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Isolation and characterization of single-chain variable fragment (scFv) antibody against polymeric immunoglobulin receptor, a potential diagnostic biomarker for *Opisthorchis viverrini*-related cholangiocarcinoma

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ABSTRACT: The Polymeric Immunoglobulin Receptor (PIGR) has been identified as a promising candidate for a diagnostic biomarker in *Opisthorchis viverrini*-related cholangiocarcinoma (CCA). Developing a single-chain variable fragment (scFv) antibody targeting PIGR could significantly advance diagnostic methodologies in CCA. Antibody phage display technology was used to screen for scFv antibodies specific to PIGR, employing the PIGR315-354 peptide as an antigen. Bio-panning was conducted using the Yamo I library. Positive phage clones were identified through phage enzyme-linked immunosorbent assay (ELISA) (indirect ELISA). The specificity of expressed scFv in *Escherichia coli* DH5α was subsequently confirmed by ELISA and dot-blot analysis. The DNA sequences of positive phage clones and their deduced amino acid sequences were analyzed. Furthermore, the scFv interaction with the PIGR peptide was predicted. The isolation of the PIGR-specific phage clone E4 was achieved. ELISA assays revealed that both the E4 bacteriophage clone and the expressed E4 scFv specifically bound to PIGR315-354 in comparison to BSA, PIGR225-268, and the PIK3CB2-43 peptide. Dot-blot analysis further confirmed the specific affinity of E4 towards PIGR315-354. The deduced amino acid sequences of the E4 bacteriophage clone indicated a full-length scFv antibody. Protein structure prediction clearly indicated the presence of the V_H and V_L domains, supporting the specificity of the isolated E4 scFv antibody for the PIGR315-354 peptide. The E4 scFv antibody, derived from the Yamo I library, exhibits specificity for PIGR315-354. This scFv antibody holds significant potential for developing diagnostic methods for CCA, presenting promising prospects for future diagnostic approaches.

KEYWORDS: polymeric immunoglobulin receptor (PIGR), antibody phage display technology, single chain variable fragment (scFv)

INTRODUCTION

Antibody phage display technology is a powerful tool for generating antibodies while eliminating the need for experimental animals. This technology involves expressing antibodies on the surface of phages. Foreign DNA fragments are inserted into gene III of filamentous phages, creating a fusion protein that is displayed on the bacteriophage surface. These recombinant phages can then be screened based on their affinity for antibodies directed against the target sequence [1] . Antibody phage display technology enables the screening of antigen-specific antibodies, providing a specific, convenient, and rapid method for detection.

Each individual phage is inserted with a different DNA fragment encoding a single-chain variable fragment (scFv), resulting in the expression of scFv antibodies on the surface of the phages. This collection of phages is called a phage display antibody library, in which each phage particle displays a single scFv antibody, allowing for the screening of target-specific scFv antibodies [2]. Therefore, antibody phage display technology is highly valuable for producing antibodies against specific targets, particularly cancer antigens, with applications in both diagnostic and therapeutic approaches.

Cholangiocarcinoma (CCA), a type of bile duct cancer, is an aggressive disease, accounting for ap-

proximately 15% of liver cancer cases, particularly in Southeast and Eastern Asia, where the incidence rates are notably high and strongly associated with infection by *Opisthorchis viverrini* [3]. Understanding the molecular drivers of CCA is critical for improving therapeutic strategies [4, 5]. Recently, the polymeric immunoglobulin receptor (PIGR) was identified as a potential biomarker for CCA diagnosis, showing a differential profile in the plasma of healthy control subjects, *O. viverrini*-infected subjects, and CCA patients [6]. The PIGR is a transmembrane glycoprotein that plays a crucial role in mucosal immunity [7]. It is a receptor responsible for the transcytosis of polymeric IgA and IgM from the basolateral surface to the apical side of the epithelium, mediating their subsequent secretion into mucosal fluids, where secreted IgA and IgM play vital roles in mucosal immunity in response to pathogenic infections [8]. The PIGR demonstrates potential as a future diagnostic tool for *O. viverrini*-related CCA, as it has been identified as a potential biomarker for differentiating CCA patients from individuals infected with *O. viverrini* and healthy subjects using sandwich ELISA [6]. Additionally, PIGR shows potential for evaluating its levels in patient plasma and other liquid biopsies as a diagnostic approach for CCA, as well as other cancers such as hepatocellular carcinoma [9], colon cancer [10], osteosarcoma [11], pancreatic cancer [12], and ovarian cancer [13]. Therefore, the development of a scFv antibody targeting PIGR holds significant potential for advancing diagnostic methodologies in CCA. The aim of the present study is to utilize antibody phage display technology to isolate and characterize PIGR-specific scFv antibodies from the Yamo I library [14]. Antibody phage display technology offers the potential to produce antibodies at a lower long-term cost, making it a suitable approach for CCA diagnosis in the future.

MATERIALS AND METHODS

Bio-panning of phage display library

The Yamo I antibody library was used for this study. The Yamo I library (with a diversity of 1.5×10^8) was constructed and provided by Prof. Dr. Montarop Yamabhai, Suranaree University of Technology [14]. The Yamo I library is derived from 140 non-immunized human donors. A wide variety of antigens have been successfully used to affinity-select specific binders [15–20].

