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School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Development of a recombinant biosimilar single-chain variable fragment antibody targeting human estrogen receptor α36

Soodabeh Shafiee[1], Sedighe Kolivand[1], Neda Jalili[1], Mohammad Abedini[1], Mahboubeh Navabi[2], Yeganeh Talebkhan[2], Rezvan Esmaeili[3,*], and Mohadeseh Haji Abdolvahab[1,*]

[1]Recombinant Proteins Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran.
[2]Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran.
[3]Genetics Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran.

Abstract

Background and purpose: The estrogen receptor alpha-36 (ER-α36) is an alternative splice variant of classical ER-α66 and is abundantly present in both ER-α66-positive and ER-α66-negative breast tumor cells. Given its clinical relevance, developing targeted strategies against this isoform is of particular significance to breast cancer research. This study aimed to develop an ER-α36-specific recombinant biosimilar single-chain variable fragment (scFv) antibody.

Experimental approach: The primary amino acid sequence of the anti-ER-α36 scFv was retrieved from patent *US20110311517A1*. An expression cassette harboring the scFv coding sequence was designed and incorporated into the backbone of the pET-28a(+) expression vector for recombinant expression in *Escherichia coli (E. coli)* BL21(DE3) cells. Expression conditions were then optimized, and the protein was purified using immobilized metal affinity chromatography. The binding of the purified scFv to ER-α36-expressing breast cancer cells was assessed using enzyme-linked immunosorbent assay (ELISA) and flow cytometry.

Findings/Results: Characterization using sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting experiments revealed a molecular weight of 29 kDa for the expressed scFv antibody. Relative quantification revealed the highest scFv protein expression level 16 h after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside at 25 °C. Flow cytometry and ELISA assays demonstrated specific binding of the scFv to ER-α36 protein on MDA-MB-231 breast cancer cells, while no interaction was detected with ER-α36-negative MCF-10A normal mammary epithelial cell line.

Conclusion/implications: The anti-ER-α36 scFv antibody fragment was successfully expressed using the *E. coli* expression system, and the purified protein was able to specifically recognize and bind to ER-α36-expressing human breast cancer cells.

Keywords: Antibody fragment; Breast cancer; Estrogen receptor; Recombinant; scFv.

INTRODUCTION

Guiding clinical decisions regarding the diagnosis, prognosis, and treatment of breast cancer relies mainly on specific biomarkers (1). As a well-known case in point, the estrogen receptor alpha (ER-α66) is one of the most established prognostic and predictive markers in the clinical management of breast cancer (2,3). ER-α36 is an alternative splice variant of classical ER-α66 and is abundantly present in both ER-α66-positive and ER-α66-negative breast tumor cells. Membrane-initiated estrogen signalling through ER-α36 has been shown to activate multiple downstream pathways implicated in tumor aggressiveness and metastasis (4).

* Corresponding authors:
M. Haji Abdolvahab, Tel: +98-2188641398, Fax: +98-2188203915
Email: mohadesehabdolvahab@gmail.com
R. Esmaeili, Tel: +98-2188641398, Fax: +98-2188203915
Email: esmaeili.rezvan@gmail.com

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Localization of ER-α36 to the plasma membrane as well as the cytoplasm suggests its involvement in both genomic and non-genomic estrogen signaling pathways. With respect to estrogen genomic signaling, this implication is chiefly related to the role of ER-α36 as a dominant suppressor of pathways mediated by ER-α66 and ER-β (5,6). In addition, the association of ER-α36 with clinical phenotype and responsiveness to endocrine therapy, especially in breast cancer, highlights its relevance as a tumor-associated ER isoform and supports its potential role as a biomarker for the diagnosis and treatment of estrogen-dependent cancers (7).

To date, a great deal of effort has been invested in improving the design, production, and application of targeted agents that selectively recognize a diverse array of molecular cancer biomarkers for both diagnostic and therapeutic purposes (8-10). In this context, not only are widely used full-length antibodies, but also multiple smaller formats, including single-chain variable fragments (scFv), variable fragments (Fv), and antigen-binding fragments (Fab), have undergone extensive engineering and evaluation (11,12).

Structurally, an scFv is a recombinant fusion protein consisting of variable heavy (VH) and light (VL) domains linked by a short peptide sequence. Having a comparatively reduced molecular size, an scFv preserves the antigen-binding specificity characteristic of intact antibodies (13). Their facile development, ease of manipulation, manufacturing scalability, as well as enhanced functional affinity have established scFvs as powerful platforms in research, preclinical, and clinical settings (14,15). These antibody fragments can be efficiently expressed in a functional form using bacterial expression systems, most notably in *Escherichia coli* (*E. coli*) host, which offers a rapid and economically feasible approach for high-yield recombinant protein production (13). The versatility of scFvs across diagnostic and therapeutic contexts has been exemplified by their application in enzyme-linked immunosorbent assay (ELISA) detection of glycolytic acid (16), positron emission tomography-computed tomography (PET/CT) imaging of mesothelin (17), magnetic resonance imaging (MRI) visualization of epidermal growth factor receptor (EGFR) (18), near-infrared (NIR) spectroscopy to identify human ether-a-go-go-related gene (hERG1) (19), fluorescent molecular tomography of prostate-specific membrane antigen (20), and a targeted therapy directed against epithelial cell adhesion molecule (EpCAM) (21).

