Cloning and Expression of a Recombinant Single-Chain Variable Fragment Antibody Specific to Hemoglobin Bart's

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ABSTRACT

Hemoglobin Bart's (γ_4), an abnormal Hb, is a homotetramer of γ-globin chains . The amount of this abnormal Hb in blood circulation can be used as an indicator for the presence of different genotypes of α-thalassemias. We successfully cloned and expressed a novel recombinant scFv antibody derived from mouse hybridoma producing monoclonal antibody highly specific to Hb Bart's. The genes encoding variable regions of the heavy (V_H) and light (V_L) chains were cloned and identified by DNA sequencing. The V_H and V_L genes were connected via a short linker to form the full length V_H-linker-V_L construct and ligated into pET28a. The His tag-scFv fusion protein was expressed in *E. coli* and purified by affinity chromatography. The recombinant scFv antibody was mostly expressed as inclusion bodies with the predicted molecular weight of 28 kDa. This scFv antibody was very specific by reacting with Hb Bart's (γ_4) but not cross-react with HbA ($\alpha_2\beta_2$), HbF ($\alpha_2\gamma_2$), HbS ($\alpha_2\beta_2^S$), HbE ($\alpha_2\beta_2^E$), HbA_2 ($\alpha_2\delta_2$), and HbH (β_4), as determined by Western blot. The detection sensitivity of this scFv antibody was 5 µg/µl of Hb Bart's by dot blot ELISA. The scFv antibody should be useful in development of an immunoassay with high sensitivity and specificity for the diagnosis of α-thalassemias.

Keywords: Hemoglobin Bart's, Recombinant antibody, Single-chain variable fragment antibody, α-Thalassemias

Introduction

α-Thalassemias are major health problem in the Mediterranean countries,the Middle East, Southern China, India, and Southeast Asia especially Thailand [1]. It is a genetic disorder caused by the reduction of α-globin chain synthesis resulting in the formation of the abnormal homotetramer hemoglobins including HbH (β_4) and Hb Bart's (γ_4). HbH disease, the outcome of the interaction of α^0- and α^+-thalassemia carriers, is the moderate severe form with marked hypochromia and microcytosis.The interaction between α^0-thalassemia carriers leads to the most severe type, Hb Bart's hydrops fetalis which die *in utero* or soon after birth because the highest affinity O_2 binding of Hb Bart's results in severe hypoxia [2]. One major way to prevent the spreading of the severe α-thalassemia diseases, including HbH disease and Hb Bart's hydrops fetalis, is to identify α-thalassemia carriers and subsequent genetic counseling. At present, standard methods such as polymerase chain reaction (PCR) [3-5] or DNA hybridization [6, 7] to identify α-thalassemia carriers require expensive equipments and tedious laboratory intervention. These methods are not suitable for screening large populations and therefore, one easy and practical method such as antigen-antibody based assay needs to be developed.Hb Bart's in blood circulation is an indicator of α-thalassemia carriers [2]. The quantity of Hb Bart's in cord blood usually correlates well with the α-thalassemia genotypes [8-11]. Low level in 1-2% range of Hb Bart's reflect heterozygous state for □$^+$-thalassemia. Higher level in 4-10% indicate the heterozygous state for □$^□$-thalassemia or homozygous □$^+$ thalssemia. Level of 12% suggest HbH disease and level up to 70-80% was found in Hb Bart's hydrops fetalis [2]. However, the amount of Hb Bart's decreases with age [12], and trace amount of Hb Bart's in adult blood is normally undetectable by routine hemoglobin electrophoresis. The more sensitive methods involved antibody against Hb Bart's, such as rabbit polyclonal antibody coupled

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with radioimmunoassay were previously reported by our research group [13, 14]. However, low specificity and batch-to-batch variation of rabbit polyclonal antibody limited their applications. To overcome the limitation of using rabbit polyclonal antibody, monoclonal antibodies (mAb) specific to Hb Bart's (hybridoma clone 2D4) was firstly established in our laboratory and successfully applied for the detection of Hb Bart's in α-thalassemia blood samples [15]. Methods involving mAb specific to Hb Bart's for the detection of -thalassemias have been further developed, including sandwich ELISA [16, 17], ELISA strip test [18], and sandwich immunochromatographic strip test [19]. Although, mAb with high sensitivity and specificity to Hb Bart's was successfully used, however, there are considerations about the cost of large scale antibody production, purification, and long-term storage. Recombinant antibody technology in bacterial cells offers a low-cost, high recovery yield, and reasonable binding affinity alternative to antibody production [20-22]. Therefore, recombinant antibody technology is a good alternative means to overcome all the mentioned problems. This research study aims to clone and express a novel recombinant scFv antibody against Hb Bart's using the variable domains of immunoglobulin V_H and V_L chains derived from a mouse hybridoma producing mAb highly specific to Hb Bart's. The specificity and sensitivity of this recombinant scFv antibody were determined for the possibility of developing an immunoassay for the detection of α-thalassemias.