The Yamo I library was used to screen for phages that specifically bind to a potential biomarker of CCA, the polymeric immunoglobulin receptor (PIGR). The PIGR315-354 peptide (LRKEDAGRYLCGAHSDGQLQEGSPIQAWQLFVNEESTIPR) (Bankpeptide, China) served as the antigen for bio-panning. To ensure specificity and stringency, 3 rounds of bio-panning were performed. Ten micrograms of PIGR peptide in 100 µl of 100 mM $NaHCO_3$ (pH 8.5) was coated on the immunotube (Nunc™, Denmark) for each round. After overnight incubation at 4 °C, the PIGR-coated immunotube was washed 3 times with 10 mM phosphate-buffered saline (PBS), pH 8.5 (PBS: 7.6 mM Na_2HPO_4, 2.4 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCL) and blocked with 2% MPBS (2% skim milk in 10 mM PBS) for 2 h at room temperature. Subsequently, the blocking solution was removed, and the immunotube was washed 3 times with PBS. The 100 µl of 2% MPBS containing 1 µl of Yamo I library was added to the immunotube and incubated at room temperature for 2 h. Unbound phages were removed by washing 3, 5, and 10 times with PBST (10 mM PBS, 0.05% Tween® 20) for first, second, and third round of bio-panning, respectively. The bound phages from each round were eluted by incubating with 100 µl of 1 mg/ml trypsin for 10 min, followed by 200 µl of 50 mM glycine (pH 2.0) for 15 min, and the solution was neutralized by adding 200 µl of 200 mM Na_2HPO_4 (pH 7.5). The eluted phages were used to infect *E. coli* strain TG1 by incubating at 37 °C for 30 min. Then, 100 µl of infected cells were spread on 2xYT plates containing 100 µg/ml ampicillin and 1% glucose, and the plate was incubated overnight at 37 °C.

To prepare the phage for the next round of bio-panning, 2xYT broth was added to agar plates. Bacteria were scraped, collected, pooled, and cultured in 10 ml of 2xYT broth with 100 µg/ml ampicillin and 1% glucose until the log phase (OD_{600} reached 0.4–0.5) at 37 °C. M13K07 helper phage (NEB, MA, USA) was then added and incubated at 37 °C without shaking for 30 min. After discarding the supernatant by centrifugation at $3000 \times g$, the pellet was resuspended in 10 ml of 2xYT broth containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 0.1% w/v glucose and cultured at 30 °C overnight (18–20 h) with shaking at 250 rpm. Then the phage was precipitated by adding 2.5 ml of PEG/NaCl (polyethylene glycol 6000 in 2.5 M NaCl) and kept on ice for 1 h, then centrifuged at $3000 \times g$ at 4 °C for 30 min. The supernatant was discarded, and the pellet was resuspended in 100 µl of PBS and centrifuged at $3000 \times g$. The supernatant was retained for the next round of screening. Bio-panning was conducted for 3 rounds.

Individual phage rescue

After bio-panning, individual phage-infected bacterial colonies from 2xYT agar were picked and cultured in 2xYT broth containing 100 µg/ml ampicillin and 1% glucose in a 96-well plate to produce phage particles. Following overnight incubation at 37 °C, 20 µl from each well was transferred to 2xYT broth containing 100 µg/ml ampicillin and 1% glucose in a 96-deep-well plate. The plate was incubated with shaking at 37 °C for 3 h, and the culture was rescued by adding M13K07 helper phage to each well. After incubating at 37 °C with shaking (150 rpm) for 1 h, the mixture was centrifuged at $3000 \times g$ for 15 min. The supernatant was

discarded, and the pellet was resuspended in 400 µl of 2xYT broth containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 0.1% w/v glucose and incubated at 30 °C overnight (18–20 h) with shaking at 250 rpm. Finally, 150 µl of the supernatant containing phage was used for phage ELISA.

Phage ELISA (indirect ELISA)

The 96-well microtiter plates (MaxiSorp Nunc-immuno, Thermo Fisher Scientific, Denmark) were coated with 10 µg/well of PIGR315-354 peptide diluted in 100 µl of 100 mM $NaHCO_3$. Controls at 10 µg/well, including BSA (Appichem, Germany), PIGR225-268 peptide (Bankpeptide, China), and PIK3CB2-43 peptide (Bankpeptide), were used as negative controls. After overnight incubation at 4 °C, plates were blocked with MPBS (3% skim milk in 10 mM PBS) at room temperature for 1 h, followed by 3 washes with PBS. Phage supernatant, diluted 50 µl in 150 µl of MPBS, was added and incubated for 2 h at room temperature. Unbound phages were removed by washing 3 times with PBST (10 mM PBS, 0.05% Tween® 20) and PBS. Binding phages were detected with 100 µl of 1:10,000 horseradish peroxidase (HRP) anti-M13 antibody conjugate (Sino Biological, China) diluted in MPBS, followed by incubation for 1 h and washing 3 times with PBS. Then, 100 µl of peroxidase substrate, 3,3′,5,5′- tetramethylbenzidine (TMB) (Sigma, Switzerland) was added to the plate. The reaction was stopped by adding 50 µl/well of 2 N sulfuric acid, and absorbance was read at 450 nm using a multifunction microplate reader (VarioSkan, Thermo Fisher Scientific, Finland). For bio-panning, clones with a phage ELISA absorbance at least two-fold greater than that of the negative controls were defined as positive clones.