Despite the clinicopathological significance of ER-α36, understanding the exact role of ER-α36 in breast cancer has been hampered by the lack of reliable antibodies on the market. ER-α polyclonal antibodies have poor specificity for ER-α36 and, therefore, limit its evaluation as a clinical biomarker. Among the ER-α36-related literature, the patent US20110311517A1 (22) discloses antibodies and antibody fragments designed for the diagnosis, prevention, and treatment of diseases involving this receptor. The introduced scFv antibodies were engineered to selectively recognize and bind amino acid residues 284-310 of ER-α36. As claimed in the patent, these scFv fragments specifically target ER-α36 while showing no binding to other ER isoforms, including ER-α66. The current study aimed to reproduce an anti-ER-α36 scFv fragment described in this patent for subsequent in-house research. We therefore produced the scFv antibody fragment in an *E. coli* expression host, optimized the expression conditions, and evaluated the antibody functional activity in breast cancer cell lines.

MATERIALS AND METHODS

Chemicals and reagents

Luria-Bertani (LB) broth and agar media, kanamycin, and reagents used for expression and analysis of protein, including isopropyl β-D-1-thiogalactopyranoside (IPTG), Tris-HCl, lactose, glycine, sodium dodecyl sulphate (SDS), acrylamide/bis-acrylamide, phenylmethylsulfonyl fluoride (PMSF), tetramethylethylenediamine, and ammonium persulfate, were purchased from Sigma-Aldrich (USA). Ni-Sepharose 6B Fast Flow affinity chromatography resin was ordered from ARG Biotech (Tabriz, Iran). Horseradish peroxidase (HRP)- and fluorescein isothiocyanate (FITC)-conjugated anti-histidine secondary

antibodies were purchased from Abcam (Cambridge, UK). Commercially available cell culture media and reagents were obtained from Gibco (Waltham, MA, USA).

Plasmid vectors, bacterial strains, and cell lines

The pBR322 plasmid vector for cloning, pET28a(+) plasmid vector for scFv antibody expression, and pUC57 plasmid carrying the anti-ER-α36 antibody coding sequence were procured from Pishgam Co. (Tehran, Iran). One Shot™ TOP10F' and BL21 (DE3) *E. coli* strains (obtained from Pasteur Institute, Tehran, Iran) were employed as bacterial hosts for cloning and expression experiments. ER-negative breast cancer cell line MDA-MB-231, which expresses high levels of endogenous ER-α36, and MCF-10A, a non-tumorigenic, and ER-α36-negative human mammary epithelial cell line, were purchased from the Cell Bank repository (Pasteur Institute, Tehran, Iran). MDA-MB-231 cell line was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin). MCF-10A cells were grown in a mixture of Ham's F-12 medium and DMEM supplemented with 5% (v/v) FBS, 10 µg/mL human insulin, 20 ng/mL human EGF, 100 ng/mL cholera toxin, and 0.5 µg/mL hydrocortisone. The cells were subsequently incubated in a humidified incubator with 5% CO_2 at 37 °C.

Design and cloning of recombinant anti-ER-α36 scFv expression cassette

The primary amino acid sequence of the fully human anti-ER-α36 scFv fragment was obtained from patent *US20110311517A1* (22). The patent details the development of antibodies and antigen-binding fragments that target ER-α36. It further includes drug formulations and strategies for the diagnosis, prevention, and treatment of ER-α36-mediated diseases. The scFv fragments introduced in the patent were obtained through panning a phage display scFv library against a target peptide matching amino acid residues 284-310 of human ER-α36. The anti-ER-α36 scFv of interest in this study, arranged in a V_L-linker-V_H orientation, comprises a V_L domain (QSVLTQPASVSGSPGQSITISCTGTSSDVGGY NYVSWYQQHPGKAPKLMIYDVSKRPSGVSN RFSGSKSGNTASLTISGLQAEDEADYYCSSYT SSSTLVFGGGTKLTVLG), a linker (SGGSTITSYNVYYTKLSSSGT), and a V_H domain (EVQLVQSGGGVAQPGRSLRLSCAASGITFNS YGMHWVRQAPGKGLEWVAVMPYDGSNEY YADSVKGRFTISRDNSKNTLYLQMNSLRAED TAVYYCAKGSGMVQLWADAFDVWGQGTM VTVSSAS).

The codon-optimized gene construct, which carries the scFv coding sequence and is 806 bp in length, was cloned into the pBR322 cloning vector and transformed into chemically competent TOP10F' cells. Amplification of the recombinant vector was performed by inoculating single colonies of the recombinant TOP10F' cells in LB medium supplemented with ampicillin and tetracycline antibiotics, and then incubating the culture at 37 °C on a shaker. The *NcoI/XhoI*-digested anti-ER-α36 scFv gene fragment was gel-extracted using a commercially available plasmid extraction kit (MEGA quick gel extraction kit, iNtRON Biotechnology, Korea). The obtained DNA fragment was then subcloned at *NcoI* and *XhoI* restriction sites within the pET28a(+) expression vector in frame with the C-terminal His tag to facilitate detection and affinity purification of the expressed recombinant protein. Chemically competent *E. coli* BL21 (DE3) cells were then subjected to transformation with the recombinant plasmid pET28a-anti-ERα36-scFv (hereafter referred to as pET28a-scFv36). The identity of the recombinant plasmid was verified through restriction enzyme digestion and sequencing.

