α^0-thalassemia; - -/- -), transfusion dependent homozygous β^0/β^0-thalassemia, transfusion dependent compound heterozygous β^0/β^+ thalassemia and transfusion dependent compound heterozygous β^0/β^E thalassemia. Red blood cell indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), one-tube osmotic fragility test (OFT), dichlorophenol indophenol precipitation (DCIP) test are among the tests performed to seek for carriers of α-thalassemia, β-thlassemia and HbE.[16-25] These screening techniques have quite high sensitivity, but all of them lack specificity with low positive predictive value (PPV).

Antibody

Antibodies (Ab), also known as immunoglobulins (Ig), are specialized serum proteins belonging to the γ-globulin fraction that identify and neutralize foreign invaders such as bacteria, viruses, and toxins. They are produced and secreted by plasma cells derived from B lymphocytes, which are part of the humoral immune system. Immunoglobulins are naturally Y-shaped proteins composed of two main regions: the Fc region at the C-terminal domain and the Fab region at the N-terminal domain. The Fc region binds to receptors on the surface of target cells, while the Fab region, or antigen-binding site, binds to epitopes on target antigens. Antibodies are formed by two types of polypeptide chains: heavy chains and light chains. The Fab region contains both heavy and light chains, whereas the Fc region is composed only of heavy chains. The tips of the "Y" structure contain variable regions that specifically bind to antigens, which represent the epitope molecules of invading pathogens (Figure 1).There are five major classes of immunoglobulins, distinguished by their heavy chains: IgG (γ-heavy chain), IgA (α-heavy chain), IgM (μ-heavy chain), IgE (ε-heavy chain), and IgD (δ-heavy chain). Both humans and mice possess all five classes, while rabbits lack IgD in their serum.[26-28] In general, antibodies can be classified into two main types: polyclonal antibodies (pAb) and monoclonal antibodies (mAb). The two differ in their production and specificity. Monoclonal antibodies are derived from a single B-cell clone, producing highly specific antibodies that bind to only one epitope on an antigen. In contrast, polyclonal antibodies are generated from multiple B-cell clones, resulting in a heterogeneous mixture of antibodies capable of binding to multiple epitopes on the same antigen. The production of polyclonal antibodies involves simpler procedures, whereas monoclonal antibody production requires the hybridoma technique, followed by limiting dilution to isolate a single hybridoma clone capable of producing the monoclonal antibody of interest. Because of their high specificity, monoclonal antibodies are particularly well suited for diagnostic applications and targeted therapies.[29]

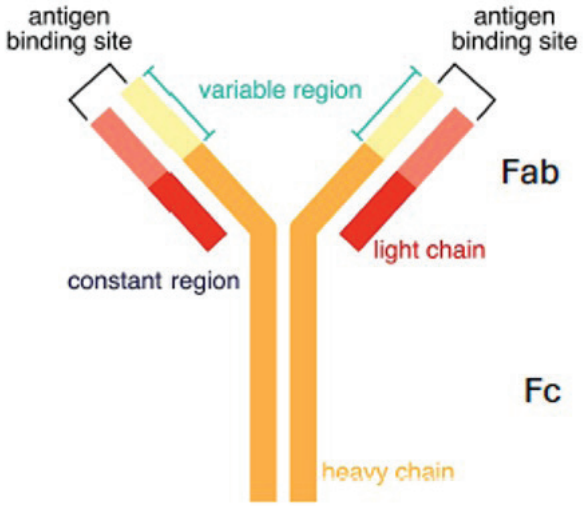

Figure 1. *Structure of antibody (Ab) or immunoglobulin (Ig).*
(modified from https://www.news-medical.net/life-sciences/Types-of-Antibodies.aspx)

Antibody-based detection of thalassemia and hemoglobinopathies

Antibody-based detection of hemoglobins can specifically identify hemoglobins that accurately indicate presence of thalassemia and hemoglobinopathies in blood samples. This capacity is superior to the conventional thalassemia screening tests which can only indicate chance of existing thalassemia and hemoglobinopathies in blood samples. Therefore, including the antibody-based detection of hemoglobins in screening protocol for thalassemia and hemoglobinopathies would increase effectiveness of the screening protocol of thalassemia and hemoglobinopathies.

