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Production and Characterization of Single Chain Nimotuzumab: An In Vitro Study

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Abstract Recombinant expression and EGFR-binding activity assay of single chain nimotuzumab (nimotuzumab scFv) is reported in this study. The scFv was produced in VH-linker-VL format in Origami™ 2(DE3)pLysS bacterial cells and purified using Ni-TNA resin column. 3-D structure prediction using I-TASSER (Iterative Threading Assembly Refinement) server and analyzing the predicted models using YASARA (Yet Another Scientific Artificial Reality Application) viewer revealed that VH and VL domains assemble into a correctly-folded single chain antibody that is able to bind EGFR. The scFv was evaluated in ELISA and western blot tests and proven to be able to bind EGFR-overexpressing cancer cells (A-431 cells in an efficient manner but unable to recognize cancer cells expressing low levels of EGFR (MCF-7 breast cancer cells).

Keywords Nimotuzumab · ScFv · EGFR · A-431 · YASARA

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Introduction

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor involved in a variety of human cancers, including breast cancer, lung cancer, head and neck squamous cell carcinoma, and so forth (Masuda et al. 2012; Maiti et al. 2013; Siegelin and Borczuk 2014). This receptor has been extensively used as a mediator for delivery of imaging agents/drugs to cancer cells for imaging or therapeutic proposes (Day et al. 2013; Limasale et al. 2015; Scott et al. 2012). Natural ligands or monoclonal antibodies that selectively react with EGFR can serve as vehicles for delivering drugs or imaging agents to EGFR-overexpressing cells. So far, several monoclonal antibodies have been developed against EGFR, including cetuximab, panitumumab, and nimotuzumab. Cetuximab and panitumumab are FDA-approved drugs (Price et al. 2014; Giusti et al. 2007), while nimotuzumab is still under evaluation in clinical trials (Yang et al. 2011; Westphal et al. 2015). Nimotuzumab has been proven to be an effective antibody in treatment of human cancers in which EGFR is overexpressed (Solomon et al. 2014; Massimino et al. 2011). Although this monoclonal antibody has lower affinity for EGFR (when compared to cetuximab), it exhibits higher specificity (Westphal et al. 2015) and therefore is of greater interest for specific targeting of EGFR-overexpressing cancers.

Sometimes, large size of full-length antibodies may be regarded as a drawback. Compared to full-length antibodies, smaller antibody fragments such as Fab fragments or single chain antibodies (scFv) can more easily enter the cells or tissues and therefore are more preferred to be used for some purposes (e.g. tumor imaging and antibody-mediated targeted drug delivery). In the current work, we present the production of single chain format of nimotuzumab

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and evaluate its activity in recognizing EGFR-overexpressing cells.

Materials and Methods

Cell Lines and Reagents

A-431 and MCF-7 cell lines were obtained from cell bank of Pasteur Institute of Iran. HRP-Protein L (Cat. Number M00098) was purchased from GenScript (NJ, USA). EGFR peptide (Catalog Number: ab167752) was purchased from Abcam. Diaminobenzidine (DAB) tablets were purchased from Sigma-Aldrich (Cat. Number: D4293). Cetuximab (Erbitux) was obtained from Red Cross Pharmacy in Gorgan (Golestan Province, Northern Iran).