Production of phage supernatant

To confirm the binding of the screened scFv antibody from the phage with positive clone results, an upscaling of the culture was performed. The positive well was spread onto a 2xYT plate containing 100 µg/ml ampicillin and 1% glucose. Single colonies were picked and cultured in 5 ml of 2xYT broth containing 100 µg/ml ampicillin and 1% glucose and incubated overnight at 37 °C with shaking at 250 rpm. Then, 50 µl of the overnight culture from each positive clone was added to 5 ml of 2xYT broth containing 100 µg/ml ampicillin and 1% glucose and incubated with shaking at 37 °C until the OD_{600} reached 0.4–0.5. M13K07 helper phage was added and incubated at 37 °C without shaking for 30 min, followed by centrifugation at $3000 \times g$ for 15 min. The pellet was resuspended in 5 ml of 2xYT containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 0.1% w/v glucose and incubated at 30 °C overnight (18–20 h) with shaking at 250 rpm. Finally, 150 µl of the supernatant containing phage was used for phage ELISA.

Expression of soluble scFv antibody

E. coli DH5α cells were infected with a positive phagemid. The infected cells were cultured in lysogeny broth containing 100 µg/ml ampicillin and 0.1% glucose and incubated with shaking at 37 °C overnight. Then, 100 µl of the culture was transferred into 5 ml of LB broth containing 100 µg/ml ampicillin and 1% glucose and incubated at 37 °C with shaking at 250 rpm until an OD_{600} of 0.9. After centrifugation at $3000 \times g$ for 15 min, the supernatant was discarded, and the pellet was resuspended in 5 ml of LB broth containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, Switzerland) and incubated at 30 °C for 16 h with shaking at 250 rpm. The supernatant containing scFv was used for ELISA and dot-blot immunoassay. To test the specificity of soluble scFv, PIGR315-354 peptide was used as an antigen, and 1:5000 His-probe conjugated with HRP (Sino Biological) was used as the conjugate in ELISA. For the dot-blot immunoassay, the soluble scFv antibody and *E. coli* cell pellets were sonicated using an ultrasonic processor for 5 min at a constant frequency of 30% amplitude, with short bursts of 30 s on and 30 s off. Both the soluble scFv antibody and *E. coli* cell pellets, which were sonicated using an ultrasonic processor, were used for further analysis.

Analysis of detection range of scFv by ELISA

To investigate the detection range of the expressed soluble scFv antibody, recombinant human PIGR (rh-PIGR) (Sino Biological) at concentrations ranging from 0.005 µg/ml (5 ng/ml) to 10 µg/ml was used as the antigen for ELISA. The plate was washed, blocked with 2% BSA to prevent non-specific binding, and incubated with a 1:5000 anti-Myc antibody conjugated with HRP (Sino Biological) in 2% BSA. TMB was used as the substrate. The absorbance at 450 nm for each concentration was used to plot a standard curve. A linear equation and R^2 were calculated.

Dot-blot immunoassays

Dot-blot immunoassays were performed on a polyvinylidene difluoride (PVDF) membrane. One microliter of 10 µg/µl PIGR peptide was spotted onto the membrane as the antigen, and 1 µl of 10 µg/µl BSA was used as a negative control. The membrane was dried at room temperature for 30 min and blocked with MTBS (2% skim milk in Tris-buffered saline, TBS) for 2 h. After blocking, the membrane was washed 3 times with TBST (TBS containing 0.05% Tween® 20), incubated with the soluble scFv antibody for 2 h, and washed 3 times with TBST. Subsequently, the membrane was incubated with a 1:5000 His-probe conjugated with HRP for 2 h, followed by detection using enhanced chemiluminescence (ECL) substrates. The signal was

visualized using a chemiluminescence imaging system (Amersham Imager 600, GE Healthcare Bio-Sciences AB, Sweden).

In the rhPIGR experiment, 6 µl of 1.5 ng/µl rhPIGR was spotted onto the PVDF membrane as the antigen. Negative controls including BSA, PIGR225-268 and PIK3CB2-43 peptides (each at 6 µl of 1.5 ng/µl) were also spotted. The membrane was dried at room temperature for 30 min and blocked with 3% BSA in TBS for 2 h. After washing 3 times with 0.05% TBST, the membrane was incubated with E4 scFv antibody and commercial anti-PIGR antibody (Sino Biological) for 2 h, followed by 3 additional TBST washes. For detection, the scFv antibody membrane was incubated with a 1:5000 anti-Myc antibody conjugated with HRP, while the anti-PIGR antibody membrane was incubated with a 1:100000 anti-mouse antibody conjugated with HRP (Abcam, UK), each for 2 h. Both membranes were subsequently incubated with ECL substrates and visualized using a chemiluminescence imaging system.

DNA sequencing and analysis

To ensure the scFv fragment was inserted into the phagemid clone, the phagemid DNA of the positive clone was extracted using a DNA miniprep kit (Qiagen, Germany), digested with *Sfi* I and *Not* I (NEB) restriction enzymes and analyzed by 2% agarose gel electrophoresis. The scFv-encoded DNA fragment of 800–900 bp was expected and subjected to DNA sequencing (Macrogen, Korea) using the M13R-pUC primer (5′-CAGGAAACAGCTATGAC-3′) and the 96 gene III primer (5′-CCCTCATAGTTAGCGTAACG-3′). The sequence was analyzed for complementarity-determining regions (CDRs) using the IGBLAST tool (http://www.ncbi.nlm.nih.gov/igblast/). The sequence was then translated into amino acids using the Expasy translate tool (https://web.expasy.org/translate/), and the three-dimensional (3D) structure of the positive clone was generated from the amino acid sequence using the SWISS-MODEL web server (https://swissmodel.expasy.org/). The interaction between the scFv antibody and PIGR was predicted by HawkDock (http://cadd.zju.edu.cn/hawkdock/), and models were visualized with the program PyMOL.

