

EGFR Specific Affibody for Diagnostic Detection and Therapeutic Targeting of Thyroid Cancer Cells

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2 **Cancer Cells**

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Abstract

Thyroid cancer is known as the most common endocrine malignancy, with an increasing incidence rate over the past decades. Although thyroid tumor represents a favorable prognosis, a minority of patients experience tumor metastasis and recurrence. Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is normally overexpressed in different types of cancers. In thyroid tumors, EGFR expression is shown to be correlated with advanced metastatic features and therapeutic resistance. Affibody molecules are the small-sized proteins that are genetically designed and engineered to have an affinity for specific target molecules. In this study, four recombinant proteins (probes) were designed for targeting EGFR receptor in thyroid cancer cells. Affibody probes were recombinantly expressed using a prokaryotic host and purified with an affinity purification column. The cytotoxic effect of EGFR-specific affibodies was determined in thyroid cell lines (BCPAP and 8305C) using MTT and apoptosis assay. To measure the properties of the designed probes, they were conjugated with FITC and binding affinity was calculated using binding assays and flow cytometry. The results demonstrate that EGFR specific affibodies exert an antiproliferative and apoptotic effect on thyroid cancer cell lines. Moreover, the engineered affibodies demonstrate high-affinity binding in thyroid cell lines that have baseline EGFR expression, but not in EGFR-negative cells. Overall, EGFR-specific affibodies might have the potential application for both detection and targeting of TC.

Keywords: Thyroid cancer, EGFR, Affibody, Cytotoxicity, Cell binding

1. Introduction

Thyroid cancer (TC) is the most frequent malignancy occurs in the endocrine system, representing about 1% to 4% of all malignant tumors ¹. Papillary and follicular cancers, known as differentiated tumors, are the most prevalent type of thyroid tumors, account for 90% of all tumors. Most of these tumors have a good prognosis with a 5-year survival rate over 90%. However, a portion of papillary tumors (PTC) can develop more aggressive and advanced stage thyroid cancer ². It has been reported that about 30% of all differentiated TC patients will experience recurrence or develop lymph node metastasis ³. The survival rate is significantly decreased in patients with initial distant metastasis. In addition, dedifferentiation of thyroid cancer cells occurs during cancer progression, resulting in developing the highly aggressive type of thyroid malignancy ⁴. The current treatment options for differentiated thyroid cancer include thyroidectomy and post-operative radioiodine ablation. PTCs might exhibit aggressive and metastatic features at the initial or late stage of tumor progression. Some PTCs harbor complex genetic mutations, notably in BRAF V600E serine/threonine kinase, RET/PTC, and telomerase reverse transcriptase (TERT), which derive tumors to acquire more aggressive metastatic behavior ⁵. Adjuvant targeted therapies are preferably used as the first-line option for patients with locally advanced and metastatic PTC ⁶. The receptor tyrosine kinases (RTKs) are emerging as key cell surface receptors that play a pivotal role in tumor biology. These receptors are involved in initiating various intracellular signaling pathways that regulate cell growth, survival, and differentiation ⁷. Aberrant expression and activation of these receptors significantly contributed to survival, metastasis, and resistance to treatment ⁸. Among these, epidermal growth factor receptor (EGFR) is a prominent member of the ErbB family of RTKs, which plays a significant role in tumor initiation and progression, particularly in invasion and metastasis ⁵. EGFR is a membrane-spanning protein consists of an extracellular ligand-binding domain, a transmembrane helix, and an intracellular tyrosine kinase domain. Following the binding to its ligand, EGFR undergoes dimerization and autophosphorylation, which subsequently induces downstream signaling pathways including mitogen-activated protein kinases (MAPKs) and PI3K/Akt signaling pathways ⁹. EGFR is often overexpressed in different types of cancers, thereby it considered as ideal biomarker for cancer detection ¹⁰.

In recent years, several methods have been developed to enhance the early detection and targeting of tumors. Convectional imaging methods, including ultrasound, magnetic resonance imaging

(MRI), and computed tomography (CT), are suited for tumor detection but can't reflect the molecular characteristics of abnormal thyroid lesions¹¹. Therefore, recent imaging techniques are designed based on using molecular probes, consisting of targeting agents (antibodies, peptides) that can bind to the molecule of interest and a signal agent (radionuclides) to produce signal^{12,13}. However, the use of these methods may be limited due to insufficient target expression, increased risk of irradiation, non-specific tissue uptake, and off-target cytotoxic effects¹⁴. Among these probes, antibody-based probes have been widely used in thyroid tumor both for diagnostic and therapeutic applications¹⁵.

Recent advancements have led to the generation of various antibody-derived molecules, such as single-chain variable fragments (scFvs), Fab fragments, small recombinant bispecific antibodies, single-domain antibodies (dAbs), and other engineered proteins with desired features¹⁶. Affibody molecules are a class of binding proteins derived from the immunoglobulin G (IgG) binding domain of *Staphylococcus aureus* protein A¹⁷. These proteins are selected from a library based on a three-helix bundle domain scaffold in which thirteen specific amino acids are randomly mutated¹⁸. Affibody molecules are engineered proteins with a small size of approximately 6-7 kDa, and exhibit high binding affinity toward specific targets¹⁹. This small size facilitates rapid tumor penetration and accumulation a significant advantage over full-length antibodies (Xia et al. 2024). Affibodies can be conjugated to radionuclides to form a probe for cancer imaging, or a cytotoxic drug for targeted therapy²⁰. This combination of properties enhances their effectiveness in various applications, particularly in diagnostic imaging and therapeutics^{21,22}.

In the present study, we first evaluated the cytotoxic effects of three EGFR specific affibodies on two thyroid cancer cell lines, and then we constructed fluorescent affibody probes for detection of EGFR expression in these cells. We describe the production and biochemical characterization of three affibody constructs (Z_{EGFR} , $Z_{EGFR-ABD}$, $Z_{EGFR2-ABD}$) and evaluate their cytotoxicity and specific binding to EGFR-expressing cells.

2. Materials and Methods

2.1 Cell lines, reagents, bacterial strains, and plasmids

Two human thyroid cancer cell lines (BCPAP, 8305C) and an epithelial cell derived from Chinese hamster ovary (CHO-K1) were purchased from the National Cell Bank, Pasteur Institute of Iran. BCPAP and 8305C were used as the EGFR-positive²³ and CHO-K1 was used as the EGFR-

negative cell line ²⁴. The cells are cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, USA) supplemented with 10% Fetal Bovine Serum (FBS) (DNA Biotec), containing 100 units/ml of penicillin (Sigma) and 100 mg/ml of streptomycin (Sigma), and incubated in humidified 37 °C incubator with 5% CO₂. All experiments were performed with second or third passage when the cell density reached 60–70% confluence. We used pET-28a (Novagen) as an expression vector, Escherichia coli (E. coli) strains DH5α and BL21 gold (DE3) (Invitrogen). Isopropyl-beta-D-thiogalactopyranoside (IPTG), kanamycin, Imidazole, 2-Mercaptoethanol, Acrylamide, TEMED (N, N, N', N' Tetramethylethylenediamine), Ammonium persulfate, SDS (Sodium dodecyl sulfate), and Tryptone-yeast extract were all purchased from Biobasic. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Fluorescein Isothiocyanate (FITC) were products of Sigma. Nickel-Sepharose column (Ni-NTA) was purchased from Arg Tajhiz (Tabriz, Iran). Cetuximab (Erbix®) 5 mg/mL solution was purchased from Merck.

2.2 Design and production of Z_{EGFR} affibody probes

The Z_{EGFR} sequence used in this study corresponds to clone Z_{EGFR} from (<https://registry.igem.org/parts/bba-k404302>), the codon usage was optimized for high expression in a prokaryotic expression vector. These affibodies included Z_{Taq}, Z_{EGFR}, Z_{EGFR}-G₄SG₂-ABD, and Z_{EGFR}-G₄SG₂-ABD-G₄SG₂-Z_{EGFR}. Z_{Taq} is a negative control affibody generated to bind to Thermus aquaticus (Taq) DNA polymerase and doesn't bind to any other surface proteins ²⁵. Z_{EGFR}-G₄SG₂-ABD contains EGFR-specific affibody (Z_{EGFR}) fused to a flexible amino-acid Glycine/ Serin linker (GGGGSGG) and a half-life-extending albumin-binding domain (ABD035) ²⁶. The linker is a sequence of non-polar aminoacids (Glycine) and polar aminoacids (Serine). In Z_{EGFR}-G₄SG₂-ABD-G₄SG₂-Z_{EGFR} two sequences of Z_{EGFR} were linked to the Gly/Ser linkers and ABD.

The DNA sequences were synthesized by Genray. (Shanghai, China). Then the genes were cloned into pET28a expression vector through NdeI/HindIII restriction sites to a construct recombinant plasmid in which a polyhistidine tag (HHHHHH) was added to the N terminus of each construct. The plasmids were transformed into Escherichia coli strain Bl21 (DE3) using the heat-shock method as previously described ²⁷. A single colony of pET28a+ recombinant plasmid was inoculated to 10 mL pre-culture TB broth medium, containing 50 µg/mL of kanamycin, and incubated at 37 °C overnight shaking at 200 rpm. Afterward, the pre-cultures were grown in 1000

ml Terrific Broth (TB), supplemented with kanamycin, until the optical density (OD) of the culture at 600 nm reached 1.2. The protein expression was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to reach the final concentration of 0.1 mM and further incubation was performed at 22 °C for 16 h shaking at 200 rpm. The bacterial cultures were then centrifuged (4500 rpm for 10 min at 4°C), and bacterial cell pellets were resuspended in appropriate volume of lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM Imidazole, pH 7.8), and disrupted by sonication with 10 cycles of 30- second bursts and 1 minute rest interval using a Sonicator (FAPAn, Tehran, Iran) on ice. The cell lysate was centrifuged and the supernatant was loaded onto a Ni-NTA resin column, followed by washing with increasing imidazole concentration (20–50 mM). The target protein was eluted from the column using elution buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.8, 250 mM imidazole). The Bradford assay was used to determine protein concentrations ²⁸. The protein size and purity were tested by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel.

2.3 Evaluation of EGFR expression level in thyroid cancer cells and epithelial cells

EGFR expression levels in cell lines were detected with reverse transcriptase qPCR. Total RNA was extracted by TRIzol (YTzol, Yektatajhiz, Tehran, Iran) using the recommended procedure. The quality and quantity of total RNA were evaluated by Nanodrop spectrophotometer (MAESTRO-Gene) and agarose gel electrophoresis, respectively. Extracted RNA was used to synthesize cDNA by using an AddScript cDNA Synthesis kit (Addbio, Korea). Real-time qPCR was performed with StepOnePlus Real-Time PCR System (Applied Biosystems, USA) using Add SYBR Green master mix (Addbio, Korea) to detect expression of the EGFR gene and Hypoxanthine Phosphoribosyl Transferase 1 (HPRT1) as the control gene. The primer sequences were EGFR: 5'- TCATGGTCAAGTGCTGGATG-3' and 5'- ACGGTAGAAGTTGGAGTCTGTAG-3', and HPRT1: 5'- CTGGCGTCGTGATTAGTGATG-3' and 5'- AGCACACAGAGGGCTACA-3'. The Real-Time qPCR reactions were incubated at 95°C for 10 min, followed by 35 cycles of 95°C for 15 seconds and 61°C for 25 seconds. All samples were assayed in duplicate, and the average Ct value was used for quantification. Ct values of EGFR were normalized to the control gene HPRT1.