As hemoglobins have globin polypeptide chains in the structure, they certainly can serve as immunogen to activate production of antibodies against them. The produced antibodies are then subsequently used to invent hemoglobin detection tools such as sandwich ELISA, flow cytometry, immunochromatographic strip test. Two types of antibodies against human hemoglobins have been produced, *i.e.* polyclonal antibody (pAb) and monoclonal antibody (mAb).

Production of pAb involves several laboratory techniques. As described by Kerdpoo and colleagues, the production of pAb against HbF ($\alpha_2\gamma_2$) involved the technique of HbF separation and purification by medium pressure liquid chromatography (MPLC), followed by immunizing the 2-month-old New Zealand White rabbits with purified HbF, mixed with complete Freund's adjuvant at the first injection and incomplete Freund's adjuvant in the second and third injections. Lastly, blood sample was collected from the marginal ear vein via incision technique and antiserum was obtained for further purification with protein G affinity chromatography (Figure 2).[30]

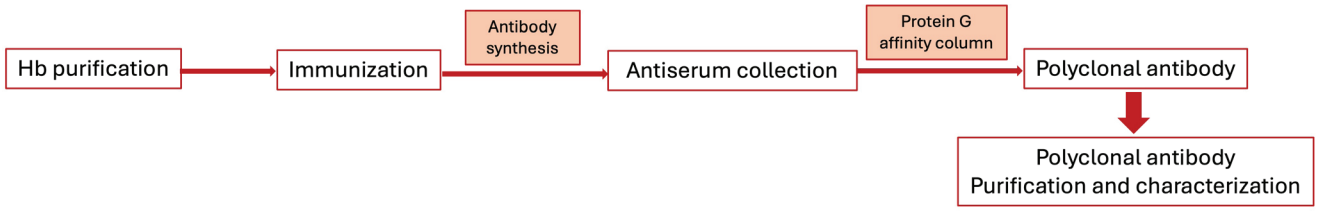

Figure 2. *Schematic presentation of procedure for production of polyclonal antibody for hemoglobin.*

Production of mAb against human hemoglobins also involves several steps as described by Pakdeepak and co-workers.[31] The first step is also separation and purification of hemoglobins by the MPLC, followed by injecting the purified hemoglobin mixed with complete Freund's adjuvant at the first injection and incomplete Freund's adjuvant in the second and third injection, into 2 six-week-old BALB/c mice, followed by spleen cell collection and fusion of spleen cells with P3-x63Ag8.653 myeloma cells to establish hybridoma clones capable of producing mAb specific to the immunized hemoglobin (Figure 3).

Figure 3. *Schematic presentation of procedure for production of hemoglobin-specific monoclonal antibody.*

Application of polyclonal antibody (pAb) in thalassemia and hemoglobinopathies

Polyclonal antibody by itself reacts with most types of hemoglobin. As shown in the study of Kerdpoo and colleagues that the pAb obtained after immunized the rabbits with purified HbF reacted with HbA ($\alpha_2\beta_2$), HbA_2($\alpha_2\delta_2$), HbE ($\alpha_2\beta_2^E$), HbF ($\alpha_2\gamma_2$), Hb Bart's (γ_4) and Hb Portand ($\zeta_2\gamma_2$).[30] With this reason, this pAb could not be applied in inventing the hemoglobin detection kit. However, Kerdpoo and co-workers used this pAb as a fluorescently labelled second antibody in the modified sandwich ELISA they invented and found that the new modified sandwich ELISA was effective in measuring HbF levels in the presence of minute amount of Hb Bart's in carriers of α-thalassemia (0.09-0.18 mg/mL).[32] Therefore, this pAb could be utilized to accurately measure HbF levels to identify a condition of hereditary persistence of fetal hemoglobin (HPFH) in area where α-thalassemia carriers are commonly encountered.