The binding affinity

The binding affinity of E4 scFv to rhPIGR was investigated. The rhPIGR was labeled with Dye RED-NHS 2nd Generation (Protein Labeling Kit RED-NHS 2nd Generation, Nanotemper, Germany) and analyzed using the Monolith system (Nanotemper) following the kit manual. Briefly, 90 µl of 1 mg/ml rhPIGR was mixed with 5 µl of Dye RED-NHS 2nd Generation and 5 µl of Labeling Buffer NHS and incubated for 30 min in the dark. The solution was loaded onto a 1xPBS (pH 7.4) equilibrated column and allowed to empty by gravity. Then, 550 µl of 1xPBS (pH 7.4) was added, and the column was allowed to empty. To elute the labeled rhPIGR, 450 µl of 1xPBS (pH 7.4) was added, and the eluate was collected. The concentration of labeled rhPIGR was measured using a NanoDrop™ One (Thermo Fisher Scientific, MA, USA).

For the ligand, scFv-E4 was buffer-exchanged into 1xPBS (pH 7.4) using a buffer exchange column (Protein Labeling Kit RED-NHS 2nd Generation, Nanotemper), and its concentration was measured by NanoDrop™ One. A 300 nM rhPIGR solution and a 1.89 µM E4 scFv solution were prepared by diluting in 1xPBS, pH 7.4, containing 0.05% Tween® 20 and 0.1% Pluronic F127. The 1.89 µM E4 scFv was serially two-fold diluted (ranging from 0.9 nM to 1.89 µM). Each dilution was mixed with 300 nM rhPIGR at a 1:1 ratio.

The mixtures were loaded into Monolith capillaries and analyzed for a dose-response curve at 670 nm, with a 10-second time frame and 30% excitation laser power. The K_d (dissociation constant) was analyzed to determine the binding strength.

Statistical analysis

All measurements were performed in triplicate, and data are presented as mean ± standard deviation (SD). Statistical significance was determined using Student's *t*-test (Independent Samples Test). A *p*-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS

Bio-panning of phage display library

To screen PIGR peptide-specific bacteriophages, three rounds of bio-panning against the PIGR315-354 peptide were performed using the Yamo I library. A total of 96 phage clones against PIGR315-354 were selected and their specificity was determined by phage ELISA. The results showed that at least 16 phage clones were isolated, namely A4, A6, B11, B12, C11, C12, D1, D8, E4, E8, F1, F9, G2, G7, H1 and H4, as shown in Fig. 1a. All 16 phage clones demonstrated greater than a two-fold increase in absorbance relative to the negative control (BSA) (Table S1). Notably, clone E4 exhibited the highest absorbance among all clones, with a value of 33.78-fold greater than the control group. Accordingly, it was selected for further investigation.

To ensure the binding specificity of the positive phage, another 2 rounds of testing were performed. In phage ELISA, clone E4 against PIGR315-354 demonstrated a positive result when compared to negative controls, including BSA, PIGR225-268, and PIK3CB2-43 (Fig. 1b, Table S2). In round 1, the absorbance at 450 nm for PIGR315-354 was 3.14-, 4.39-, and 4.50-fold higher than that of BSA, PIGR225-268, and PIK3CB2-43 (negative controls), respectively. Similar patterns were observed in round 2, with fold changes of 2.89-, 3.47-, and 3.35-fold, respectively. Statistical

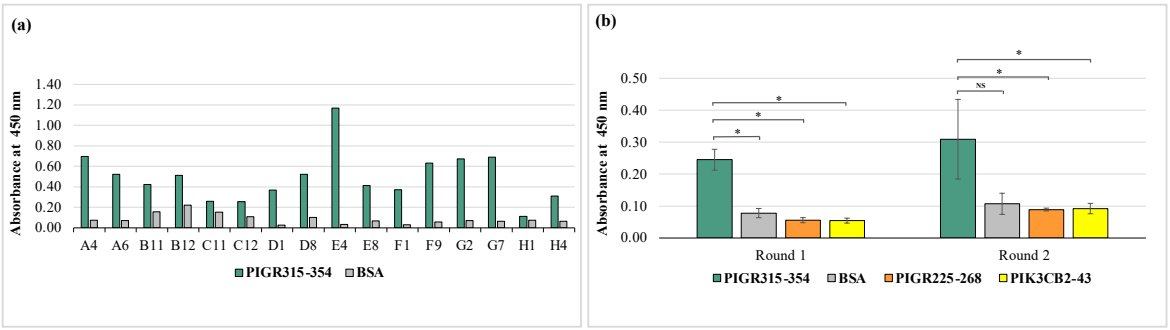


Fig. 1 Phage ELISA of bio-panning bacteriophage positive clones specific to the PIGR315-354 peptide (a). The bacteriophage clones are represented on the x-axis. The green bar indicates the bacteriophage against the PIGR315-354 peptide, while the grey bar indicates BSA, which serves as a control. Confirmation of isolated E4 bacteriophage clones against the PIGR315-354 peptide performed using 2 large-scale productions and phage ELISA to check consistency (b). The green bar indicates the E4 bacteriophage clones against the PIGR315-354 peptide, while the grey, orange, and yellow bars are negative controls, including BSA, PIGR225-268, and PIK3CB2-43, respectively. Statistical analysis was performed using Student's *t*-test (Independent Samples Test) to compare PIGR315-354 with BSA, PIGR225-268, and PIK3CB2-43, respectively. The asterisk (*) indicates significant differences ($p < 0.05$).