2.4 Assessment of cytotoxicity of Z_{EGFR} affibody probes on thyroid cancer cells

The MTT cell cytotoxicity assay was performed as previously described²⁹. Thyroid cancer cell lines, BCPAP and 8305C, were seeded in triplicate in 96-well plates at the density of 3×10^3 and 5×10^3 cells/well, respectively, and incubated at 37 °C for 24 hours. Then the cells were treated with Z_{EGFR}, Z_{EGFR}-ABD, Z_{EGFR2}-ABD, and Z_{Taq} affibodies and cetuximab at varying concentrations of 1, 2.5, 5, 10, 50, and 100 µg/ml for 24, 48, and 72 hours. After each time period, 100 µl MTT solution, at the concentration of 0.5 mg/ml, was added to each well and incubated for a further four hours. The MTT solution was then replaced with 100 µl dimethyl sulfoxide (DMSO), and the optical density (OD) was measured by a microplate ELISA reader at a wavelength of 540 nm. The percentage of viable cells was normalized to non-treated control cells.

2.5 Evaluation of apoptosis induction by Z_{EGFR} affibody probes

Thyroid cancer cells were cultured in 12-well plates at the density of 2×10^5 cells/well and allowed to adhere overnight. Following the treatment with different concentrations of each probe (1, 5, and 10 µg/ml), and Cetuximab (1, 5, and 10 µg/ml) as positive control, were done. After 48 hours, cell apoptosis was performed using an Annexin V/PI Apoptosis Detection kit (Mahboub Bioresearch, Tehran, Iran) according to the manufacturer's instructions³⁰. First, the cells were trypsinized, harvested, and centrifuged at 1000 rpm for 5 minutes. The cell pellet was washed twice with ice-cold PBS and then resuspended in 500 µl annexin binding buffer. Finally, 5 µl Annexin V/PI conjugate and 2.5 µl propidium iodide (PI) were added to the sample for 20 and 5 minutes, respectively. Then cell analysis was done using FACSCalibur flow cytometer (BD, California, USA). Data were analyzed with Flow Jo software (Treestar Inc, Ashland, OR, USA).

2.6 Construction of Z_{EGFR} affibody- Fluorescein Isothiocyanate (FITC) conjugate

To preparation of labeled probes, FITC conjugation was performed according to the manufacturer's instruction. For conjugation, 2 mg of purified protein was dialyzed in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.5, at 4°C overnight. The proteins were mixed with 1 mg of FITC, dissolved in 100 µl DMSO, and stirred at room temperature for 4 hours in the dark. Afterwards, the FITC-conjugated proteins were dialyzed in phosphate-buffered saline (PBS) using 6-8 kDa molecular-weight cut-off (MWCO) dialysis membrane to remove unbound FITC with twice buffer exchange³¹.

2.7 Determination of binding ability of Z_{EGFR} affibody probes

The binding ability of affibodies to the EGFR receptor was determined by ELISA and Flowcytometry. In enzyme-linked immunosorbent assays (ELISA), BCPAP, 8305C, and CHO-K1 were seeded in 96-well plates at the density of 1×10^4 and 8×10^3 cells/well, respectively. After 24 hours of incubation, the cells were washed twice with ice-cold PBS and fixed with 4% formaldehyde for 15-20 minutes. Cells were then blocked with 1% bovine serum albumin (BSA) in PBS for one hours at room temperature. After washing with PBS, cells were treated with different concentrations of probes (0.1, 1, 2.5, 5, 10, 15, 25, 50, 100, 150, and 200 $\mu\text{g/ml}$) and incubated with the cells for 2 hours at RT. Then cells were washed three times with PBS and anti-His tag antibody conjugated with Horseradish peroxidase (HRP) (1:500) was added to each well. After 1.5 hours of incubation at RT, the plates were washed with PBS three times and 100 μl 3,3',5,5'-tetramethylbiphenyldiamine (TMB) was added to each well, and incubated for 30 minutes at room temperature in the dark, with shaking. The reaction was stopped by adding 100 μl 2N H_2SO_4 and the absorbance was determined using an ELISA plate reader (Stat Fax 2100 Microplate Reader, Awareness Technologies, USA) at 450 nm. Binding curves were fitted using a one-site specific binding nonlinear regression model (GraphPad Prism 9.0.2). The equilibrium dissociation constant (K_d) was calculated from the fitted curve.

The flow cytometric binding assay was performed as previously described³². First, cell suspension was incubated with different concentrations of FITC-labeled probes (1, 50, 100 $\mu\text{g/ml}$) and incubated at 4 °C for 30 min in the dark. Next, cells were washed with PBS twice to remove the excess unbound probes and eliminate non-specific binding. Finally, the fluorescence intensity was measured by flowcytometric analysis.

2.8 Immunofluorescence microscopy

To analyzed the localization of probes with EGFR on thyroid cancer cells, immunofluorescence cell binding assay was performed. In brief, BCPAP, 8305C, and CHO-K1 cells were seeded and incubated overnight. The cells were detached and washed with PBS three times. Then, cells were exposed to FITC-labeled probes (1 $\mu\text{g /mL}$) and incubated at 37 °C in the dark condition for one hour. After washing with PBS, 100 μl of 10 $\mu\text{g/ml}$ 4',6-diamidino-2'-phenylindole (DAPI) was added to cell suspension. Finally, a 20 μl of each cell suspension was observed under a fluorescence microscope.

2.9 Statistical analysis

The experiments were performed in triplicate, otherwise it was mentioned. Data are presented as the mean $\pm$ SEM. GraphPad Prism version 9.0.2 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. Statistical analysis between groups was calculated using one-way/two-way analysis of variance (ANOVA). NS, non-significant; *** $p < 0.001$.

3. Results

3.1 Design, expression and purification of Z_{EGFR} probes

As mentioned previously, the genes encoding affibody were designed, cloned into pET28a expression vector and then expressed in *E. coli* BL21 (DE3) the optimum conditions. In this study, four structures were designed: anti-EGFR affibody, anti-EGFR affibody fused into ABD₀₃₅, and two anti-EGFR affibodies fused together with ABD, and the negative control affibody (Z_{Taq}) (Figure 1A). ABD₀₃₅ is an engineered albumin binding domain with high affinity for human serum albumin which is often used to prolong the serum half-life of therapeutic molecules. The flexible linker (G₄SG₂) can reduce the interface and keep a proper distance between two domains of affibody structures¹⁷. The constructs used in the present study, Z_{EGFR}, Z_{EGFR}-ABD, and Z_{EGFR2}-ABD, have molecular weights of approximately 7.8, 13.2, and 20.1 kDa, respectively, as ABD domain (5–6 kDa) was fused to Z_{EGFR} unit. The sequence and structure of Z_{EGFR} affibodies are presented in Figure 1A. SDS-PAGE confirmed successful purification of all constructs (Figure 1B). The observed band sizes (~12, 16, and 22 kDa for Z_{EGFR}, Z_{EGFR}-ABD, and Z_{EGFR2}-ABD, respectively) were higher than the theoretical molecular weights calculated from the amino acid sequence. The difference of molecular weight might be due to the anomalous SDS-PAGE migration of helix-rich secondary structure³³.

3.2 Evaluation of EGFR expression level in thyroid cancer cell lines

EGFR expression in three cell lines (BCPAP, 8305C, and CHO-K1) was evaluated by reverse transcriptase Real-time qPCR. The CHO-K1 cell line exhibited a lower EGFR mRNA level than the other cell lines as compared with control gene expression (Figure 2). BCPAP cell line demonstrated higher level of EGFR expression than CHO-K1 cell lines ($p = 0.0008$).

3.3 Cytotoxicity of ZEGFR affibody probes on thyroid cancer cell lines

The cell lines BCPAP and 8305C were cultured at a density of 10,000 cells per cm², and after incubation for 24 hours, they were treated with various concentrations of purified probes. Cell viability was assessed using the MTT assay after 24, 48, and 72 hours. As shown in Figure 3, no significant cytotoxicity was observed for any probe at low concentration in both cells. The result shows that cell viability was not significantly changed in 8305C cells treated with different concentrations of Z_{EGFR} affibodies. However, a dose and time dependent cytotoxicity of Z_{EGFR} and Z_{EGFR2}-ABD was observed in BCPAP cells, which was more pronounced at the high concentration of 50 and 100 µg/ml. Z_{EGFR} and Z_{EGFR2}-ABD induced a significant reduction in cell viability to 30% and 60% of control, respectively, at a concentration of 100 µg/ml after 72 hours. The treatment with Z_{Taq} (control affibody) did not induce any significant cell death at any concentration in both cell lines. In BCPAP cells, cetuximab didn't change cell viability at low concentration, but a significant reduction was only observed at the highest concentration (500 µg/ml) after 72 hours. Treatment with cetuximab led to a slight decrease in the viability of 8305C cells.

3.4 Evaluation of apoptosis induction by Z_{EGFR} affibody probes

The measurement of apoptotic and necrotic cell death resulting from treatment of thyroid cancer cells with each of the purified probes was performed using dual staining with Annexin/Propidium iodide. As shown in Figure 4A and B, there was no significant level of apoptotic or necrotic cell death at low concentrations of probes. However, the percentage of viable cells decreased with increasing concentration of probes and the maximum apoptotic and necrotic cell death was observed at the higher concentrations. The results showed that Z_{EGFR} and Z_{EGFR2}-ABD affibodies exhibited a stronger cytotoxicity against BCPAP compared to 8305C cells, which is consistent with the results of MTT assay. The percentage of necrotic cells was increased to about 60% in BCPAP cells treated with 50 µg/ml of Z_{EGFR} and Z_{EGFR2}-ABD, while a slight increase in the percentage of necrotic cells was observed in 8305C at the same concentration (Table 1). Z_{EGFR}-ABD increased the percentage of apoptotic and necrotic cells in both cell lines, which was more pronounced in 8305C compared to BCPAP cells. The control affibody Z_{Taq} did not induce significant apoptosis or necrosis in either BCPAP or 8305C cells (Figure 4C). Moreover, cells treated with cetuximab showed a higher apoptotic effect than affibodies. The percentage of viable cells was significantly decreased in both cells treated with 1 µg/ml cetuximab (40.7% and 60.4% for BCPAP and 8305C, respectively).