Application of monoclonal antibody (mAb) in thalassemia and hemoglobinopathies

In contrast to polyclonal antibodies (pAb), monoclonal antibodies (mAb) are highly suitable for detecting thalassemia and hemoglobinopathies due to their high specificity. Several studies have

attempted to produce monoclonal antibodies against hemoglobins, the levels or presence of which may indicate thalassemia or hemoglobinopathies.

For α-thalassemia, presence of HbH (β_4) or Hb Bart's (γ_4) indicates α-thalassemia. Shyamala and co-workers in 1992 produced mAb against HbH used for diagnosis of HbH disease by the technique of enzyme immunoassay.[33] The mAb they produced was highly specific to HbH and was able to quantify HbH level in HbH disease. However, there was no study utilizing mAb to HbH for detecting α-thalassemia carriers. It might be because HbH which is unstable has low level in the carriers of α-thalassemia. In contrast, significant amount of Hb Bart's was demonstrated in neonates with α^0-thalassemia carriers (--/αα).[7] Therefore, mAb to Hb Bart's was produced, followed by inventing the diagnostic tests utilizing this mAb. Monospecific antibody to Hb Bart's was produced by Garver and colleagues in 1984.[34] The established ELISA test utilizing this mAb to Hb Bart's showed that the carriers of α-thalassemia had average level of Hb Bart's was 6.10%, being significantly higher than that in normal individual whose Hb Bart's level was 0.25%. Monoclonal antibody against Hb Bart's was also produced by Tayapiwatana and co-workers in 2009 and immunochromatographic (IC) strip test was developed.[35] The IC strip test developed by Tayapiwatana and colleagues showed positive results in α^0-thalassaemia carriers (- -/αα), HbH disease (- -/- α), HbH-Constant Spring (H-CS) disease (- -/α^{CS}α), Hb Constant Spring EABart's disease (- -/α^{CS}α + $\beta^E\beta^N$), and homozygous α^+-thalassaemia (-α/-α). This IC strip test was not able to detect heterozygous α^+ thalassemia (- α/αα) and some α-structural variants such as Hb Westmead (HBA2: c.369C>G), Hb Jax (HBA2: c.44G>C), and Hb J-Buda [α61(E10)Lys → Asn, AAG > AAT].[36-38] The IC strip test did not show a negative result with all β-thalassemia and β-hemoglobinopathies. It could show positive results in individuals with elevated HbF levels or other coexisting hemoglobinopathies such as δβ-thalassemia and β-thalassemia (heterozygotes, homozygotes and HbE/β-thalassemia).[35,39,40] This IC strip test for Hb Bart's has been evaluated by several centers across the world. Wanapirak and colleagues evaluated this IC strip test in diagnosis of α^0-thalassemia in 499 pregnant women visiting antenatal care clinic at Maharaj Nakorn Chiang Mai hospital, Chiang Mai, Thailand and demonstrated 100% sensitivity and 89% specificity of this test. The falsely positive results observed in this study should be due to α^+-thalassemia (heterozygote or homozygote) which is also common in the northern Thailand.[41] In addition, Prayalaw and co-workers assessed this IC strip in 300 blood samples having positive thalassemia screening results and found that this IC strip had 100% sensitivity and 73.1% specificity in detecting α^0-thalassemia. This group postulated that all forms of α^+-thalassemin caused falsely positive results as this genotype also had Hb Bart's in blood.[42] Also, Sudjaroen and co-workers tested this IC strip in 414 pregnant women for α^0-thalassemia at Kudjab Hospital located in Udornthani Province, Thailand and found that this test had 92.6% sensitivity, 95.1% specificity and 94.9% efficiency.[43] This IC strip was also used by 4 laboratories in Thailand and Australia in screening for α-thalassemia by Winichagoon and colleagues who demonstrated 97% sensitivity and suggested this test to replace HbH inclusion body test in screening for α-thalassemia.[44] Recently, Bunkall and colleagues compared capability of the IC strip test and HbH inclusion body test in detecting α thalassemia in 67 blood samples. They showed that the IC strip test was extremely more sensitive than the HbH inclusion body test (76% vs 24% sensitivity, respectively) in screening for the α-thalassemia and blood samples stored in 4 °C was still good for the IC strip test.[45]