Expression of recombinant anti-ER-α36 scFv protein

Recombinant *E. coli* BL21 (DE3) colonies were inoculated into 30 µg/mL kanamycin-supplemented LB broth selection medium and incubated at 37 °C with continuous agitation at 180 rpm. Incubation proceeded until the culture optical density at 600 nm (OD_{600}) reached 0.4-0.6 (mid-log phase) when the recombinant protein expression was induced through the introduction of 0.5 mM IPTG into the culture medium, followed by overnight incubation under the above-mentioned conditions. For subsequent protein expression analysis, 1 mL of aliquots was collected as pre- and post-

induction samples, centrifuged for 3 min at 9,000 rpm, and the resulting bacterial pellets were thereafter cryopreserved at -20 °C. Next, anti-ER-α36 scFv was subjected to characterization by SDS-PAGE and Western blotting. The molecular weight of the expressed protein was determined on 12% SDS-PAGE and confirmed by Western blotting. Pre- and post-induction of bacterial samples, as well as a negative control sample (*E. coli* cells carrying the empty vector), were examined. HRP-conjugated anti-His tag antibody (Cat No. ab1187, Abcam, USA) diluted 1:1000 in PBS was applied to the nitrocellulose membrane, and signal development was achieved by subjecting the membrane to a solution of 3,3′-diaminobenzidine tetrahydrochloride following a standardized protocol.

Optimization of anti-ER-α36 scFv expression in E. coli BL21 (DE3) strain

To optimize scFv protein expression, key parameters including temperature (15, 20, 25, 30, and 37 °C), lactose concentration (1, 2.5, and 5 g/L), IPTG concentration (0.25, 0.5, and 1 mM), and post-induction incubation time (6, 8, 12, 16, 24, and 48 h) were evaluated. To ensure consistency, cell cultures were initiated with identical cell densities across all conditions. SDS-PAGE was utilized to investigate the protein content of harvested bacterial cells. The relative band intensities of the recombinant protein were determined using ImageJ software.

Purification and refolding of the recombinant anti-ER-α36 scFv protein

The bacterial pellet was washed with PBS, resuspended in the lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 50 mM dithiothreitol (DTT), 5 mM EDTA, and 1 mM PMSF; pH 8.0), and sonicated for 20 cycles of 45 s sonication (each cycle consisted of 30 s “on”, followed by 15 s “off”) on ice. The bacterial lysate was centrifuged, and the pellet containing the recombinant protein was washed 3 times with the washing buffer (2 M urea, 100 mM Tris, 5 mM EDTA, and 2% v/v Triton X-100), followed by a single rinse with Triton X-100-free washing buffer. The inclusion bodies (IBs) were then solubilized by adding solubilization buffer (50 mM Tris-HCl, 8 M urea, and 1 mM DTT; pH 10.0) and then subjected to the immobilized metal affinity chromatography resin. A solution containing denatured His-tagged anti-ER-α36 scFv protein was loaded onto the Ni-NTA Sepharose 6B Fast Flow affinity column and purified under denaturing conditions. In brief, following equilibration of the column with the washing buffer (20 mM Tris-HCl, 8 M urea, 10 mM imidazole, and 25 mM NaCl; pH 8.0), unbound proteins were removed from the chromatography column. A gradient of elution buffer (washing buffer with increasing imidazole concentration) was applied to the column to elute the target protein. The fractions containing the protein were collected and characterized by SDS-PAGE. Following protein purification, the eluates were dialyzed (10 kDa molecular weight cut-off dialysis tubing; Sigma-Aldrich, Germany) against dialysis buffer at a 1:10 sample-to-buffer volume ratio for 17 h. The buffer was then refreshed, and dialysis continued for a further 7 h. Subsequently, the samples were diluted to 300 g/mL using a borate saline buffer and renatured through a 10-fold dilution dialysis with buffer exchange at each step.

Analysis of the anti-ER-α36 scFv antibody binding properties

Indirect ELISA

An indirect cell-based ELISA was performed to evaluate the binding specificity of the anti-ER-α36 scFv antibody (23). ER-α36-positive MDA-MB-231 and ER-α36-negative MCF-10A cell lines were grown in triplicate wells of a 96-well plate and incubated overnight. Then, cells were fixed in 100 µL of 4% (v/v) formaldehyde in PBS for 30 min at room temperature. Following fixation, the solution was removed by centrifugation at 200 × *g* for 4 min. Subsequently, 200 µL of blocking buffer containing 3% bovine serum albumin (BSA) was added to each well, and the plates were kept at room temperature for 30 min. Following removal of the blocking buffer, cells were incubated for 1 h at room temperature under gentle agitation with varying concentrations (0.001, 0.01, 0.1, 1, 10, and 100 µg/mL) of the anti-ER-α36 scFv antibody or an irrelevant anti-VEGF

antibody. Cells were washed twice with washing buffer (0.1% BSA in PBS), followed by incubation with 50 µL of HRP-conjugated anti-His antibody (1:2000 dilution) for 1 h at room temperature. Plates were then washed twice with washing buffer, incubated with 100 µL of 3,3',5,5'-tetramethylbenzidine substrate in the dark at room temperature for 15 min, and the reaction was stopped with 1 M H_2SO_4. Finally, the absorbance was quantified spectrophotometrically at 450 nm with a microplate reader.