3.5 Determination of binding ability of Z_{EGFR} affibody probes

To assess the binding of affibodies to EGFR receptors, a cell-based ELISA method was employed. The binding was assessed in BCPAP and 8305C cells (EGFR-positive) and CHO-K1 cells (EGFR-negative). Cells were incubated with different concentrations of each affibody. Based on the ELISA results, Z_{EGFR}-ABD showed the strongest binding affinity to BCPAP and 8305C among the three affibody probes, with a K_d of approximately 1.8 µg/ml in both BCPAP and 8305C cells. The K_d values of 3.48 µg/ml and 2.39 µg/ml were calculated for Z_{EGFR} in BCPAP and 8305C, respectively. Z_{EGFR2}-ABD exhibited a higher (weaker) K_d (15.93 µg/ml in BCPAP and 9.133 µg/ml in 8305C), indicating moderate affinity for EGFR-expressing cells. As expected, three affibody probes showed a minimum binding affinity to CHO-K1 as an EGFR-negative cells (Figure 5A). Binding of Z_{EGFR} affibody probes to EGFR receptors was also evaluated using flow cytometry to determine both the level and specificity of the binding (Figure 5B and supplementary Figure1). The results indicated that the EGFR probes bind specifically to the receptor, as shown by left shift in the fluorescence intensity compared to control cells. This binding increased with increasing concentration of affibodies, ranging from 1 to 100 µg/ml (Figure 5B). No obvious change was observed in fluorescence peak when cells were treated with control affibody (Z_{Taq}). CHO-K1 cells didn't exhibit change in the fluorescent intensity, reflecting lack of EGFR expression. Moreover, a monoclonal antibody, cetuximab, was used as a positive control for EGFR binding.

3.6 In Vitro binding of Z_{EGFR} affibodies

To confirm the results from ELISA and flowcytometric analysis, we investigate whether Z_{EGFR} affibodies could specifically bind to EGFR-expressing positive and negative cells using immunofluorescent staining. Figure 6 represents the immunofluorescent images of BCPAP and 8305C cells were treated with a concentration of 10 µg/ml of FITC-conjugated affibodies. As it clear, Z_{EGFR} affibodies were accumulated in BCPAP and 8305C showed a green fluorescent signal. Cells were subjected to nuclear staining with DAPI. The merge of two fluorescent images showed the bright green fluorescence on the cell membrane.

4. Discussion

The incidence of distant metastasis is a major challenge in the treatment of thyroid cancer, particularly in papillary thyroid carcinoma (PTC)³⁴. This challenge may lead to a delay in diagnosis and treatment, which results in a low survival rate in thyroid cancer patients.

The EGFR is one of the main oncogenes and a key driver of tumor progression and metastasis in thyroid cancer, and well-studied target for tumor cell imaging and therapy⁵. Development of EGFR specific targeting approach including anti-EGFR antibody or similar scaffolds with high specificity and favorable pharmacokinetic has emerged as a promising approach for both diagnostic and therapeutic applications in EGFR-expressing cancer cells³⁵. EGFR is normally upregulated in metastatic thyroid cancer and associated with a high risk of recurrence³⁶. Therefore, monitoring the expression pattern of EGFR and its targeting might provides an approach for management of PTC³⁷. In this study, we generated fluorescent-affibody probes for imaging of EGFR receptors. We designed and produced three different structures of affibodies, both unmodified Z_{EGFR} affibody and ABD₀₃₅-fused variants.

Affibodies are the small, single-domain protein derived from a non-immunoglobulin three-helix scaffold. Affibodies exhibit several unique features such as high stability, low immunogenicity, and rapid tumor localization, making them suitable for various medical applications^{38,39}. These molecules can be designed to have high specificity and affinity toward various targets such as human epidermal growth factor receptor type 2 (HER2), vascular endothelial growth factor (VEGF), and EGFR⁴⁰. Affibodies can be fused to the ABD, which extend the circulatory half-life through binding to the serum albumin⁴¹. We evaluate the effect of Z_{EGFR} monomer as well as bivalent constructs for further extended half-life in imaging and therapeutic applications. The G₄SG₂ flexible linker enhances the spatial arrangement of functional protein domains and strengthens the binding affinity⁴². The successful purification of affibodies is confirmed by SDS-PAGE analysis which shows three protein bands corresponding to molecular weights of approximately 12 kDa (Z_{EGFR}), 16 kDa (Z_{EGFR}-ABD), and 22 kDa (Z_{EGFR2}-ABD).

Based on the cytotoxicity analysis, affibody probes exerted cytotoxicity against thyroid cancer cell lines. Among three different affibodies Z_{EGFR} and Z_{EGFR2}-ABD induced substantially greater cytotoxicity and apoptotic effect in BCPAP than in 8305C cells. While Z_{EGFR}-ABD did not change cell viability, it caused more apoptotic and necrotic cell death in both BCPAP and 8305C. It is possible that Z_{EGFR}-ABD induces an early apoptotic phenotype detectable by Annexin V/PI at the without a considerable mitochondrial metabolic change which can be measured by MTT assay.

Schardt et al. demonstrated that multivalent form of HER3-binding affibodies inhibited Neuregulin-induced HER3 and Akt phosphorylation, which was compared to monovalent form, suggesting that engineered multivalent form enhanced cancer cell growth inhibition⁴³. Another study revealed that Z_{LMP2A-N}, an affibody against Epstein-Barr virus latent membrane proteins, inhibit nasopharyngeal carcinoma cells⁴⁴. The cytotoxic effects of anti-EGFR affibodies were compared to cetuximab, an FDA-approved monoclonal antibody that can bind to EGFR. Cetuximab is routinely used for imaging and treatment of different types of cancers. These engineered constructs exhibit cytotoxic effects on thyroid cancer cells comparable to cetuximab at higher concentrations.

To assess binding ability to EGFR, we used three cell lines with low to high expression level of EGFR to assess whether different affibody probes could bind to EGFR receptor expressed by thyroid cancer cells. Consistent with previous results⁴⁵, we observed the high expression of EGFR in BCPAP (papillary thyroid cancer cells) and 8305C (anaplastic thyroid cancer cells). CHO-K1 has been previously shown as a cell line with a lack of EGFR²⁴. The specificity of a molecular probe for molecular imaging is dependent on the greater ligand affinity for the target¹³. The lower dissociation equilibrium constant (K_d value) implies stronger binding affinity to target⁴⁶. The cell-based ELISA showed a lower K_d for the BCPAP and 8305C cells compared to CHO-K1 cell, indicating a stronger binding affinity. Among the three affibody probes Z_{EGFR2}-ABD demonstrated the lowest K_d value, indicating a high affinity for binding to EGFR. Z_{EGFR}-ABD showed the strongest binding affinity to BCPAP and 8305C among the three affibody probes, with a K_d of 1.8 μ g/ml (cell-based ELISA). Z_{EGFR2}-ABD exhibited a higher K_d , indicating moderate affinity for EGFR-expressing cells despite its bivalent design. Flow cytometry results showed that the binding of FITC-conjugated affibodies was greater in BCPAP cells, which express higher levels of EGFR receptor. Interestingly, all affibody probes bind to EGFR receptors with a similar affinity as cetuximab. Several studies have already examined the binding affinity of anti-EGFR affibodies^{47,48}. In the study by Friedman et al., Z_{EGFR} affibodies exhibited a high binding affinity to cells expressing EGFR⁴⁹. In another study by Jia et al., they designed and produced an affibody against tumor necrosis factor-related apoptosis ligand (TRAIL) fused to Z_{EGFR:1907} to increase its binding ability and cytotoxicity⁵⁰. A recent study showed that dual-targeting affibody against AxI–EGFR was able to target both respective targets⁵¹. To investigate the localization of anti-EGFR affibodies, thyroid cancer cells were treated with 10 μ g/mL of FITC-conjugated affibodies. Both

cells showed a green fluorescent signal around the cell membrane following nuclear staining. The result of fluorescent staining was consistent with binding analysis.

In this study, we suggest that Z_{EGFR} affibody could be used as an imaging probe or potential target in thyroid cancer. However, this study has some limitations that should be addressed in future research. The precise molecular size of affibodies should be measured with mass spectrometry. It is also necessary to clarify whether affibodies bind to the same epitope on EGFR as cetuximab to compare cytotoxic and binding potency. In addition, the specificity of these affibodies could be tested in other EGFR-overexpressing tumor types. We demonstrated that these probes have toxicity and high binding ability and specificity against EGFR, however, further investigations are required to evaluate the effect of ABD fusion in an *in vivo* cancer model.

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Data Availability Statement

All data generated or analysed during this study are included in this published article and its supplementary information files. No datasets were deposited in a public repository for this study. The raw data are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have approved and have consented to the publication of the manuscript.

Competing interests

The authors have no relevant financial or nonfinancial interests to disclose.

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Table 1. Percentages of apoptotic and necrotic cells treated with different concentration of Z_{EGFR} probes.

Treatment	Concentration (µg/ml)	BCPAP			8305C		
		Early apoptotic	Late apoptotic	Necrotic	Early apoptotic	Late apoptotic	Necrotic
Z _{EGFR}	1	17.6	0.22	0.66	2.83	1.98	7.55
	10	2.27	19.5	37.6	2.55	1.09	5.43
	50	3.19	16	59.3	1.38	8.29	14.3
Z _{EGFR} -ABD	1	0.19	18.4	11.4	2.96	23.6	35.3
	10	4.51	13.6	35.6	3.23	20.5	45.7
	50	2.87	18	54.9	0.7	3.77	79.6
Z _{EGFR2} - ABD	1	2.27	19.5	37.5	2.59	0.728	5.24
	10	5.08	25.4	46.2	2.15	4.39	7.26
	50	4.72	15.8	58.2	32.7	2.33	33.7
Cetuximab	1	0.88	31.3	27.1	23.9	1.91	13.6
	10	3.85	29.2	28.6	3.23	20.5	45.7
	50	11.3	10.3	63.0	9.67	14.1	46.7

Figures captions

Figure 1. (A) Schematic representation of Z_{EGFR} affibodies and Z_{Taq}. Amino acid sequences of anti-EGFR genes, a G4SG2 linker, albumin binding domain were constructed with an N-terminal histidine tag for subsequent purification using Ni-affinity chromatography. **(B)** Coomassie stain analysis of protein expression of Z_{EGFR} affibodies in soluble fractions. Protein expression was induced by overnight incubation at 37°C, 25°C and 16°C with 0.1mM IPTG concentrations as indicated and protein samples were run on 12% SDS-PAGE.

Figure 2. Quantitative real-time PCR was employed to confirm the expression of EGFR in the thyroid cancer cell lines. Expression of HPRT1 was used as the internal control. Data are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. NS, non-significant. ***p < .001.

Figure 3. The cytotoxicity of Z_{EGFR} affibody probes on (A) BCPAP and (B) 8305C thyroid cancer cells. Cells were treated with different concentration of affibodies for 24, 48, and 72 hours and the MTT assay was performed to measure the percentage of viable cells. The results represent means $\pm$ SEM of three independent experiments.