analysis revealed that in round 1, PIGR315-354 exhibited a statistically significant difference when compared to all control groups, including BSA ($p < 0.05$; 95% CI: 0.109–0.224), PIGR225-268 ($p < 0.05$; 95% CI: 0.135–0.243), and PIK3CB2-43 ($p < 0.05$; 95% CI: 0.136–0.244). In round 2, PIGR315-354 remained significantly different from PIGR225-268 ($p < 0.05$; 95% CI: 0.020–0.419) and PIK3CB2-43 ($p < 0.05$; 95% CI: 0.015–0.418); however, no significant difference was observed in comparison to BSA ($p = 0.053$; 95% CI: −0.005 to 0.408). Notably, despite the lack of statistical significance between PIGR315-354 and BSA in Round 2, the fold-change analysis indicated that PIGR315-354 still demonstrated a greater than two-fold increase relative to BSA. Therefore, the E4 phage clone was selected for further investigation.

Expression of soluble scFv antibody

The E4-positive phage clone derived from the bio-panning was used to express the soluble scFv antibody. The E4 scFv antibody was expressed from the pMOD1 vector in *E. coli* DH5α. The results showed that E4 scFv exhibited absorbance values for PIGR315-354 that were 7.01-, 7.10-, and 1.93-fold higher than those of the negative controls, including BSA, PIGR225-268, and PIK3CB2-43, respectively (Fig. 2a, Table S3). Statistical analysis revealed that PIGR315-354 was significantly different from BSA ($p < 0.05$; 95% CI: 1.529–3.589) and PIGR225-268 ($p < 0.05$; 95% CI: 1.395–3.723); however, no significant difference was observed when compared to PIK3CB2-43 ($p = 0.060$; 95% CI: −0.097 to 2.967). Although the difference between PIGR315-354 and PIK3CB2-43 was not statistically significant, fold-change analysis indicated that PIGR315-354 still demonstrated an approximately 2-fold increase relative to PIK3CB2-43.

Further investigation by dot-blot immunoassay confirmed the specificity of E4 scFv to PIGR315-354, consistent with the ELISA result. These findings suggest that E4 is specific to PIGR315-354 and a candidate for a PIGR-specific antibody (Fig. 2b). Dot-blot analysis was performed to evaluate the specificity of E4 scFv antibody binding compared to the anti-PIGR antibody. A signal was observed only with E4 scFv antibody and anti-PIGR antibody against rhPIGR, but it did not react with the negative controls including BSA, PIGR225-268 peptide, and PIK3CB2-43 peptide, indicating that the E4 scFv specifically binds to rhPIGR. The membrane incubated with the anti-PIGR antibody served as a positive control (Fig. 2c).

Detection range of PIGR by soluble expressed scFv antibody

The detection of rhPIGR concentration using the expressed soluble E4 scFv antibody showed a linear range from 0.63 to 5.00 μg/ml (Fig. 3). The linear equation was rhPIGR concentration = (Absorbance/0.0937) − 0.1521 with an R^2 value of 0.9882 (Fig. 3, Table S4).

DNA sequencing and analysis

The isolated phagemid was assessed for a scFv insertion of 800–900 bp using *Sfi* I and *Not* I digestion prior to sequencing. The inserted scFv DNA fragment was approximately 800 bp (Fig. S1). The three-dimensional (3D) structure, illustrated in Fig. 4, showed the interaction between the scFv antibody and the PIGR peptide. The deduced amino acid sequence revealed complementarity-determining regions (CDRs), as presented in Table 1. The encoded 289-amino-acid sequence contained the full-length scFv fragment, which included CDR1, CDR2, and CDR3 of the variable regions for both the heavy (V_H) and light (V_L) chains (Fig. 4a).