Flow cytometry

The specific binding of anti-ER-α36 scFv to its target antigen was assessed by a fluorescence-activated cell sorting flow cytometer (FACSCalibur, BD Biosciences, USA), and the fluorescence intensity was measured in the FL1 channel. In brief, ER-α36-positive and ER-α36-negative breast cancer cells were incubated with 3 µM anti-ER-α36 scFv for 1 h at 4 °C. Following a thorough PBS washing step, cells were stained with FITC-labelled anti-His antibody (1:250) on ice. Cells were then rinsed, resuspended in PBS, and subjected to flow cytometry analysis. As a negative control, cells incubated with FITC-labelled anti-His antibody alone (without primary antibody) were included in the experiments.

Statistical analysis

Data were expressed as mean ± standard deviation and analysed utilizing GraphPad Prism (V. 8.0, USA) software. Student's T-test was applied to determine intergroup differences. *P*-values < 0.05 were considered significant differences.

RESULTS

Cloning of anti-ER-α36 scFv cassette

The anti-ER-α36 scFv coding sequence was incorporated into the pET-28a (+) plasmid using *NcoI/XhoI* restriction sites controlled by the IPTG-activated T7 promoter (Fig. 1A). The cloning procedure was confirmed by restriction digestion of the vectors in which an 806-bp fragment corresponding to the anti-ER-α36 scFv coding sequence, along with the residual vector backbone fragments of 4.361 kb (pBR322) (Fig. 1B) and 5.369 kb (pET28a) (Fig. 1C), were observed. DNA sequencing reconfirmed the cloned anti-ER-α36 scFv gene fragment within the expression vector.

Recombinant scFv expression

Following recombinant protein expression, cell lysates were run on SDS-PAGE. As depicted in Fig. 2A, a distinct protein band (approximately 29 kDa) was observed in bacterial lysate after induction, corresponding to anti-ER-α36 scFv protein. Successful expression of His-tagged recombinant protein was revealed through Western blotting utilizing an anti-His tag secondary antibody (Fig. 2B).

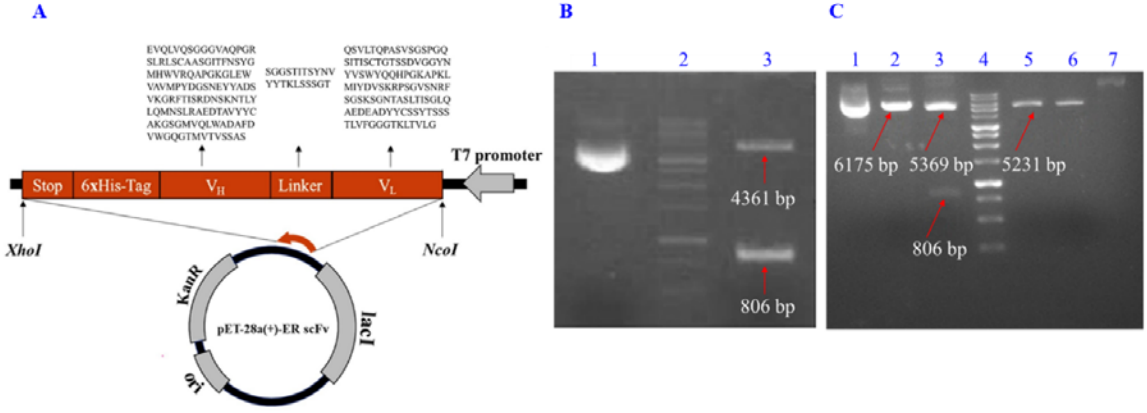

Fig. 1. Design and confirmation of the pET-28a(+)-anti-ER-α36 scFv vector. (A) A schematic illustration of anti-ER-α36 scFv coding sequence incorporated into the pET-28a(+) expression vector; (B) restriction digestion of cloning vector. Lane 1, undigested pBR322 plasmid; lane 2, 1 kb DNA marker; lane 3, *NcoI/XhoI* double-digested pBR322-anti-ER-α36 scFv plasmid; (C) restriction digestion of expression vector. Lane 1, undigested recombinant pET-28a(+)-anti-ER-α36 scFv plasmid; lane 2, *XhoI* digested recombinant expression vector; lane 3, *NcoI/XhoI* double-digested recombinant expression vector; lane 4, 1 kb DNA marker; lane 5, *NcoI/XhoI* double-digested pET-28a(+) (control); lane 6, *XhoI* digested pET28a(+); lane 7, undigested pET-28a(+) plasmid. ER-α36, Estrogen receptor alpha-36; scFv, single-chain variable fragment.