Figure 4. Effects of on Z_{EGFR} affibodies on cell apoptosis measured by flow cytometry. **A)** BCPAP, **B)** cells were treated with (1, 10, and 50 μ g/ml) of each affibodies and subjected to flowcytometry analysis using the Annexin V/PI staining method.

Figure 5. Affinity binding assay of anti-EGFR affibodies to EGFR. The binding of purified Z_{EGFR} affibodies against EGFR expressed on the surface of thyroid cancer cells was evaluated by **A)** Cell-based ELISA, **B)** Flow cytometry binding assay of Z_{EGFR} affibodiy to EGFR. Competitive binding of affibody probes to thyroid cancer cells. BCPAP and 8305C cells were detached and harvested followed by washing with PBS tweic, then, cells were treated with increasing concentration of FITC-conjugated affibodies (1, 5,10, and 100 μ g/ml. Unstained cells were used as control.

Figure 6. Fluorescence staining images of EGFR-expressing positive and negative cells were stained with FITC-conjugated Z_{EGFR} affibodies. **A)** BCPAP, and **B)** 8305C.

A



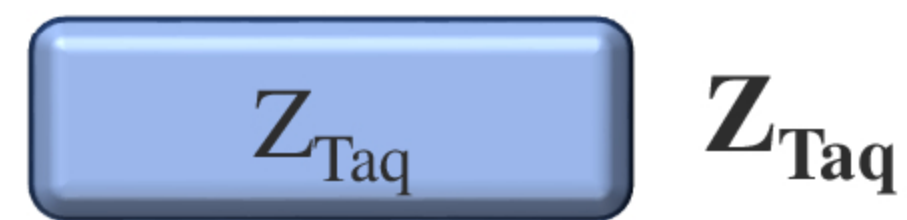
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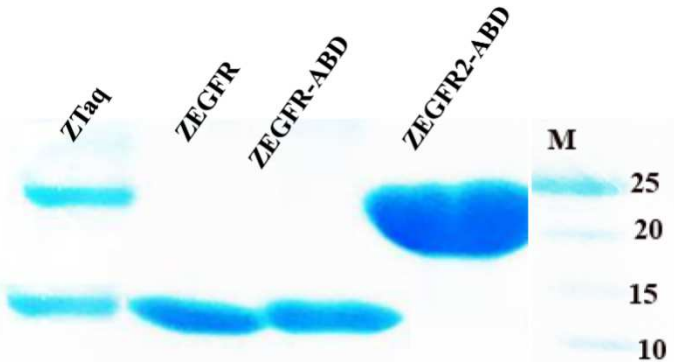
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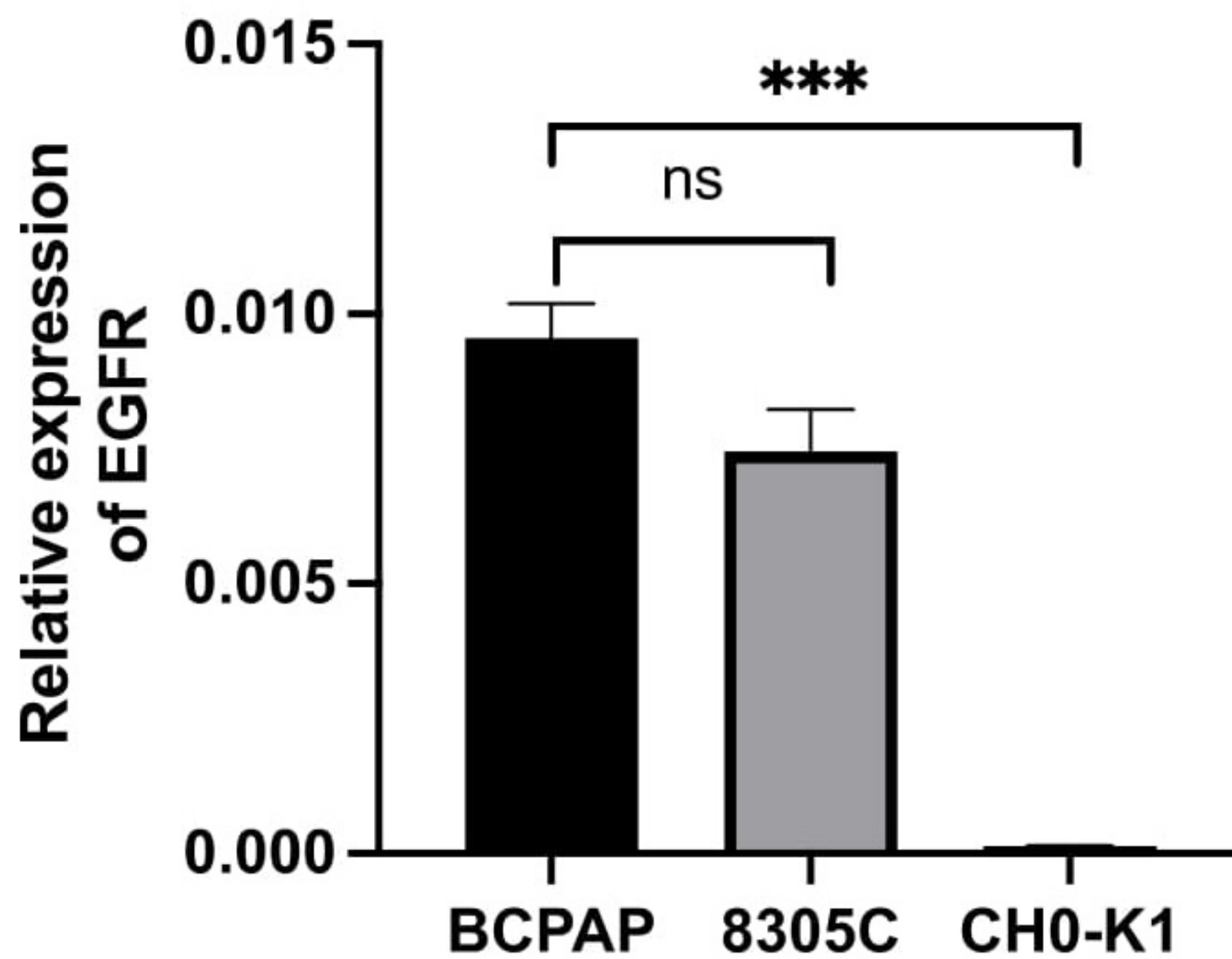


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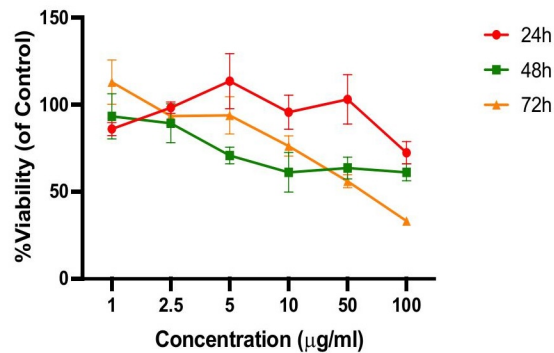
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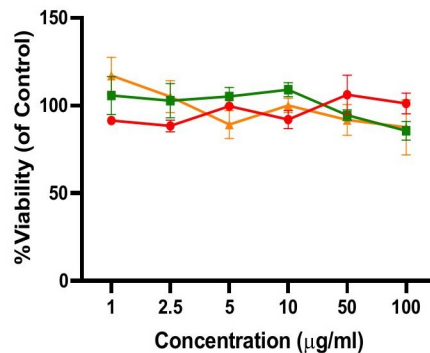


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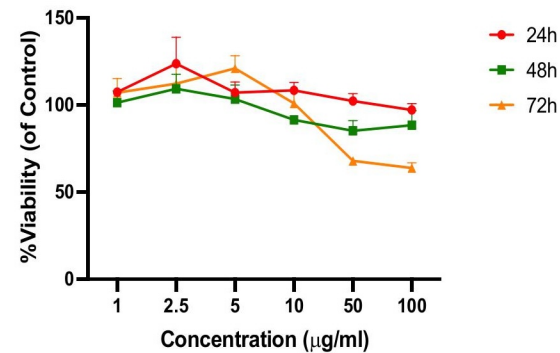
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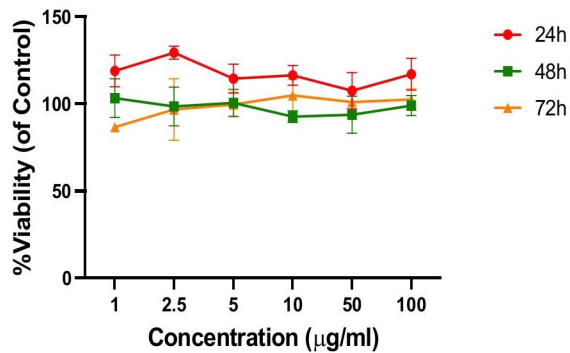
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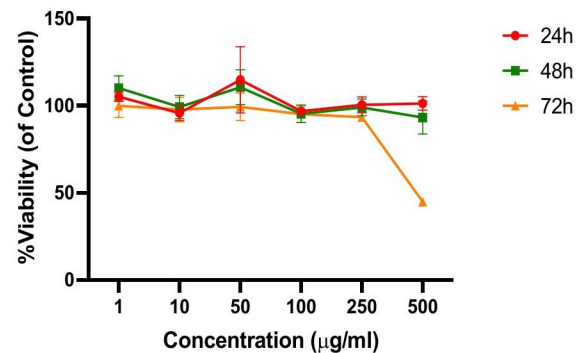
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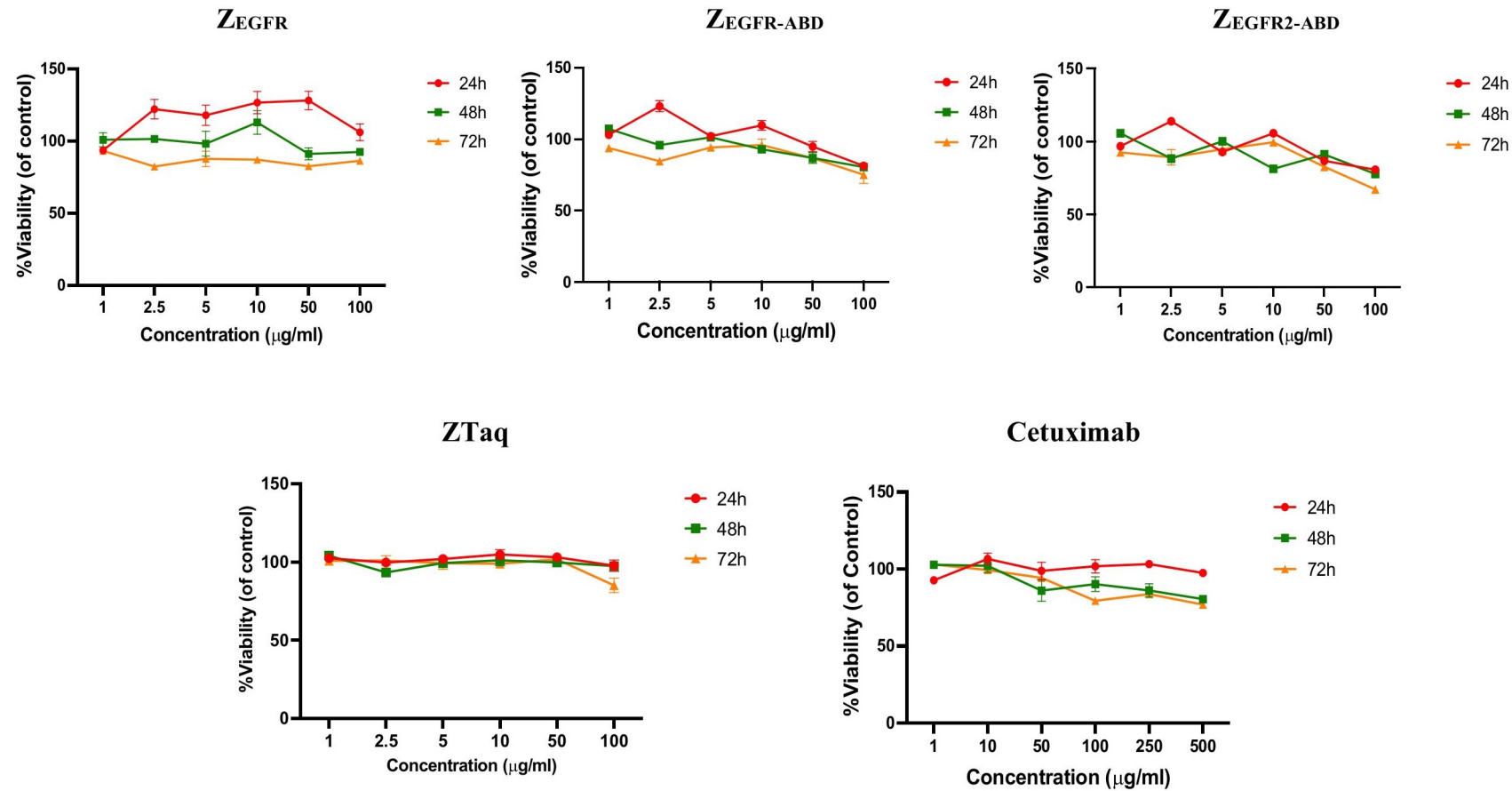