Materials and Methods

Reagents, vectors, bacterial strains, and enzymes

All reagents were purchased from well established companies: Iscove's Modified Dulbecco's Medium (IMDM) and fetal bovine serum (FBS) were purchased from Gibco, USA; RNeasy mini kit from Qiagen, Germany; RobusT I RT-PCR kit and DNA polymerase from Finnzymes, Finland; PCR clean-up gel extraction kit from Macherey-Nagel, Germany; pGEM-T vector from Promega, USA; pET28a vector, *E. coli* strain TOP10F and BL21(DE3), nickel-nitrilotriacetic acid (Ni-NTA) resin from Novagen, USA; *Nde* I and *Bam*H I from Amersham, USA; lysozyme from Armesco, USA; Horseradish peroxidase (HRP) conjugated mouse anti-His-tag mAb from USBiological, USA; HRP conjugated rabbit anti-mouse Igs from Dako, Denmark; standard hemoglobin S from Sigma, USA. Standard hemoglobins; A, F, E, A_2, and HbH disease and Hb Bart's hydrops fetalis hemolysate were obtained in our previous works [15, 17, 18]. Primers were synthesized by Bio Basic Inc., Canada. DNA sequences were commercially analyzed by dye termination method from Applied Biosystems, USA. All other common chemicals are of molecular biology grade.

RNA isolation and antibody gene amplification

Mouse hybridoma (clone 2D4) producing mAb highly specific to Hb Bart's [15] was grown in IMDM supplemented with 20% FBS and incubated at 37°C, 5% CO_2 for 2 days. Total RNA was isolated from 10^6 hybridoma using RNeasy mini kit. Complementary DNA was subsequently synthesized by RobusT I RT-PCR kit and used as template for the V_H and V_L gene amplification by the established antibody-specific primers [23]. The amplification profiles were as follows; denaturation of RNA secondary structure at 65°C for 5 min, reverse transcription at 48°C for 30 min, 30 cycles of 94°C for 30 s; 50°C for 30 s; 72°C for 1 min, and final extension at 72°C for 7 min. The amplified products were purified by PCR clean-up gel extraction kit, ligated into pGEM-T vector and transformed into *E. coli* TOP10F. Colony PCR was performed for screening of the inserted gene using universal T7 and SP6 primers. Ten positive colonies of either V_H or V_L gene inserted were randomly selected for direct DNA sequencing. Complementarity determining regions (CDRs) of V_H and V_L chains were identified using Kabat and Chothia numbering scheme (http://www.bioinf.org.uk/abs/).

Construction of scFv gene

DNA manipulation was based on the standard methods [24]. The V_H and V_L genes were amplified, and linked together to form the full length V_H-linker-V_L scFv gene construct by splice overlapped extension-polymerase chain reaction (SOE-PCR). Primers containing *Nde* I and *Bam*H I restriction sites, peptide linker $(Gly_4Ser)_3$, and a stop codon were designed for cloning into pET28a expression vector (Table 1).

Table 1: Primers designed for construction of the full length scFv gene

Primers	Sequence	Annealing T°
V_H forward	5′-GCGCATATGCAGGTTCAGCTGCAGCAGTCTGGG-3′	72°C
V_H reverse	5′-*GGAGCCGCCGCCGCCAGAACCACCACCACC*GGAGGA-GACGGTGACTGA-3′	81°C
V_L forward	5′-*GGCGGCGGCGGCTCCGGTGGTGGTGGTTCA*ATTGTG-ATGACCCAGTCT-3′	78°C
V_L reverse	5′-CGCGGGATCC**TTA**TTTCCA GCTTGGTCCCCCCTC-3′	73°C

V_H forward primer contained the *Nde* I restriction site (underlined). V_H reverse and V_L forward primers contained the linker sequence(italic).V_L reverse primer contained the *Bam*H I restriction site (underlined) and a stop codon (bold).

PCR conditions were as follows: initial denaturation at 98°C for 30 s, 30 cycles of 98°C for 10 s; 72°C for 25 s, with final extension at 72°C for 10 min. The amplified products were digested with *Nde* I and *Bam*H I, gel purified, and ligated into pET28a as shown in Figure 1. After incubation, the ligation product was transformed into *E. coli* BL21(DE3). Colony PCR was conducted for screening of inserted scFv gene using T7 promoter and T7 terminator primers. The integrity of the full length scFv gene was confirmed by DNA sequencing.