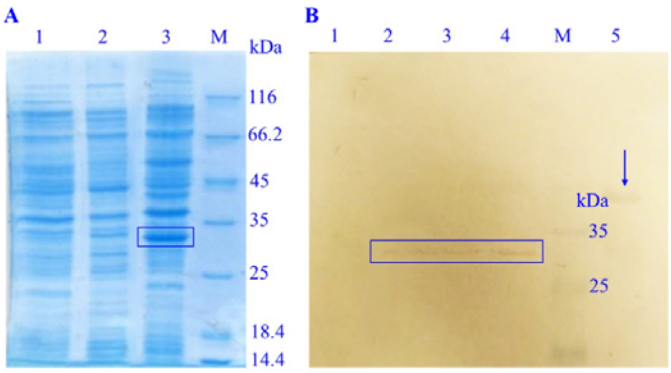

Fig. 2. Analysis of the recombinant anti-ER-α36 scFv expression. (A) SDS-PAGE analysis. Lane 1, lysate of *E. coli* BL21(DE3) cells without plasmid (negative control); lane 2, lysate of *E. coli* BL21(DE3) cells harboring pET28a(+)-anti-ER-α36 plasmid before induction; lane 3, lysate of *E. coli* BL21(DE3) cells harboring pET28a(+)-anti-ER-α36 plasmid after induction with 0.5 mM IPTG, 4 h at 37 °C; M, unstained protein marker. (B) Western blotting analysis using anti-His-tag antibodies. Lane 1, lysate of *E. coli* BL21(DE3) cells harboring empty pET28a(+) vector; lanes 2-4, lysate of *E. coli* BL21(DE3) cells harboring pET28a(+)-anti-ER-α36 plasmid after induction; M, protein marker; lane 5, His-tagged protein (positive control). ER-α36, Estrogen receptor alpha-36; scFv, single-chain variable fragment; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Optimization of culture conditions for anti-ER-α36 scFv expression

Physicochemical parameters, including temperature, IPTG concentration, and post-induction incubation time, were assessed to determine the optimal conditions to maximize the expression of anti-ER-α36 scFv protein (Fig. 3A-F). Relative quantification of scFv protein expression demonstrated that the highest expression level was achieved with 1 mM IPTG following 16 h incubation at 25 °C (Fig. 3G and H). Taken together, as Fig. 3B represents, a significantly increased scFv expression was achieved in longer post-induction times compared with 6 h incubation. It was noticed that protein expression level did not significantly change when higher temperatures were tested. Given that the protein expression level at 25 °C remained relatively constant at 16, 24, and 48 h, an incubation time of 16 h was chosen for subsequent protein expression experiments. Furthermore, increasing the IPTG concentration from 0.2 to 1 mM enhanced the expression of the scFv protein, as expected. In contrast, applying higher lactose concentrations resulted in lower expression levels (Fig. 3F and H). After establishing the optimal conditions for maximizing protein expression, the produced scFv protein was extracted and purified. As illustrated in Fig. 4, SDS-PAGE and Western blotting analyses verified expression and purification of the scFv antibody fragment by a distinct high-purity band at the predicted molecular weight of ~29 kDa.

Anti-ER-α36 scFv binding specificity

The purified scFv antibody was evaluated for binding to ER-α36 by cell-based ELISA analysis. As shown in Fig. 5A, the anti-ER-α36 scFv antibody demonstrated binding specificity to ER-α36-expressing MDA-MB-231 cells, yielding a significantly higher signal compared to negative controls. As expected, no binding signal was detected in negative control wells containing the MCF-10A cells incubated with the anti-ER-α36 scFv antibody or the MDA-MB-231 cells incubated with the irrelevant anti-hVEGF scFv antibody.

The binding specificity of the scFv antibody against ER-α36 was further assessed by flow cytometry (Fig. 5B-D). Contour plots showed a distinct subpopulation (29.1%) of MDA-MB-231 cells with increased fluorescence after incubation with anti-ER-α36 scFv, indicating surface binding to ER-α36 (Fig. 5D). In contrast, contour plots of MCF-10A cells showed only a negligible subpopulation (2.41%) with increased fluorescence intensity, consistent with their lack of ER-α36 expression (Fig. 5C). The presence of the small cell population in the higher-fluorescence region is likely attributable to non-specific binding. The negative control experiment comprising cells incubated with only the secondary FITC-labeled anti-His antibody showed no background fluorescence in both cell types (Fig. 5B).