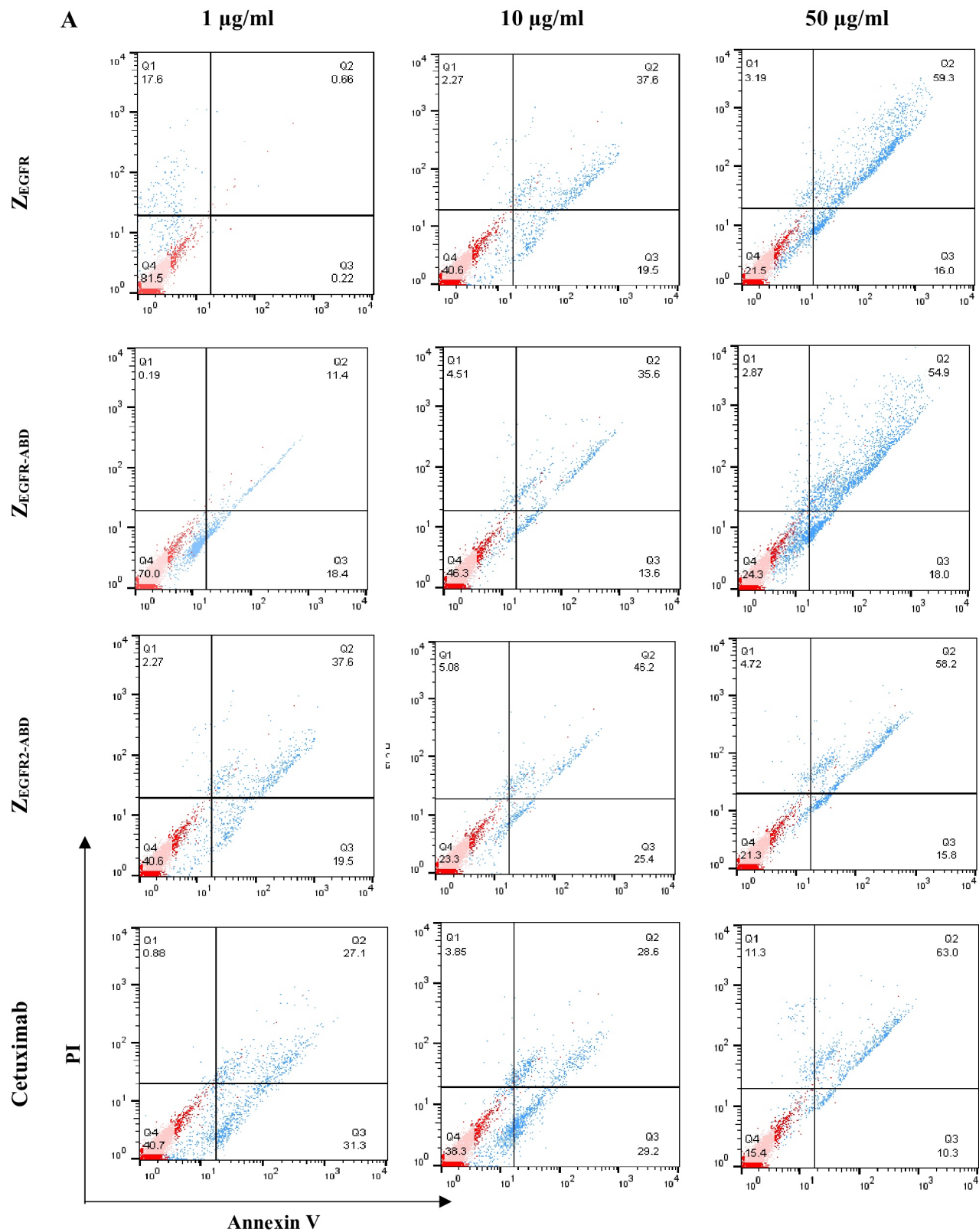
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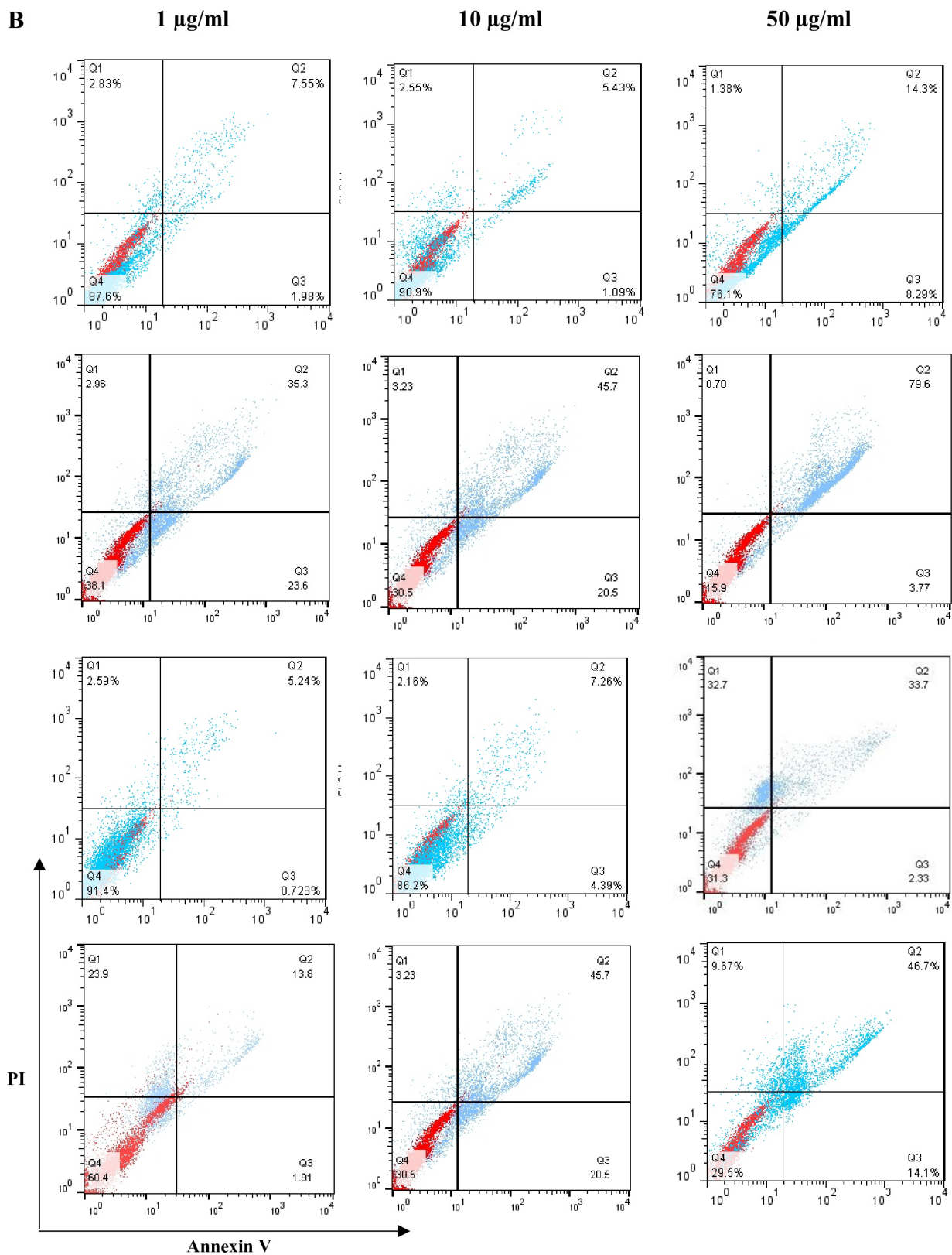