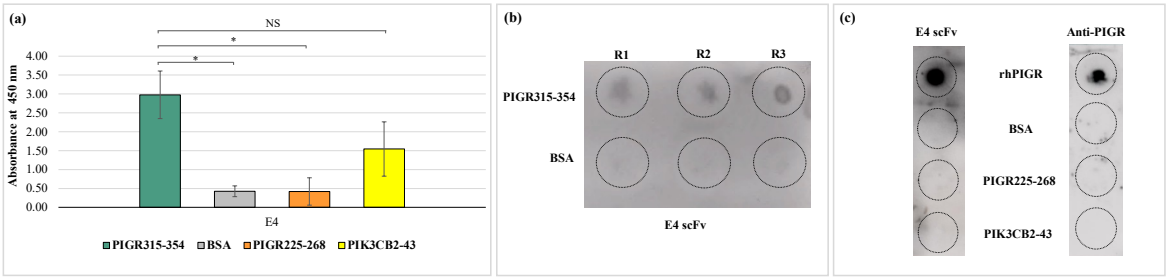


Fig. 2 ELISA of secreted E4 scFv antibody against the PIGR315-354 peptide (a). The x-axis displays the PIGR315-354 peptide and the negative controls. The green bar indicates the E4 scFv antibody against the PIGR315-354 peptide, while the gray, orange, and yellow bars represent negative controls, including BSA, PIGR225-268, and PIK3CB2-43, respectively. Triplicate dot-blot analysis of soluble E4 scFv antibody against the PIGR315-354 peptide (upper row) and BSA control (lower row) (b). Positive chemiluminescence signals are observed in the upper row (PIGR315-354 peptide). Dot-blot analysis of E4 scFv antibody and anti-PIGR antibody against rhPIGR and controls including BSA, PIGR225-268, and PIK3CB2-43 showing positive chemiluminescence signals of rhPIGR protein (c). Statistical analysis was performed using Student's *t*-test (Independent Samples Test) to compare PIGR315-354 with BSA, PIGR225-268, and PIK3CB2-43, respectively. The asterisk (*) indicates significant differences ($p < 0.05$).

Table 1 The amino acid sequence of CDR1-3 of E4 scFv fragment.

	V family	% identity	CDR1	CDR2	CDR3
V_H	IGHV1-69*01	100% (294/294)	GGTFSSYA	IIPIFGTA	ARSGGRYCSSTSCYAGVYYYGMDV
V_L	IGLV6-57*01	96.5% (297/289)	SGSIASYY	ADN	QSYDNNNRAV

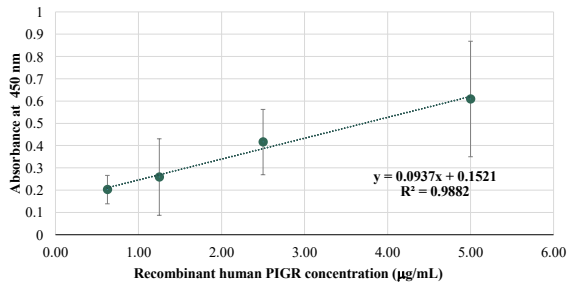


Fig. 3 ELISA of the E4 scFv antibody against rhPIGR. The E4 scFv antibody exhibits a linear relationship between absorbance at 450 nm and recombinant human PIGR concentration ranging from 0.625 to 5.000 µg/ml.

Table 2 The top five of the binding free energy of the E4-scFv antibody and PIGR peptide.

E4-scFv antibody	Binding free energy (kcal/mol)	PIGR peptide	Binding free energy (kcal/mol)
ILE-212	−3.49	TYR-9	−5.43
SER-166	−3.08	GLN-18	−3.01
TYR-178	−2.52	LEU-10	−2.25
PRO-152	−2.49	CYS-11	−1.61
ALA-110	−2.23	GLY-17	−1.34

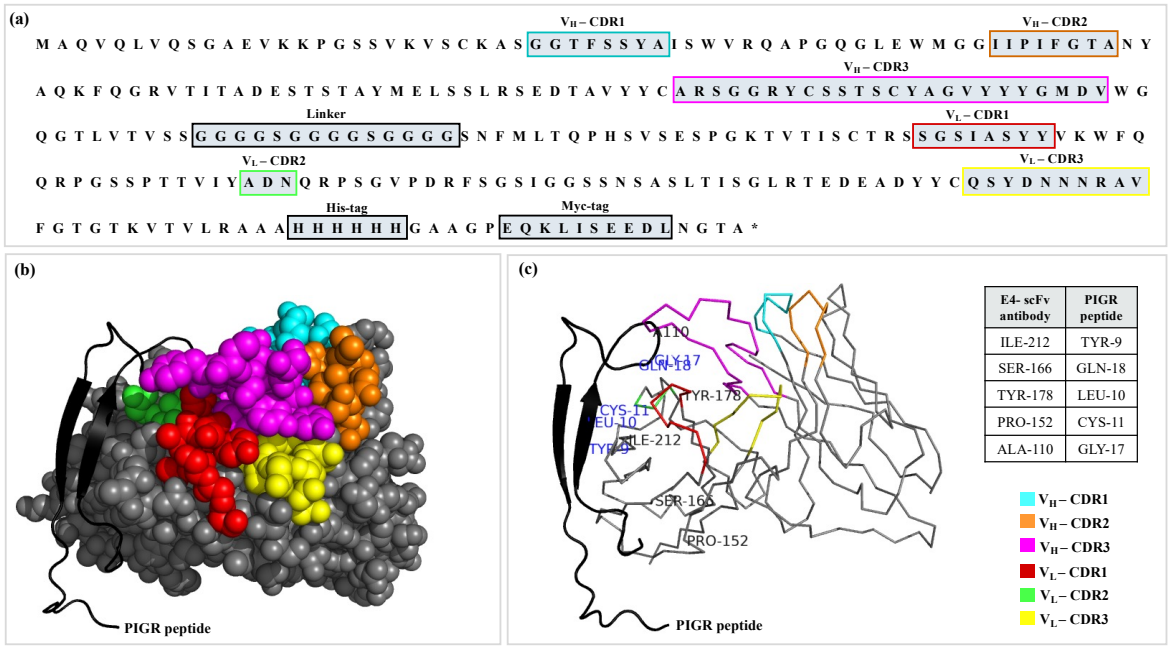
Analysis of the predicted interaction between the E4 scFv antibody and the PIGR peptide demonstrated that the E4 scFv residues ILE-212, SER-166, TYR-178, PRO-152, and ALA-110 interacted with the PIGR peptide residues TYR-9, GLN-18, LEU-10, CYS-11, and GLY-17. The top 5 residues from the binding free energy analysis were shown in Table 2, with the lowest binding energy corresponding to the most favorable complex formation. The binding free energy for the E4 scFv antibody and PIGR peptide interaction was −28.99 kJ/mol, indicating a strong and stable interaction. This negative value suggested that the formation of the ligand-protein complex was thermodynamically favorable, releasing energy and resulting in a more stable complex compared to the separated components.