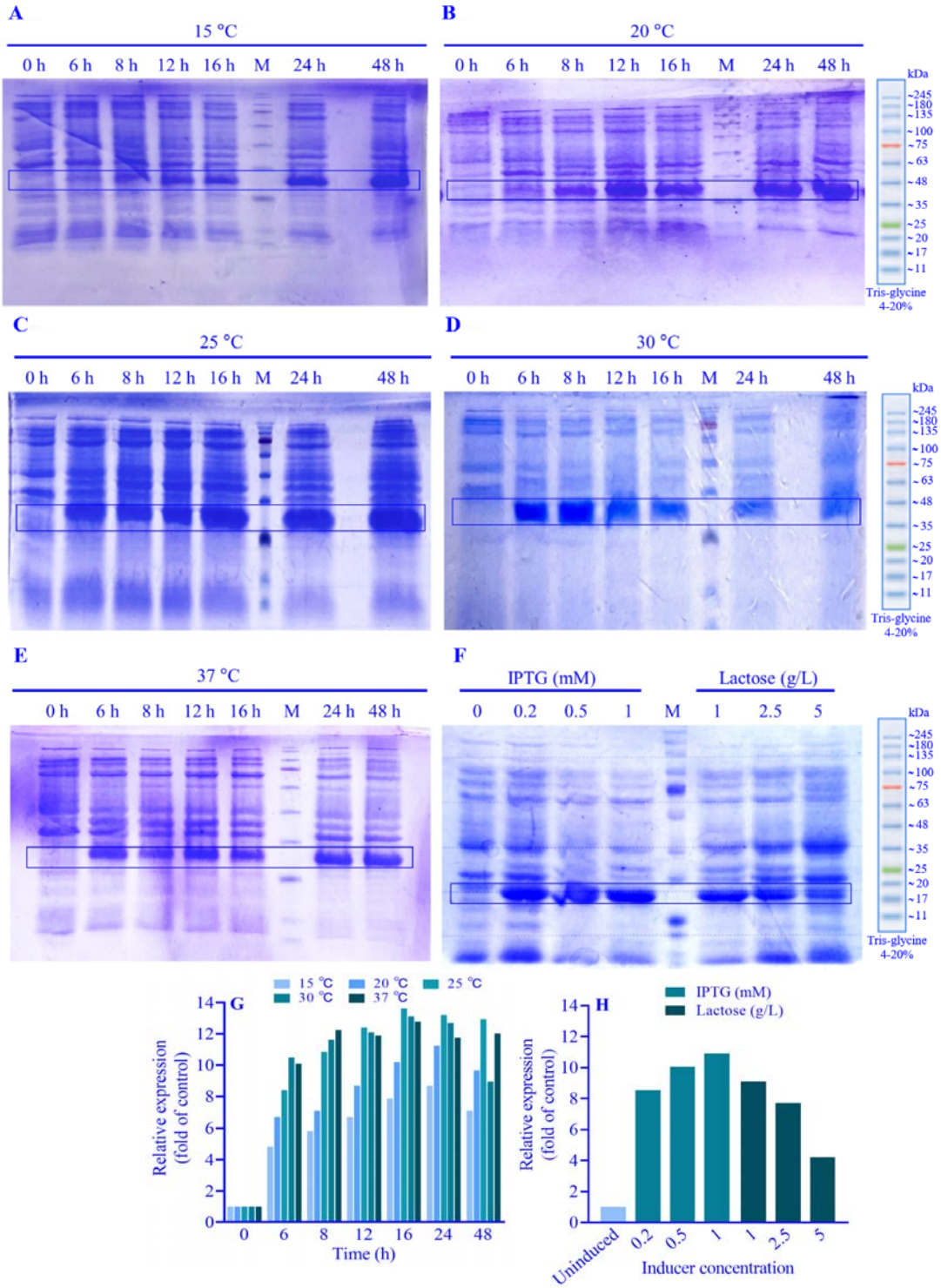

Fig. 3. SDS-PAGE analysis of the anti-ER-α36 scFv protein expression in *E. coli* BL21(DE3) cultures grown at various post-induction temperatures and inducer concentrations. Following induction, the cultures were incubated at 5 distinct temperatures of (A) 15, (B) 20, (C) 25, (D) 30, and (E) 37 °C with samples collected at various time points (0, 6, 8, 12, 16, 24, and 48 h). Lane M, protein marker; (F) a comparative analysis of IPTG and lactose induction on anti-ER-α36 scFv protein expression in *E. coli* BL21(DE3) cultures. The band intensity of anti-ER-α36 scFv protein was quantified by densitometry analysis using ImageJ software. Lane 1, uninduced control (intensities were normalized to it); lane M, protein marker. Relative band intensities for (G) various time points and (H) various concentrations of IPTG and lactose. SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; ER-α36, estrogen receptor alpha-36; scFv, single-chain variable fragment; IPTG, isopropyl β-D-1-thiogalactopyranoside.

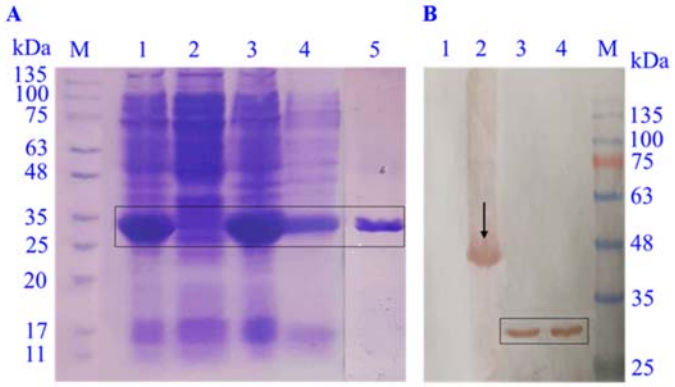

Fig. 4. Analysis of the optimized, purified recombinant scFv protein by SDS-PAGE and Western blotting. (A) Lane M, protein marker; lane 1, lysate of *E. coli* cells; lanes 2 and 3, soluble and insoluble fractions after centrifugation, respectively; lane 4, washed inclusion bodies; lane 5, the purified recombinant scFv protein. (B) Lane 1, lysate of *E. coli* BL21(DE3) before induction; lane 2, His-tagged protein (positive control); lanes 3 and 4, lysates of *E. coli* BL21(DE3) cells after induction; M, protein marker. SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; scFv, single-chain variable fragment.