Beside mAb against Hb Bart's, mAbs against ζ-globin chain was also produced. Presence of the ζ-globin chain in hemolysate indicates the α^0-thalassemia of Southeast Asian (SEA) type.[46,47] Chui and co-workers produced murine hybridoma cells secreting mAb to the ζ-globin chain which was subsequently used in slot blot immunobinding assay in to screen for the SEA-α^0 thalassemia.[47] In 1993, Ireland and colleagues successfully used anti-ζ-immunobinding tetrazolium dye test to identify the α^0-thalassemia of SEA type in 225 blood samples they tested. [48] In 2008, Lafferty and colleagues employed commercial ζ-globin enzyme-linked immunosorbent assay (ELISA) to screen for SEA-α^0-thalassemia and found its sensitivity and specificity to be 1 and 0.94, respectively. [49] Pata and colleagues, in 2014, were successful in producing mAb to ζ-globin chain. This mAb was used to set up special platform of poly-l-lysine ELISA test which was proven to specifically detect the α^0-thalassemia of SEA type.[50] Immunostick coated with mAb to ζ-globin chain to detect the ζ-globin chain in hemolysate was subsequently developed by Pata and co-workers.[51] They demonstrated 100% sensitivity and 82% specificity of this novel immunostick test for the ζ-globin chain in identifying the SEA-α^0-thalassemia. Falsely positive results of this immunostick test was assumed to be due to cross reactivity of anti-ζ globin chain mAb with other globin chain.[51] In the same year, the IC strip test for detecting the ζ-globin chain was invented by Pata and colleagues and tested in combination with IC strip test for Hb Bart's in blood samples having MCV <80 fL and MCH <27 pg.[52] The analysis showed that the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of ζ-globin chain IC strip test in identifying the SEA-α^0 thalassemia in these blood samples were 100%, 65.2%, 90.7%, 100%, respectively. This result indicated that the IC strip test for the ζ-globin chain can only be used as the screening test, not diagnostic test.

This study then proposed that blood samples to be screened for the SEA-α^0 thalassemia must be first evaluated for mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and Hb typing results.

Only samples having low MCV, low MCH and Hb typing of A_2A were assayed by the IC strip test for the ζ-globin chain. Only samples having positive result for the IC strip test for the ζ-globin chain are further assayed by Gap-PCR.[52] Cross reaction of anti-ζ globin chain mAb to other globin chains was expected as a cause of falsely positive results.

In addition to identifying presence of HbH and Hb Bart's in α-thalassemia, however, Pakdeepak and colleagues showed by using mAb against α-globin chain containing hemoglobins (HbA; $\alpha_2\beta_2$, HbA_2; $\alpha_2\delta_2$, HbF;$\alpha_2\gamma_2$) that the levels of these hemoglobins in HbH disease were less than normal significantly.[31] The authors anticipated that this platform should be applied in detecting Hb Bart's hydrops fetalis babies in which no α-globin chain is produced at all.

As state previously, Hb Constant Spring (Hb Cs) is the abnormal hemoglobin that can cause severe transfusion-dependent Hb H disease. Therefore, identifying Hb Cs by antibody-based assay would improve efficiency in screening this abnormal hemoglobin. So-far, no study was found to describe production of antibody against Hb Constant Spring. However, with advancement of hemoglobin purification and hybridoma technologies at present, production of antibody against Hb Constant Spring is challenging.