Expression of recombinant scFv antibody

E. coli BL21(DE3) carrying the full length scFv gene were cultured in 1 liter of Luria-Bertani (LB) medium containing 30 μg/ml kanamycin at 37°C with shaking at 200 rpm until the OD_{600} reached to 0.6-0.8. For induction of scFv gene expression, a final concentration of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added and incubated with shaking at 37°C for 4 h. Bacterial cells were pelleted by centrifugation at 4,000 rpm, 4°C for 20 min, washed once with 40 ml of 50 mM Tris-HCl pH 8.0 and stored at -70°C until use. To analyze the recombinant protein expression, we performed Western blot analysis. Proteins of IPTG-induced and un-induced in bacterial cell lysate were analyzed by 12% SDS-PAGE [25] and blotted onto a PVDF membrane using Trans-Blot semi-dry electrophoretic transfer cell. The membrane was blocked with 10% (w/v) skim milk at room temperature for 2 h. After washing 3 times with PBS-0.05% Tween 20 (PBS-T),
the membrane was incubated with HRP conjugated mouse anti-His-tag mAb (1:1,000) at room temperature for 1 h. After washing, 4-chloro-1-naphthol substrate was added and the color reaction was subsequently developed at room temperature for 10-15 min. The reaction was stopped by repeatedly washing with distilled water.

Purification and refolding of recombinant scFv antibody

Four grams of bacterial cell pellet were resuspended in 80 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM β mercaptoethanol, 1 mg/ml lysozyme) and incubated at room temperature for 30 min. Bacterial cells were added with final concentration of 1% Triton X-100 and 0.5 M NaCl and incubated at room temperature for another 30 min. After sonication, inclusion bodies were collected by centrifugation at 14,000 rpm, 4°C for 20 min. The inclusion bodies were washed with 30 ml of 50 mM Tris-HCl pH 8.0 for 3 times, solubilized in 100 ml of 8 M urea, 50 mM Tris-HCl pH 8.0, 1 mM phenylmethyl sulfonyl fluoride (PMSF) and purified under denaturing conditions by 20 ml Ni-NTA resin affinity chromatography
according to the manufacturer's instructions. After purification, recombinant protein was renatured in redox refolding buffer (0.1 M Tris-HCl pH 7.4, 0.4 M L-arginine, 0.2 mM PMSF, 0.5 mM oxidized glutathione, 5 mM reduced glutathione) [26] and stirred at 4°C

overnight. After centrifugation at 12,000 rpm, 4°C for 20 min, the aggregated protein precipitated and was discarded. The refolded recombinant scFv antibody in supernatant fluid was concentrated using Centriprep centrifugal filter. The refolded scFv antibody was dialyzed against PBS at 4°C overnight. The protein was quantified using Bradford assay [27] and evaluated by SDS-PAGE.

Determination of specificity

Specific binding of the recombinant scFv anti-Hb Bart's was investigated by Western blot analysis. Parent mAb (clone 2D4) specific to Hb Bart's was included as a positive control. Fifty micrograms of each standard hemoglobin (HbA, HbF, HbS, HbE, or HbA_2), and approximately 120 µg of HbH disease and Hb Bart's hydrops fetalis hemolysates, were run in 12% native-PAGE. The gel was blotted onto a nitrocellulose membrane and blocked with 10% (w/v) skim milk at room temperature for 2 h. After washing, the membrane was incubated with 100 µg/ml of the purified scFv antibody at room temperature for 1 h. The following processes were conducted as previously described.

Determination of sensitivity

Sensitivity of the recombinant scFv antibody against Hb Bart's was analyzed by dot blot ELISA. One microliter, of serial 2-fold dilutions of Hb Bart's hydrops fetalis hemolysate with the final concentration of Hb Bart's of 40-0.31 mg/ml, was spotted onto a nitrocellulose membrane. Therefore, each spot contains 40, 20, 10, 5, 2.5, 1.25, 0.63 and 0.31 µg of Hb Bart's respectively. The membrane was subsequently blocked with 10% (w/v) skim milk, washed with PBS-T, and incubated with 100 µg/ml of the purified scFv antibody at room temperature for 1 h. After washing, the membrane was then incubated with HRP conjugated mouse anti-His-tag mAb (1:500) at room temperature for 1h. Finally, 4-chloro-1-naphthol substrate was added and color reaction was developed as previously described.