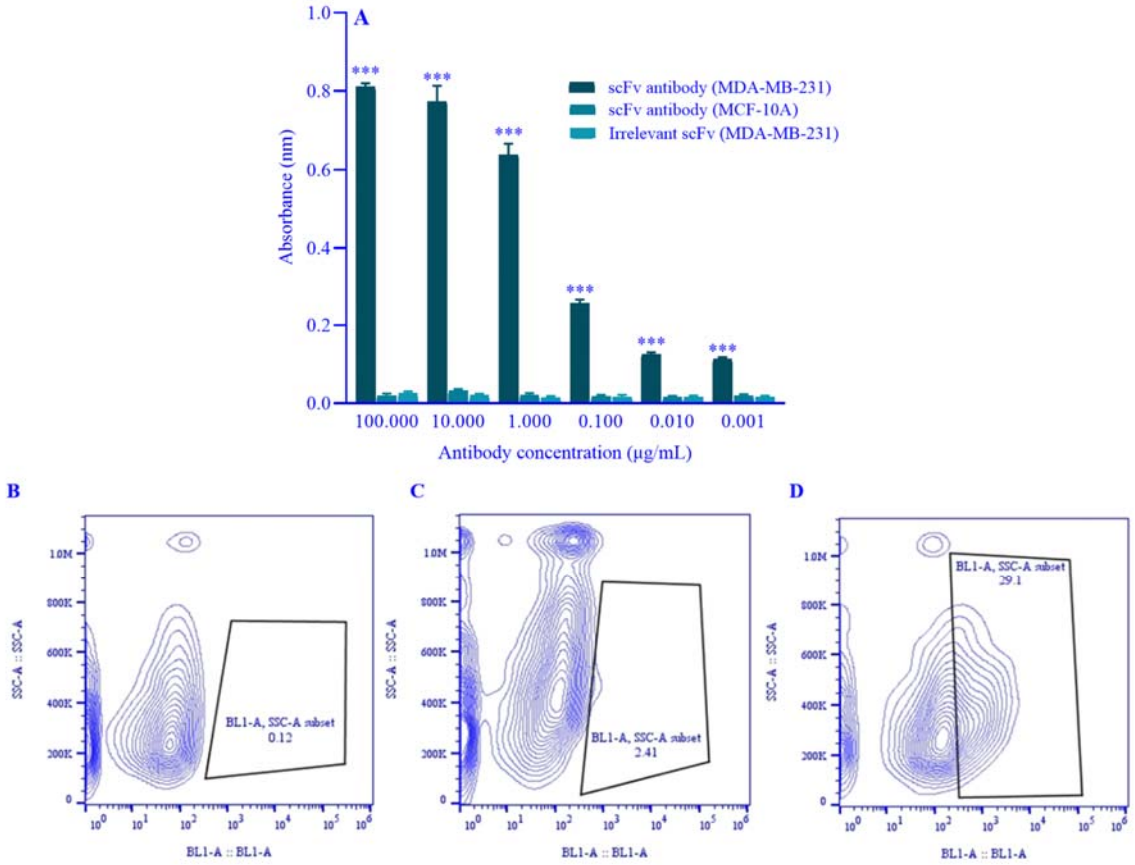

Fig. 5. Antigen-binding specificity of the anti-ER-α36 scFv antibody fragment. (A) ELISA results showing the binding of purified anti-ER-α36 scFv antibody to ER-α36-positive cancer cells at absorbance 450 nm. A linear increase in optical density was observed with escalating concentrations of the anti-ER-α36 scFv antibody. Data were expressed as mean ± SD, n = 3. $^{***}P < 0.001$ demonstrates a significant difference compared to the respective negative control groups, irrelevant scFv or anti-ER-α36 scFv tested in the MCF-10A cell line. (B-D) Flow cytometry analysis of binding the scFv antibody to ER-α36-positive MDA-MB-231 cancer cells, which was indicated by a shift in fluorescence intensity within the FL1 channel. (B), Contour plot representing background staining with secondary FITC-labelled anti-His antibody only (no primary antibody), included as a negative control; (C) and (D), contour plots showing anti-ER-α36 scFv antibody staining of MCF-10A and MDA-MB-231 cancer cells, respectively. ER-α36, Estrogen receptor alpha-36; scFv, single-chain variable fragment; FITC, fluorescein isothiocyanate.

DISCUSSION

In the current study, we produced and optimized the expression of a recombinant biosimilar scFv antibody fragment in *E. coli* BL21 (DE3) strain with ER-α36 ligand binding activity toward the ER-α36-expressing breast tumor cells. Following successful protein production, the scFv antibody was characterized using *in vitro* assays to elucidate its binding properties.

Currently, due to their great specificity, antibody-based assays relying upon antibodies raised against ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) (24) markers are among the most reliable diagnostic tools for accurate identification and characterization of specific breast cancer subtypes. The particular significance of ER-α to breast tumor treatment and diagnosis has promoted multiple studies to focus on generating full-sized monoclonal antibodies (mAbs) targeting human ER-α at the tumor site. Greene *et al*. exploited hybridoma technology, which involved the fusion of splenic lymphocytes derived from ER-α-immunized rats with mouse myeloma cells to develop the first panel of 3 mAbs exhibiting specificity for human ER-α (25). Also, Hassanzadeh Makoui's study reported the development of 2 highly specific and sensitive mAbs against ER and PR to enhance the reliability of their detection in cancer diagnostics, suggesting their capacity as advantageous tools for improved clinical decision-making and diagnostic accuracy (26). Unlike ER-α66, which is routinely detected in clinical settings, there are almost no recent reports on the development of antibodies against ER-α36, to the best of our knowledge. Advancements in recombinant DNA technology, jointly with the engineering and generation of antibody fragments using highly efficient and high-yielding bacterial expression systems, have notably revolutionized this field (27). In this regard, fully functional antigen-binding fragments produced in *E. coli* cells have emerged as promising therapeutic and diagnostic agents in oncology (28). scFv antibodies have notable advantages over full-length mAbs due to their small size (~27 kDa versus 150 kDa), which allows deeper tissue penetration and access to hidden epitopes. Furthermore, their compact structure facilitates faster clearance from blood, efficient low-cost production in microbial systems, and easy genetic manipulation. Absence of the Fc domain reduces immunogenicity, while maintaining full antigen-binding capacity and permitting conjugation for enhanced stability, affinity, and therapeutic potential (29,30). Nevertheless, scFvs still encounter limitations in specificity, stability, immunogenicity, and large-scale production. For instance, scFv-based theranostics require large-scale, high-quality production for clinical use. Yet, low and variable expression, combined with structural complexity and poor solubility, make their production and purification challenging (31). In the present study, SDS-PAGE and Western blotting analyses confirmed efficient production of the desired scFv fragment in *E. coli* cells after optimizing factors affecting protein expression yield. Evaluating the expression level of recombinant scFv fragment using densitometry analysis demonstrated that the normalized intensity of scFv protein band was the highest at 25 ℃. When the temperature was reduced from 37 ℃ to 15 ℃, cellular metabolism was so slow that induction had minimal effect on the protein production rate. It was, however, found that induction at 37 °C, which was supposed to boost protein expression, resulted in decreased protein levels compared to 25 ℃. Moreover, densitometry results showed that the protein expression level reached its maximum after 16 h of incubation following induction with 1 mM IPTG. Gholizadeh *et al*. optimized key expression parameters to improve the soluble yield of anti-CD22 scFv in *E. coli* Rosetta (DE3). They tested IPTG concentrations between 0.05 and 1 mM and showed that 0.5 mM yielded the best soluble expression. Further post-induction temperature screening (20, 25, 30, and 37 °C) combined with different incubation times indicated that 25 °C for 8 h maximized solubility, whereas extended induction reduced it (32). In another work, Behravan *et al*. optimized culture conditions for the production