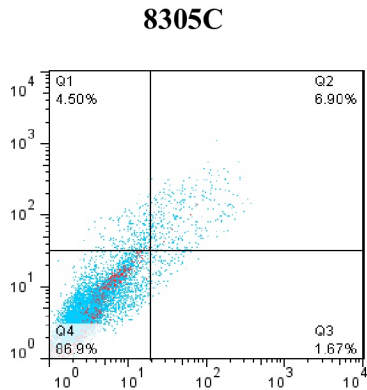
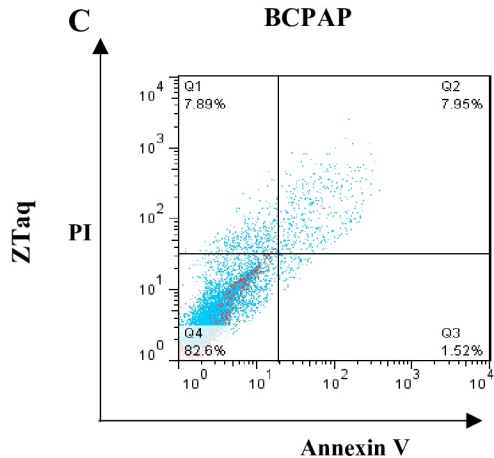
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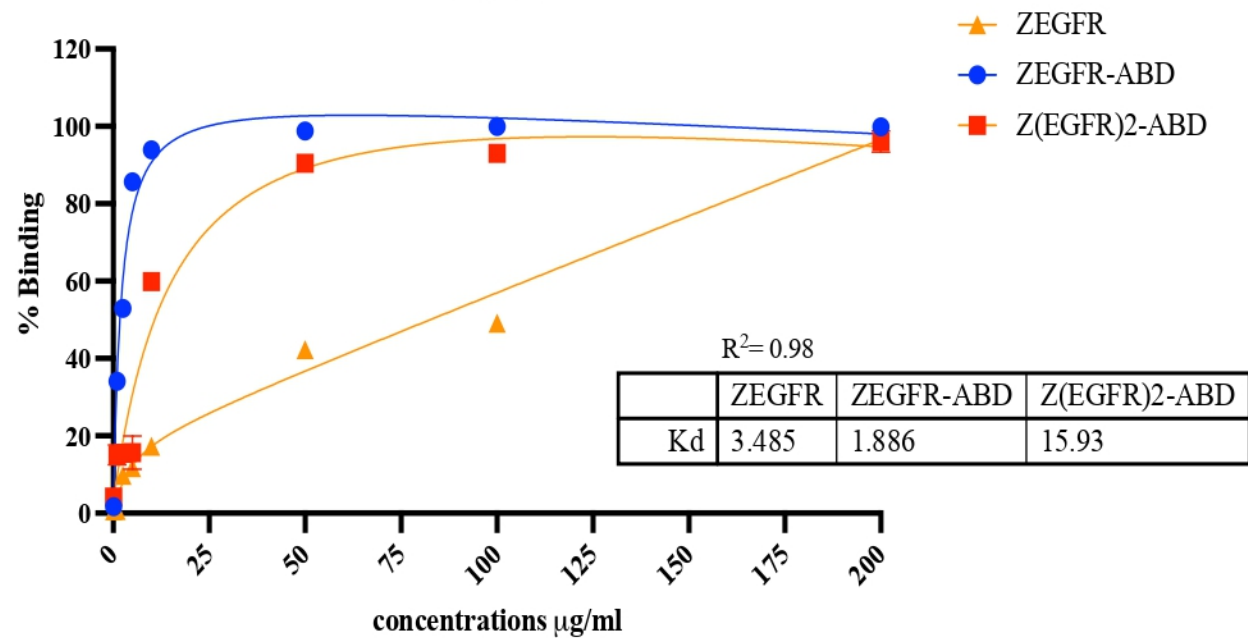
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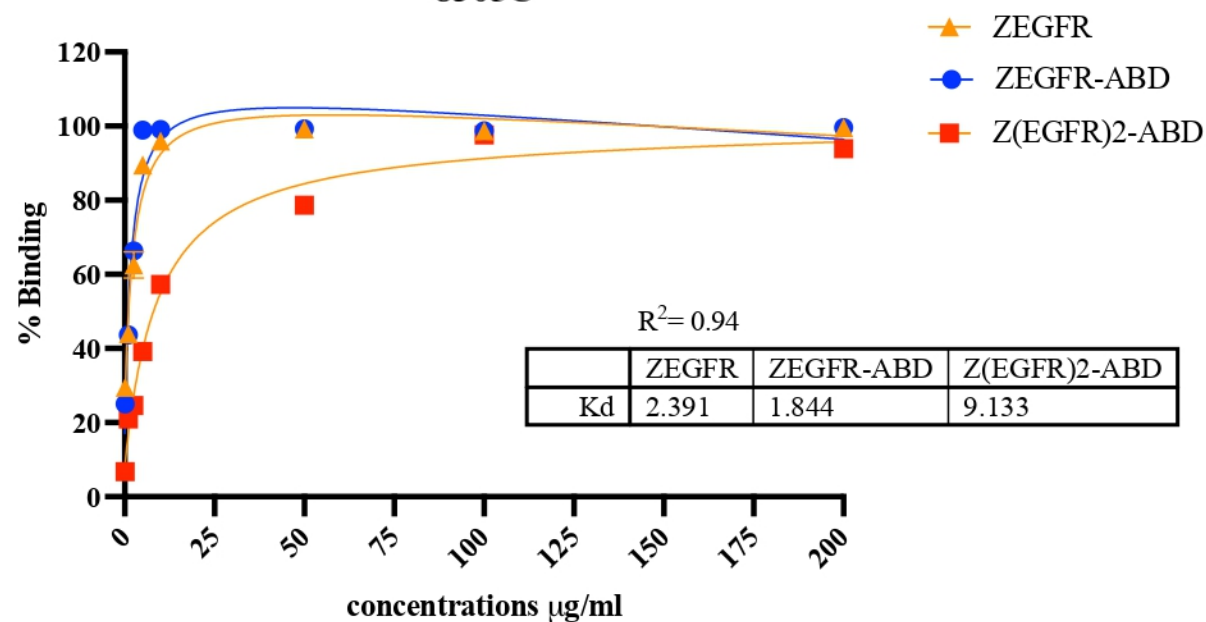