Results and Discussion

Construction of scFv gene and sequence analysis

Total RNAs from the mouse hybridoma (clone 2D4), secreting mAb highly specific to Hb Bart's, were isolated and then RT-PCR to obtain cDNA for the construction of V_H and V_L genes. Selection of V_H and V_L genes from cDNA of mouse hybridoma, we separately amplified the cDNA, inserted into pGEM-T vector, analyzed the DNA sequences, and identified the CDRs. The results indicated that all of ten V_H gene sequences were identical whereas two populations of V_L gene were observed. One of the V_L populations (5/10), deduced amino acid sequences represented the premature termination and causing of shortened polypeptide products. The deduced amino acid sequences of the other population (5/10) were retrieved from translated nucleotides and subsequently aligned with available sequences in GenBank public database using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Interestingly, amino acid sequences of the latter V_L were identical to mouse anti-chloramphenicol immunoglobulin kappa light chain variable region (accession no. ACV40677), and anti-hTERT single-chain variable fragment (99%) (accession no. ABW90123). The V_H and V_L genes were individually reamplified, and assembled into a full length scFv by SOE-PCR. The reamplified products of V_H, V_L, and full length scFv genes were shown in Figure 2. The reamplified product of V_H was 402 bp (Figure 2, lane 1) derived from the initial product of 363 bp, 9 bp *Nde* I restriction site and 30 bp linker. The product of V_L was 364 bp (Figure 2, lane 2) came from the initial product of 321 bp, 10 bp *Bam*H I restriction site, 3 bp stop codon and 30 bp linker. The full length scFv gene was 751 bp (Figure 2, lane 3) composed of the V_H (402 bp) and V_L (364 bp) subtracted by 15 bp complementary linker between V_H and V_L. The full length scFv gene was then inserted into pET28a expression vector locating after His-tag sequence. The DNA sequence and deduced amino acid sequence of the His-tag-scFv fusion protein was shown in Figure 3. The three V_H CDRs (CDR-H1, H2 and H3), same as three V_L CDRs (CDR-L1, L2 and L3), are also indicated. The DNA sequence and deduced amino acid sequence of scFv (V_H-linker-V_L format) were also submitted to GenBank public database (accession no. KF663616**).**

Expression and purification of recombinant scFv antibody

The expressed proteins, and the purified recombinant scFv antibody were analyzed by SDS-PAGE and Western blot analysis. Analysis of proteins from the IPTG-induced and un-induced bacterial cell lysate, soluble fraction of bacterial cell lysate, inclusion bodies, and purified recombinant scFv antibody were shown in Figure 4. After IPTG induction of *E. coli*, the recombinant protein was mostly expressed as inclusion bodies with predicted molecular weight of approximately 28 kDa (Figure 4A, lanes 2, 4, 5, and Figure 4B1-B2, lane 2). The recombinant scFv antibody was in the precipitate (Figure 4A, lane 2) but not in the supernatant fluid (Figure 4A, lane 3) of the bacterial cell lysate from the IPTG-induced *E. coli* while the precipitate of bacterial cell lysate of the un-induced bacteria had no recombinant scFv antibody (Figure 4A, lane 1). It was clearly shown that the scFv antibody should be in the inclusion bodies of the bacterial

cells. Although, the expression of recombinant proteins in *E. coli* can be in soluble form or in inclusion bodies depending on the nature of proteins or the expression system [26, 28]. However, most of recombinant scFv antibody tends to form inclusion bodies when expressed in *E. coli* [29-31]. The scFv antibody was further purified by Ni-NTA affinity chromatography under denaturing condition and directly refolded in redox refolding buffer. Approximately 1 mg of refolded recombinant scFv antibody was recovered from 1 liter of bacterial culture. However, the refolding and recovery efficiency from various studies were varied in different recombinant antibodies [32-35].