of a recombinant humanized anti-EpCAM scFv in *E. coli* BW25113 (DE3). The authors applied response surface methodology with a central composite design to evaluate the effects of 4 key parameters, IPTG concentration, cell density at induction, post-induction temperature, and post-induction time, on protein yield. The optimized conditions for the maximal protein yield were determined to be 0.8 mM IPTG, $OD_{600} = 0.8$, 37 °C, and 24 h (33).

The target scFv protein was mainly found in the form of insoluble IBs. There is a well-documented propensity for the formation of IBs in roughly 70% of cases of heterologous recombinant protein production using *E. coli* as the host (34). It has also been well known that the IBs formed at lower temperatures are softer, making them easier to solubilize and handle. Recovery of the active recombinant protein from the fraction of IBs was accomplished through sequential washing steps to remove unwanted proteins, followed by protein purification by means of Ni-affinity chromatography. SDS-PAGE and Western blotting analyses further verified the high purity of the recombinant scFv fragment.

Following purification, we assessed the biological activity of the constructed recombinant anti-ER-α36 scFv antibody *in vitro*, using MDA-MB-231 and MCF-10A breast cancer cell lines to confirm that the protein was functional and appropriately refolded. The differential binding behaviour demonstrated by the recombinant anti-ER-α36 scFv antibody when interacting with cancer cells exhibiting high ER-α36 protein abundance established that the antibody could specifically bind to its target ER-α36 molecule. Evaluating the binding specificity of the antibody, applying flow cytometry- and ELISA-based binding assays, demonstrated good binding affinity towards MDA-MB-231 cells when compared to the MCF-10A negative control cell line. In a study performed by Mahgoub *et al*., indirect ELISA data revealed that their developed scFv exhibited superior performance in comparison to the parental mAb molecule. Although the signal yielded from one of the clones was slightly lower than that from the mAb, its binding specificity to MCF-7 cells was notably high, suggesting that the reduced signal did not compromise its functional capabilities (35). In addition, Kim *et al*. constructed an FITC-labeled scFv-Cκ antibody fragment targeted at CCSP-2 for imaging of colorectal tumor lesions and exhibited a high degree of affinity and specificity *in vitro* and *in vivo*, equivalent to those observed with full-length IgG antibodies (36). In subsequent flow cytometry analysis, the results of the ELISA assay were further confirmed. Therefore, in the same vein, these data verified the correct folding of the anti-ER-α36 scFv antibody since improper folding of the antibody hampers its function.

CONCLUSION

This current study successfully produced a recombinant biosimilar anti-ER-α36 scFv antibody, previously reported in patent *US20110311517A1*, and improved its expression level by establishing optimal conditions. The binding specificity of the developed scFv antibody fragment for its target receptor was confirmed through ELISA and flow cytometry techniques. This study could be considered a first step toward a deeper exploration of this scFv for therapeutic or diagnostic applications. Although further functional characterizations are needed, the evidence provided here can serve as a reference point for subsequent studies on the produced anti-ER-α36 scFv antibody. In practice, this scFv has the potential to be used as one of the components of an immunohistochemistry kit for ER-α36 detection in cancerous tissues. Compared to whole antibodies, small antibody fragments can be more advantageous in specific clinical settings. The targeted scFv of the present study can be conjugated with therapeutic payloads, including potent cytotoxins, to deliver them to the cancer cells and consequently destroy these cells without affecting normal cells. As another promising application, this scFv can be further employed as a theranostic tool in the context of oncology.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

S. Shafiee contributed to the data collection, interpretation, and drafting of the manuscript; S. Kolivand guided the project with consistent feedback and advice; N. Jalili, M. Abedini, and M. Navabi contributed to the data collection; R. Esmaeili, M. Haji Abdolvahab, and Y. Talebkhan contributed to the conception and design of the study. All authors have read and approved the finalized article. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

AI declaration

During the preparation of this work, the authors used Grammarly to improve readability and language. After using this tool, the authors reviewed and edited the content and take full responsibility for the content of the publication.

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