Binding affinity

The binding affinity of E4 scFv to rhPIGR was investigated using a dose-response curve at a target concentration of 300 nM rhPIGR. The K_d value was 722 nM, indicating a moderate binding affinity between rhPIGR and scFv-E4.

DISCUSSION

PIGR has been identified as a potential biomarker for CCA, capable of distinguishing CCA patients from *O. viverrini*-infected patients and healthy subjects using a sandwich ELISA, with *O. viverrini* being a known risk factor for CCA [6]. At least 5 peptides of PIGR were specifically identified in all CCA plasma proteomes, including PIGR193-211, PIGR232-268, PIGR297-316, PIGR323-354, and PIGR526-536, serving as biomarkers for CCA [6]. The PIGR 323-354 peptide region was predicted to contain an epitope using the Immune Epitope Database (IEDB) Analysis Resource and was



E4- scFv antibody	PIGR peptide
ILE-212	TYR-9
SER-166	GLN-18
TYR-178	LEU-10
PRO-152	CYS-11
ALA-110	GLY-17

Fig. 4 Predicted 3D structure of the E4 scFv antibody and the PIGR peptide. The amino acid sequences of the variable heavy (V_H) and variable light (V_L) chains of the E4 scFv antibody (a). The predicted interaction between the E4 scFv antibody and the PIGR peptide presented as a sphere structure (b). The same interaction depicted as a ribbon structure to show interacting amino acid residues (c).

found to belong to the extracellular domain. Therefore, the PIGR 315-354 region, which encompasses the predicted epitope, was selected and utilized in this study. For diagnostic approaches to CCA, scFv antibodies will reduce costs with ease of large-scale production through antibody phage display technology. This research utilized the synthetic PIGR peptide (amino acids 315-354) as an antigen to isolate the anti-PIGR scFv antibody, specifically E4, from the Yamo I library using phage display technology.

The E4 scFv-positive clone was obtained from the Yamo I library. The Yamo library has successfully yielded several scFv antibodies, with a diversity of approximately 1.5×10^8 clones. However, the affinity of scFv antibodies derived from a naïve library may be limited compared to those from an immunized library. The Yamo I library is a compact phage-displayed human scFv library comprising antibodies with high diversity obtained from 140 non-immunized donors, and it has been effective in affinity selection of specific antibodies against a wide variety of antigens such as amylase, cobra snake venom, and CCA cell line KKU-100 [14]. Moreover, the Yamo I library has been utilized to select antibodies against various targets, including the rabies virus (for neutralization activities) [15], zearalenone (for the detection of ZEN contamination) [18], and cholangiocarcinoma cells [21]. Its broad capacity to select specific antibodies against a wide range of antigens makes it a valuable tool for both diagnostic antibody development and research applications. Therefore, the diversity and success of this library in screening against diverse targets make it possible to successfully isolate antibodies against the PIGR315-354 peptide.

Application of antibody phage display technology is useful but has limitations, especially for cancer therapeutics and diagnostics [22]. Previous studies have utilized phage display to identify small peptide motifs with specific affinity for human PIGR. One such selected phage display peptide, CVVWMGFQQVC, demonstrated binding to the free secretory component (PIGR) and may be exploited for PIGR-mediated epithelial transport without interfering with secretory immunity [23]. Similarly, in this research, the E4 scFv antibody against the PIGR315-354 peptide identified as a biomarker of CCA was successfully isolated. Phage display enables *in vitro* selection of mono-specific scFv antibodies with virtually any specificity, greatly facilitating recombinant production of reagents for research, clinical diagnostics, and therapeutic pharmaceuticals [24]. During bio-panning, a diverse phage library is exposed to the target antigen, and phage clones with weak or no binding are washed away, while those with higher affinity remain bound. To enrich high-affinity antibody fragments, bio-panning cycles are repeated 2–4 times with increasingly stringent washing steps [25]. After 3 rounds of bio-panning, the affinity of the screened phage for PIGR315-354

increased significantly. Phage ELISA analysis showed that E4-positive antibodies had binding values against PIGR315-354 more than twice those against BSA, PIGR225-268, and PIK3CB2-43 in both rounds of testing. These results indicate that bio-panning successfully selected E4 clones with specific affinity for PIGR315-354.