Specificity and sensitivity of recombinant scFv antibody

The native-PAGE of hemoglobins was either stained with Coomassie brilliant blue (Figure 5A) or blotted onto a nitrocellulose membrane (Figure 5B). The specific binding of scFv antibody to Hb Bart's was determined by Western blot analysis. Results revealed that the scFv antibody could bind specifically to Hb Bart's (γ_4) (Figure 5B, lanes 1 and 8) without cross-reactivity to HbA ($\alpha_2\beta_2$), HbF ($\alpha_2\gamma_2$), HbS ($\alpha_2\beta_2^S$), HbE ($\alpha_2\beta_2^E$), HbA$_2$ ($\alpha_2\delta_2$), and HbH (β_4) (Figure 5B, lanes 2-7). Similar results were observed when specificity test was undertaken in the parent mAb (clone 2D4) (data not shown). It was clearly shown that the recombinant scFv antibody only react to the specific folding region of the tetrameric γ_4 globin chains in Hb Bart's but not any folding regions or side chains of the dimeric γ_2 globin chains in HbF. The recombinant scFv antibody generated in this study was highly specific to Hb Bart's comparable to its parent mAb.Dot blot ELISA was developed for quantitative determination of Hb Bart's to determine the sensitivity of the recombinant scFv antibody. The detection sensitivity of Hb Bart's by the scFv antibody was 5 µg/µl as indicated in Figure 6. Same results were obtained when using the parent mAb (data not shown). The detection sensitivity of scFv antibody is quite interesting for the determination of α^0-thalassemia carriers. Previous report of Munkongdee *et al*. [9] showed that the level of Hb Bart's in cord blood of 17 cases α^0-thalassemia carriers were 6.2 µg/µl (4.6% of Hb Bart's in 134 g/L Hb). Fucharoen et.al. [36] reported the level of Hb Bart's in cord blood of 9 cases α^0-thalassemia carriers were 12.2 µg/µl (9.2% of Hb Bart's in 133 g/L Hb). From these results, we are attempting to develop an immunoassay screening test for determination of α-thalassemia using the recombinant scFv antibody. The ability to identify α^0-thalassemia carriers and HbH disease in infants will be very useful for health care of the affected children. When the identification of α-thalassemias was coupled with appropriate genetic counseling, it is also very helpful for the family planning to prevent or avoid having α-thalassemia disease children.

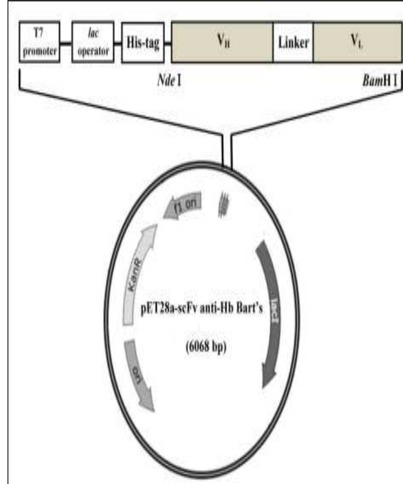

Fig 1:Expression vector map of pET28a-scFv anti-Hb Bart's.

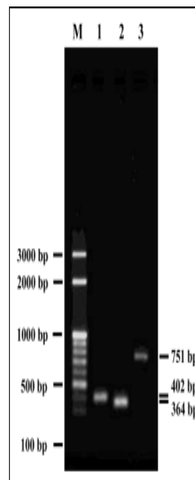

Fig 2:Splice overlapped extension-PCR (SOE-PCR) for construction of the full length scFv antibody encoding gene.

Fig 3:DNA sequence and deduced amino acid sequence of the His-tag-scFv fusion protein.

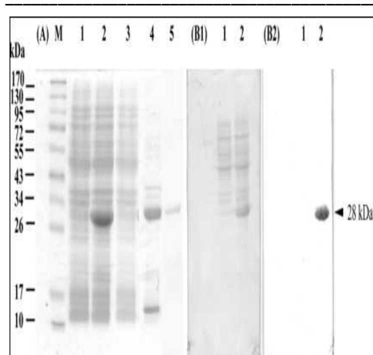

Fig4:Analysis of expression and purification of the recombinant scFv anti-Hb Bart's.

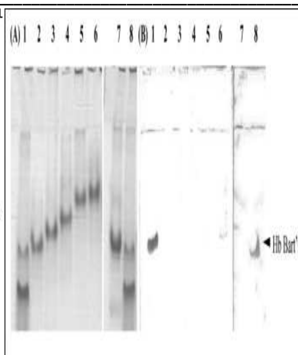

Fig 5:Analysis of specific binding of the recombinant scFv anti-Hb Bart's.

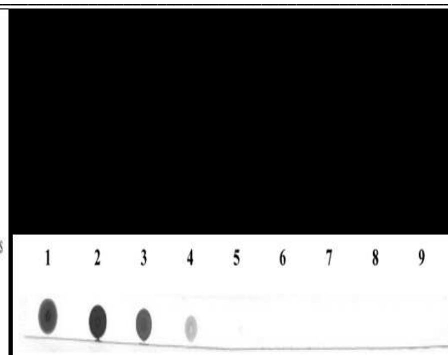

Fig6:Dot blot ELISA for sensitivity determination of the recombinant scFv anti-Hb Bart's.

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