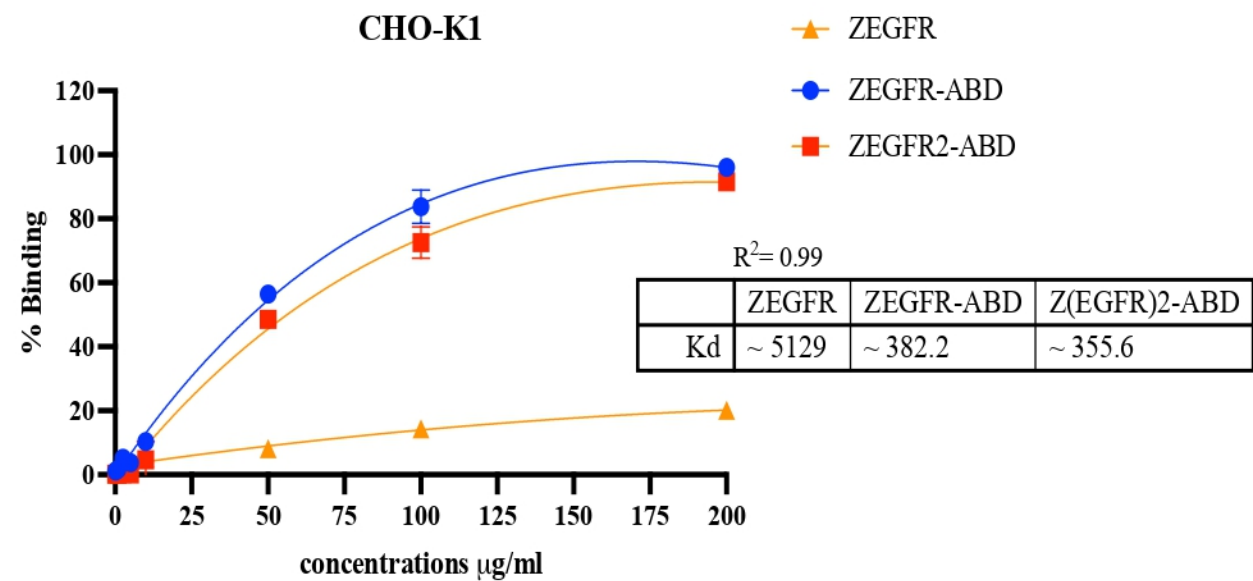
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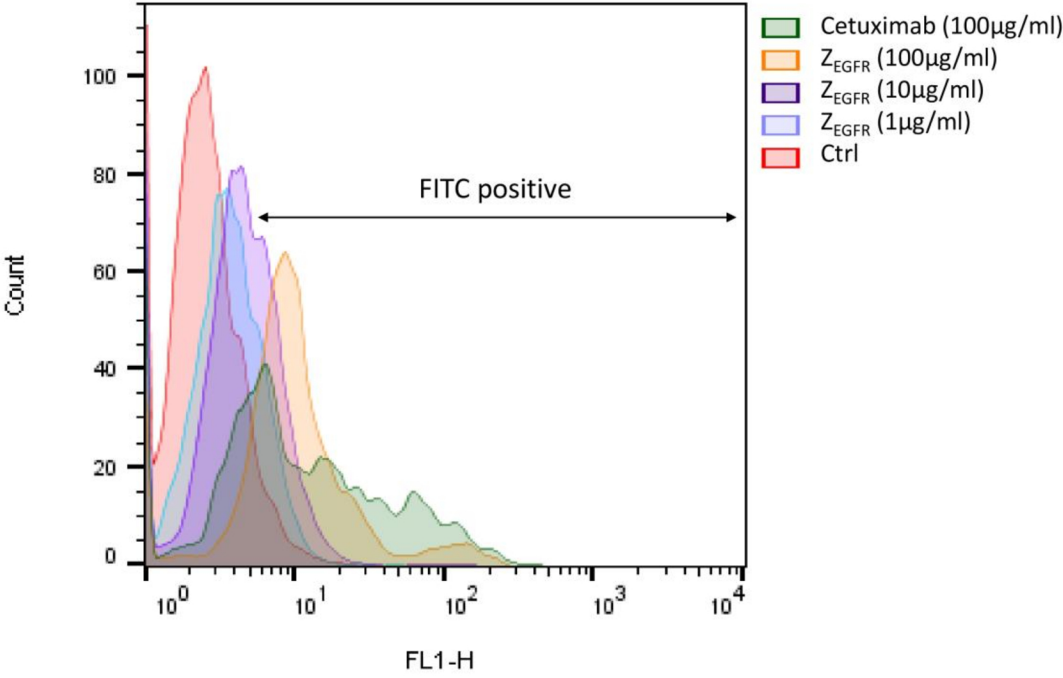
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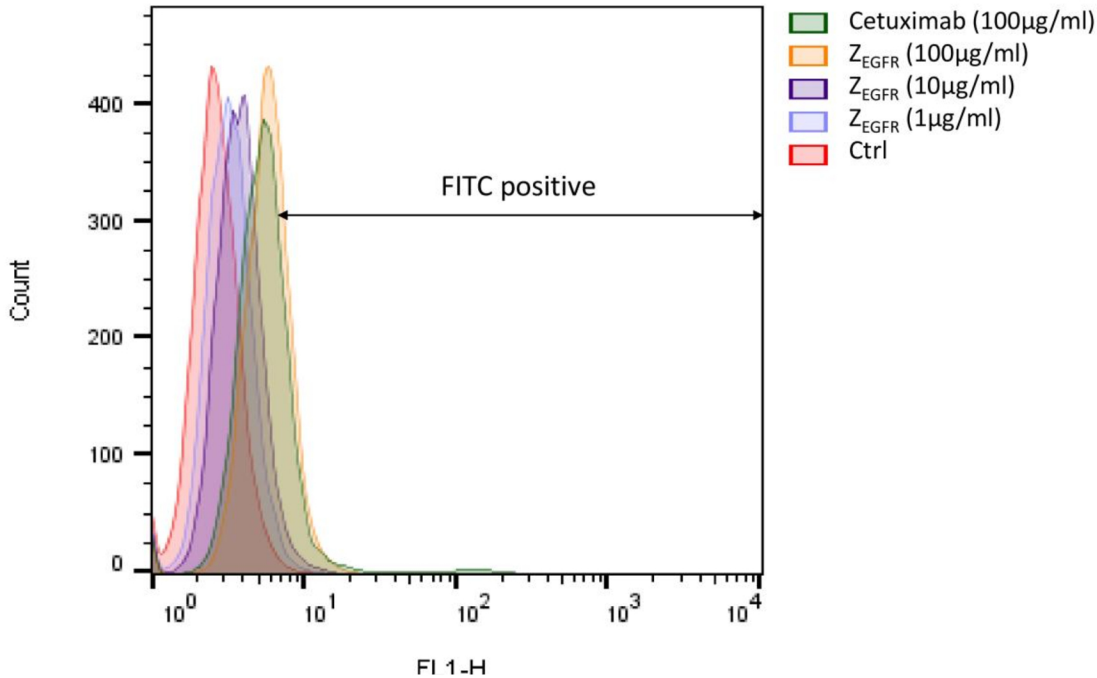
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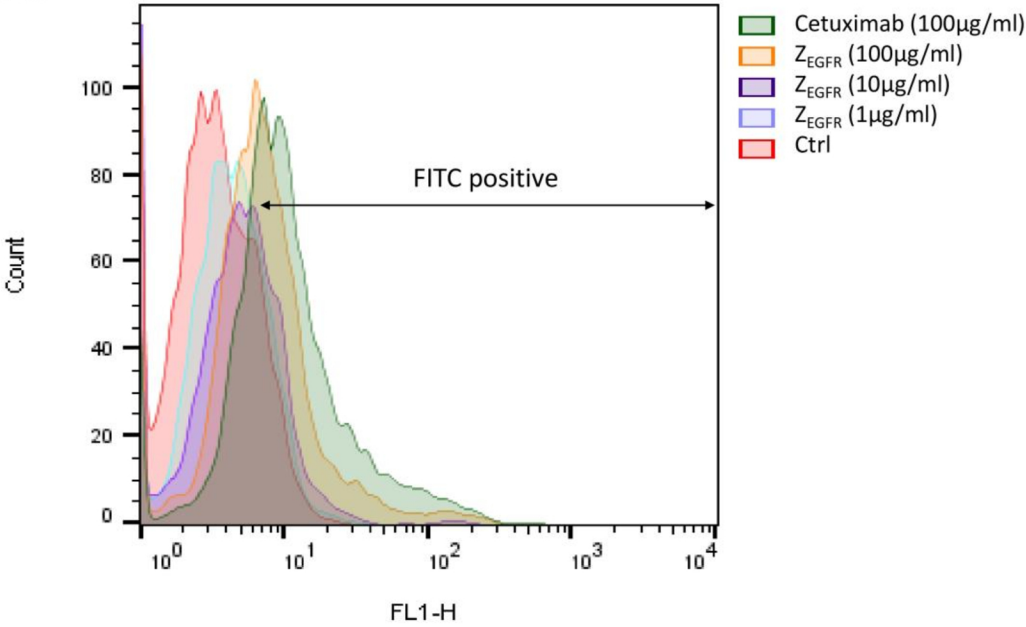
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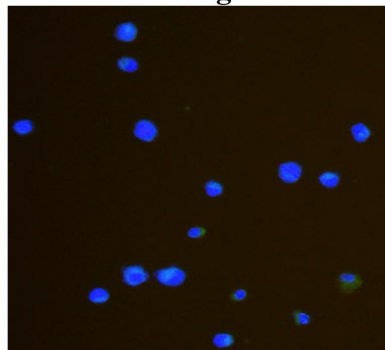
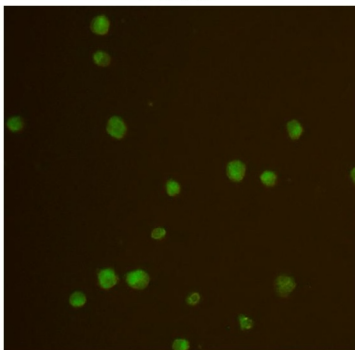
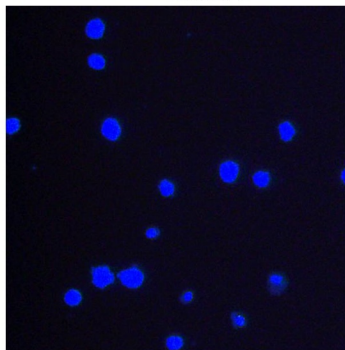
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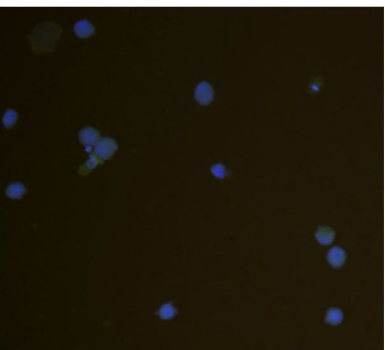
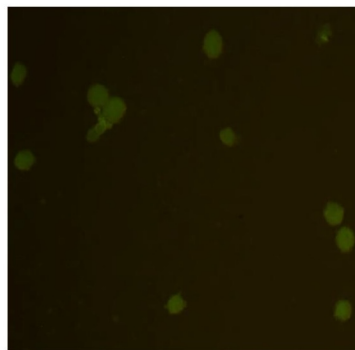
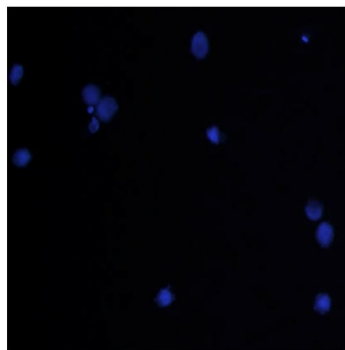
FITC

Merge

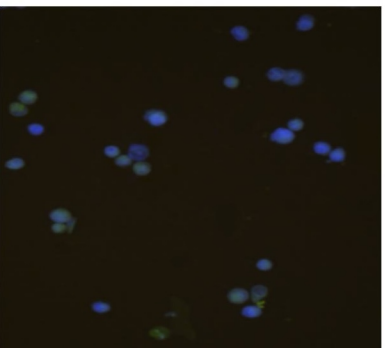
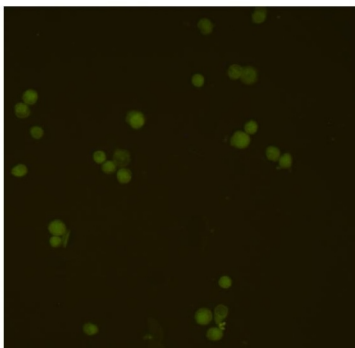
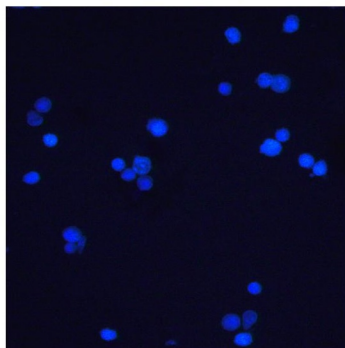
Z_{EGFR}