For detection of β-thalassemia/hemoglobinopathies, mAb to HbA_2 was produced by several laboratories. Shyamala and co-workers successfully produced murine mAb against δ-globin chain of HbA_2 and the rapid ELISA test was invented.[53] They showed by this rapid ELISA test that the mean HbA_2 levels in β-thassemia carriers was 5.4%, being higher than normal individuals whose mean HbA_2 levels by this platform was 2.5%. Recently, Kuntaruk and colleagues produced murine mAb against HbA_2 which was utilized subsequently to set up the sandwich ELISA for measuring Hb A_2 in hemolysate.[54] They showed that under their developed sandwich ELISA, the mean HbA_2 levels in β-thalassemia carriers was 4.4%, being higher than the non β-thalassemia carriers (HbE carriers, HbE homozygote and α thalassemia carriers) whose mean HbA_2 levels by this platform ranged from 1.5% to 1.9%. They established 2.5% of HbA_2 level as cut-off values for identifying β-thalassemia carriers whose HbA_2 levels are higher than 2.5%. This 2.5% HbA_2 cut-off value was shown to be effective in detecting the β-thalassemia carriers by 100% sensitivity, 95% specificity, 82.3% positive predictive value and 100% negative predictive value. Therefore, when sandwich ELISA is performed, 2.5% cut-off HbA_2 values are used. However, for the routine laboratory diagnosis of the β-thalassemia carriers, the conventional HbA_2 cut-off value of 3.5% is still reliable.[55,56] Table 1 summarizes the studies that applied mAb to hemoglobin for detecting α-thalassemia and β-thalassemia.

Table 1. Summary of application of antibody-based detection of thalassemia/hemoglobinopathies.

Thalassemia	mAbs against	Invented diagnostic tools	Limitation	References
α	HbH	Sandwich ELISA	A	33
	Hb Bart's	Sandwich ELISA	A	34
	Hb Bart's	Immunochromatographic (IC) strip	B	35, 41, 42, 43, 44, 45
α	Hemoglobins that contain α-globin chain	Sandwich ELISA	B	31
	ζ-globin chain	Poly-l-Lysine ELISA	C	50
	ζ-globin chain	Immunostick test	C	51
	ζ-globin chain and Hb Bart's simultaneously	Immunochromatograpgic (IC) strip test	C	52
β	HbA_2	Sandwich ELISA	A	53, 54

Note: *A; many steps of operation, B: cannot detect specific type of α-thalassemia, C: not completely specific to SEA-α^0-thalassemia.*

The techniques for α-thalassemia listed in Table 1 had very high sensitivity for detecting the α^0-thalassemia. Therefore, in the national thalassemia screening Guideline of Thailand, they should be used in replacement of the conventional HbH inclusion body test which is the screening test for α-thalassemia. Modifying the α-thalassemia screening protocol using the mAb-based approach will increase efficiency and accuracy of α^0-thalassemia screening protocols. In contrast, the sandwich ELISA techniques were developed to quantify HbA_2 levels in blood lysates to help diagnosis of β-thalassemia carriers. Although this approach should replace cation-exchange HPLC or CZE in determining HbA_2 levels, the sandwich ELISA was suitable for only population-based screening for β-thalassemia carrier and testing in an individual is less possible. Therefore, at present, HbA_2-based diagnosis of β-thalassemia solely relies on HPLC-based or CZE-based enumeration of HbA_2 levels.

Limitations

Antibody-based identification of hemoglobins for the diagnosis of thalassemia and hemoglobinopathies currently focuses only on the qualitative analysis of

hemoglobins. Quantitative determination using this approach remains challenging, and further studies on this platform are encouraged.

Conclusion

Monoclonal antibodies (mAbs) against human hemoglobins and the ζ-globin chain become increasingly attractive for identifying α-thalassemia and β-thalassemia in both carrier and disease forms. With the invention of lateral flow IC strip or cassette-based immunological test kit utilizing these mAbs, the screening for carriers of thalassemia/ hemoglobinopathies will become reliable and greatly beneficial to the patients.

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Conflict of interest

None

CRediT authorship contribution statement

Thanusak Tatu: conceptualization (lead), writing: original draft (lead), review and editing (equal); **Watcharapong Jugnamang**: writing: original draft (supporting), review and editing (equal).

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