Currently, ultrasonography is the primary method used to screen for CCA [26]. It places a burden on expert ultrasonographic doctors and staff and increases the costs associated with ultrasonography equipment. Therefore, there is an urgent need for an easy, inexpensive, and rapid screening method, such as an immunochromatographic assay (lateral flow strip), to enable CCA screening in community settings. Moreover, using a rapid-format PIGR-based diagnostic biomarker for CCA in a liquid biopsy, a non-invasive approach, may be more useful than tissue-based biopsy due to its lower cost and greater ease compared to imaging techniques. The application of an scFv antibody derived from the Yamo I library is an important component in the production of antibodies for the diagnosis of CCA. The process can involve bioprocess optimization for large-scale production [27], affinity maturation of scFv antibody to increase the affinity [16], and the generation of scFv-alkaline phosphatase fusions. However, false-positive results from the screening test can occur and need to be confirmed by ultrasonography, imaging techniques, or histopathological analysis. Nevertheless, this approach can reduce the burden currently placed on ultrasonography, as well as lower the overall cost and time required in community settings. This format can be used as a convenient one-step detection antibody in ELISA, as previous studies have shown the binding of scFv-AP from the Yamo I library against zearalenone [18]. All of these efforts will contribute to obtaining antibodies that can be used for the diagnosis of CCA.

CONCLUSION

The Yamo I library was utilized in conjunction with antibody phage display technology to screen for PIGR315-354 specific scFv antibodies. The E4-positive scFv antibody showed promising binding to the PIGR peptide using ELISA and dot-blot immunoassay. Its application in a rapid and simple detection format indicates its potential as a diagnostic tool for this condition, which will be further developed in the next step.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.069.

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Appendix A. Supplementary data

Table S1 ELISA of small-scale bio-panning. Bacteriophage clones are screened against the PIGR315-354 peptide, with BSA used as a control. Absorbance is measured at 450 nm. Fold-change is calculated as the ratio of absorbance against the PIGR315-354 peptide to that against BSA (PIGR/BSA).

Clone	Absorbance 450		Fold-change
	PIGR315-354	BSA	
A4	0.698	0.075	9.290
A6	0.524	0.072	7.225
B11	0.425	0.157	2.705
B12	0.514	0.221	2.330
C11	0.259	0.153	1.695
C12	0.255	0.110	2.317
D1	0.370	0.026	14.330
D8	0.522	0.104	5.047
E4	1.168	0.035	33.777
E8	0.415	0.068	6.097
F1	0.373	0.029	12.906
F9	0.634	0.059	10.720
G2	0.672	0.073	9.254
G7	0.690	0.065	10.689
H1	0.112	0.074	1.504
H4	0.312	0.064	4.858

Table S2 ELISA of large-scale bio-panning of E4-phage clone. Absorbance values at 450 nm are obtained from 2 rounds of large-scale bio-panning. E4-phage clone is screened for binding to the PIGR315-354 peptide using ELISA. BSA, PIGR225-268, and PIK3CB2-43 serve as negative controls. Each condition is tested in triplicate, and the corresponding mean and standard deviation (SD) are shown for each target.

Round 1					
	Absorbance 450			Mean	SD
PIGR315-354	0.279	0.241	0.214	0.245	0.033
BSA	0.063	0.079	0.092	0.078	0.014
PIGR225-268	0.065	0.053	0.050	0.056	0.008
PIK3CB2-43	0.057	0.061	0.046	0.054	0.008
Round 2					
	Absorbance 450			Mean	SD
PIGR315-354	0.434	0.308	0.185	0.309	0.125
BSA	0.085	0.092	0.145	0.107	0.033
PIGR225-268	0.093	0.091	0.084	0.089	0.004
PIK3CB2-43	0.083	0.111	0.083	0.092	0.004

Table S3 ELISA of expressed E4-scFv antibody against PIGR315-354 peptide. Absorbance values at 450 nm, along with the mean and standard deviation (SD), are shown for E4-scFv antibody binding to PIGR315-354 and to negative controls (BSA, PIGR225-268, and PIK3CB2-43).

	Absorbance 450			Mean	SD
PIGR315-354	2.304	3.551	3.080	2.978	0.630
BSA	0.297	0.579	0.398	0.435	0.143
PIGR225-268	0.240	0.182	0.836	0.429	0.362
PIK3CB2-43	0.903	2.321	1.405	1.543	0.719

Table S4 Detection range of the E4-scFv antibody against PIGR315-354. Different concentrations of the PIGR315-354 peptide (μg/ml) are tested to evaluate binding by the soluble scFv antibody using ELISA. Absorbance at 450 nm is measured in triplicate, and the mean and standard deviation (SD) are reported.

Concentration of PIGR315-354 (μg/ml)	Absorbance 450			Mean	SD
5.000	0.364	0.582	0.881	0.609	0.259
2.500	0.247	0.500	0.501	0.416	0.147
1.250	0.139	0.183	0.456	0.259	0.172
0.630	0.133	0.217	0.258	0.203	0.064

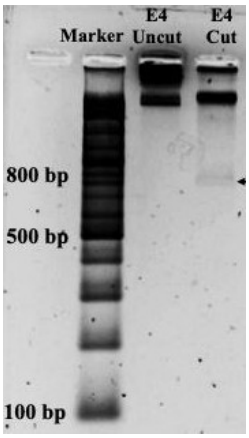

Fig. S1 Agarose gel electrophoresis of the E4 scFv DNA fragment. The E4 scFv DNA fragment is observed at approximately 800 bp after digestion with *Sfi* I and *Not* I enzymes. The arrow indicates the scFv DNA fragment. The image is shown in inverted black and white.