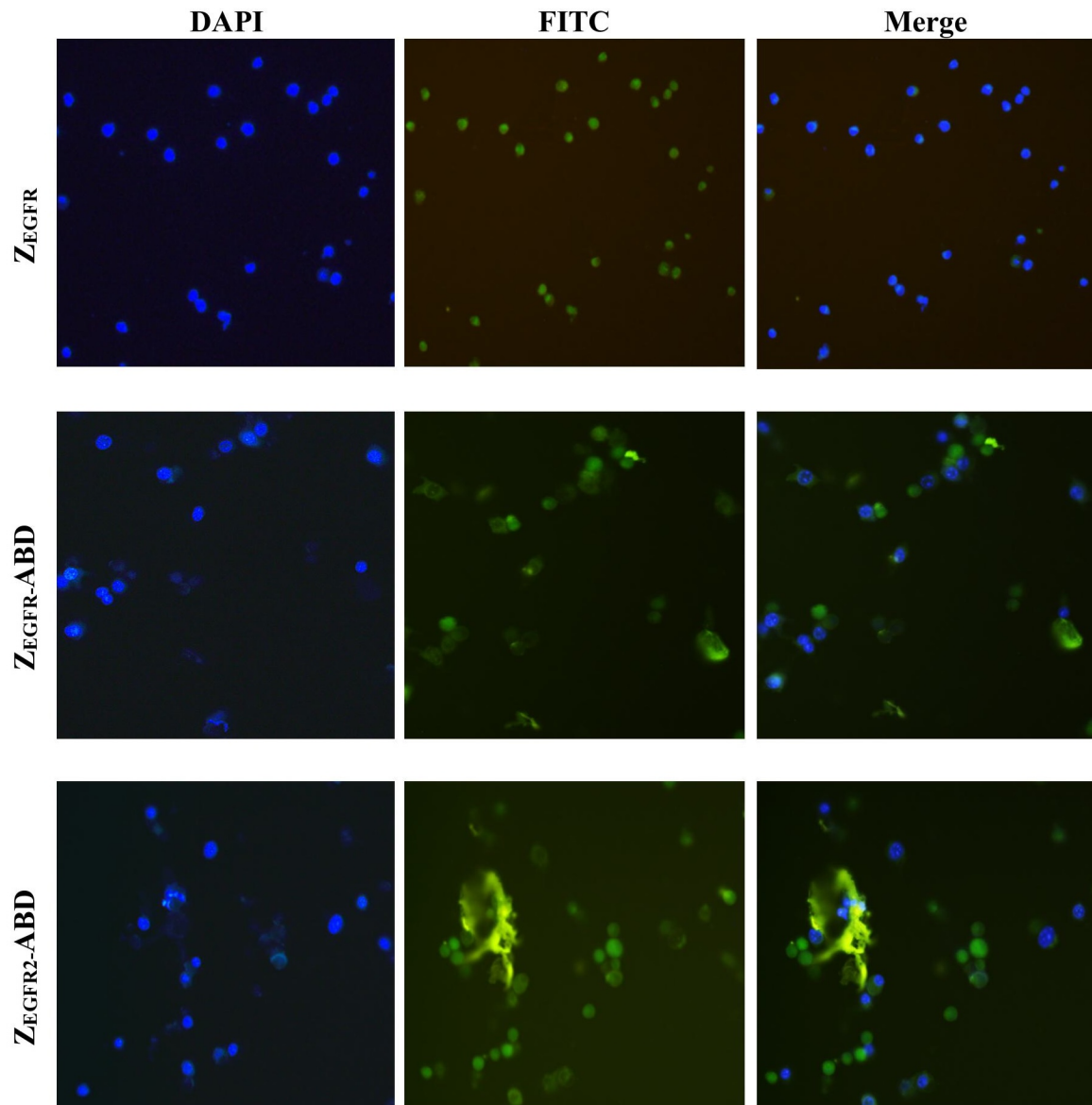


$Z_{EGFR-ABD}$

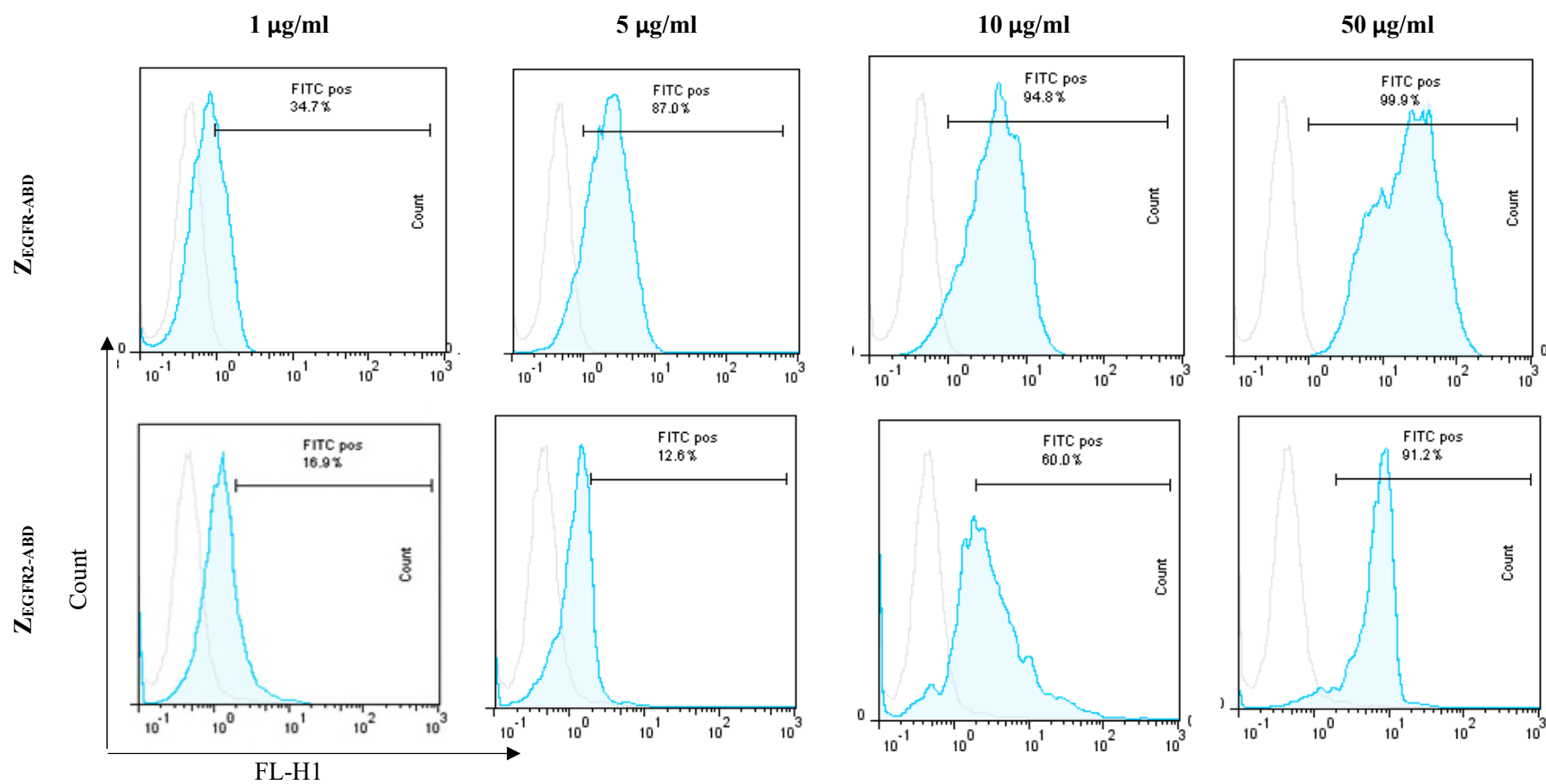


$Z_{EGFR2-ABD}$

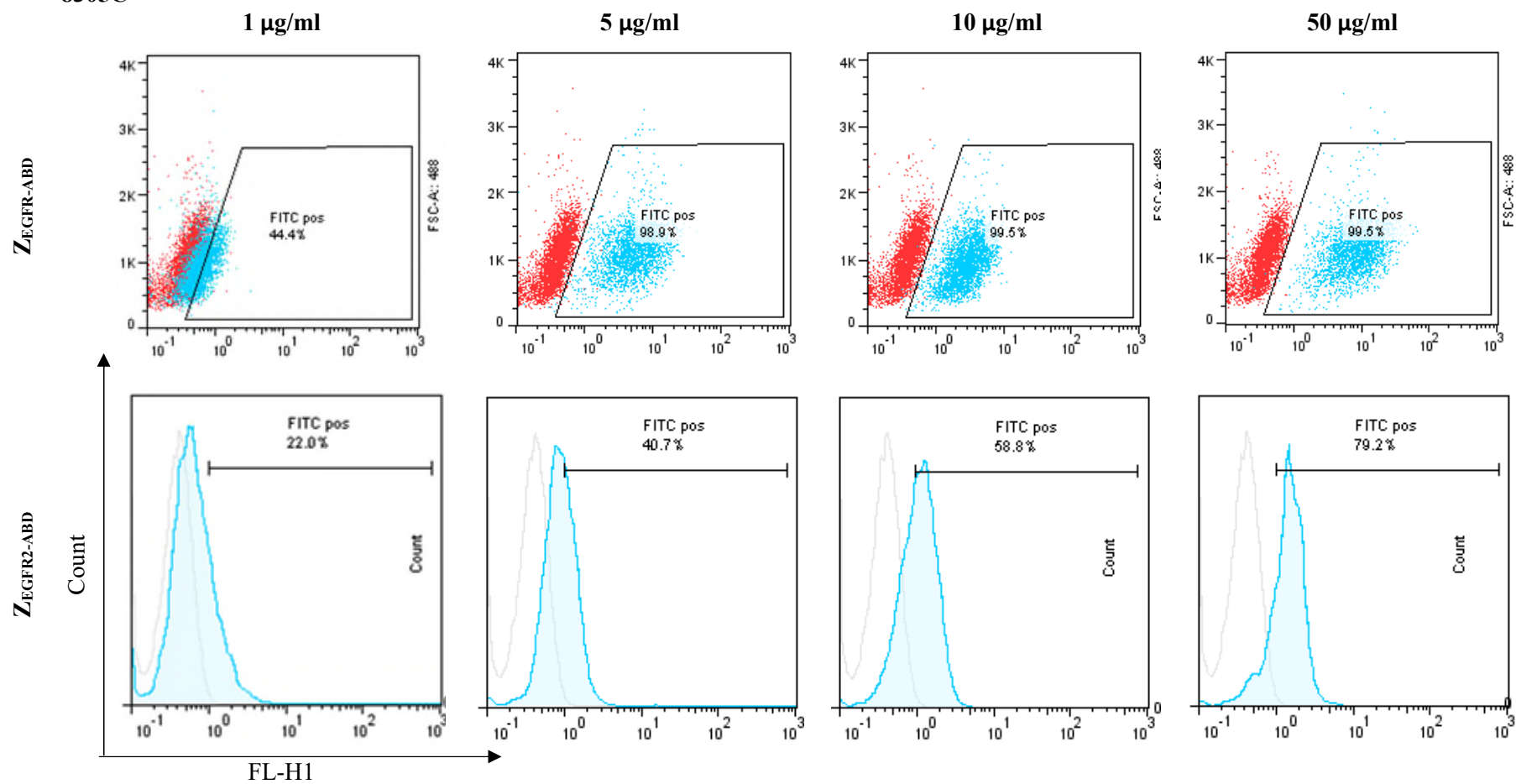




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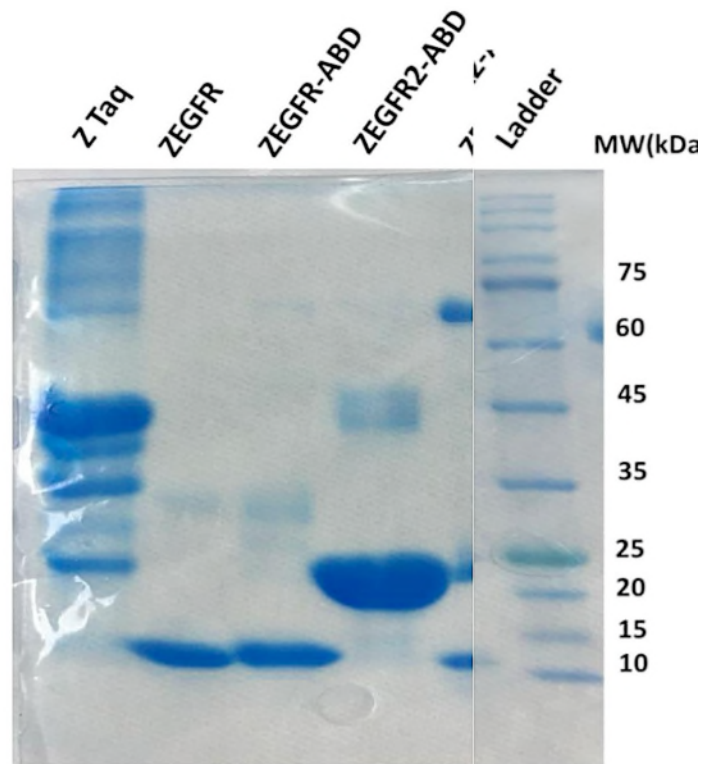


8305C



Supplementary figure 1

Flow cytometry binding assay of Z_{EGFR}-ABD and Z_{EGFR2}-ABD affibodies to EGFR. Competitive binding of affibody probes to thyroid cancer cells. BCPAP and 8305C cells were detached and harvested followed by washing with pbs. Then, cells were treated with increasing concentration of FITC- conjugated affibodies (1, 5,10, and 100 µg/ml). Unstained cells were used as control.



Supplementary figure 2

Full SDS-PAGE gel.