Design, Expression, and Purification of Single Chain Nimotuzumab

The amino acid sequence of Nimutuzumab Fab fragment was derived from PDB (PDB ID: 3GKW), from which VH and VL domains were determined using AbNum, an online tool for numbering amino acids in variable domains of antibody molecules (http://www.bioinf.org.uk/abs/abnum/). Using a 15- amino acids glycine-serine linker (GGGGSGGGGSGGGGS), the VH and VL domains were linked together to form a single chain antibody in NH_2-VH-linker-VL-6His-COOH format. The resultant protein sequence (Nimotuzumab scFv) was reverse-translated using "optimizer" online tool (http://genomes.urv.es/OPTIMIZER/) into a nucleotide sequence with codons optimized for *E. coli* expression. The nucleotide sequence was in frame flanked by two restriction sites (NcoI at N-terminal, and XhoI at C-terminal) to allow subcloning in expression vector, pET22b(+). The gene construct (nimotuzumab scFv coding sequence) was synthesized by Bioneer Company (South Korea) in pBHA cloning vector. The scFv gene was isolated from pBHA with the same enzymes and inserted into pET22b(+) expression vector for further expression in bacterial cells. The recombinant plasmid was inserted into Origami™ 2(DE3)pLysS bacterial cells using calcium chloride- heat sock method. Bacterial cells were cultured on conical flasks containing LB medium and allowed to growth up to $OD_{600}=0.5$. After addition of IPTG (final concentration of 100 µM), the flasks were incubated overnight at 21 °C. Bacterial cells were precipitated by centrifuging in 4 °C at 9000 g and then sonicated at 80 Amp for 30 cycles (30 s on/off) while cooled on ice. The sonication yield was centrifuged again in 4 °C at 9000 g for 8 min to precipitate bacterial pellet. After proving the presence of recombinant antibody (nimotuzumab scFv) in supernatant fraction by SDS-PAGE analysis, affinity chromatography (Ni-TNA resin column) was used to purify the protein.

Prediction of Three-Dimensional (3-D) Structure of Nimotuzumab scFv

3-D structure of nimotuzumab scFv was predicted using I-TASSER server [(Iterative Threading Assembly Refinement). The predicted models were visualized and analyzed using YASARA (Yet Another Scientific Artificial Reality Application) program (http://yasara.org/).

ELISA Assay with EGFR Peptide

Serial dilutions of EGFR peptide (250, 125, and 62.5 ng) were prepared in 50 mM carbonate/ bicarbonate buffer (0.159 g Na2CO3 + 0.293 g $NaHCO_3$ in 100 mL distilled water, pH 9.6) and coated on ELISA wells (100 µl each well) overnight at 4 °C. The wells were washed three times (each 5 min) with washing buffer (PBS buffer containing 0.05% Tween 20, pH 7.4) and then blocked with blocking buffer (PBS butter with 5% Bovine Serum Albumin, BSA) for 2 h. The wells were washed again and then filled with different concentration of recombinant nscFv (serial dilutions of 50, 25, 6.25, and 3.125 µg/mL) or cetuximab (50, 25, 12.5, 6.25, and 3.125 µg/mL). After 1 h, the wells were washed five times (each 5 min) with washing buffer and filled with HRP-Protein L (final concentration of 0.1 µg/mL, 100 µl each well). 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added to each well and OD values measured using spectrophotometer at wavelength of 490–630 nm.

ELISA Assay with A-431 and MCF-7 Cells

A-431 and MCF-7 cells were grown on RPMI culture medium and then precipitated by centrifuging at 5 °C in 3000 g. The cells were serially diluted by suspending in PBS buffer (400,000, 200,000, and 10,000 cells/mL) and then sonicated at 80 Amp (15 cycles of 10 s on/off) to prepare cell lysate. Each ELISA well was filled with 100 µL of cell lysates. Ten repetitions were considered for each antigen (cell lysate) concentration, five for treating with cetuximab and five for treating with nimotuzumab scFV. After coating overnight at 4 °C, the wells were washed three times with wash buffer and then filled with 300 µL BSA 5% to block non-specific binding. After 2 h, the wells were washed again and each filled with 100 µl of cetuximab (50, 25, 12.5, 6.25, and 3.125 µg/mL) or nimotuzumab scFv (50, 25, 12.5, 6.25, and 3.125 µg/mL). After 1.5 h incubation with the antibodies, the wells were washed again (for three times) and filled with 100 µL of HRP-Protein L. Our previous study has shown that HRP-Protein L can

efficiently recognize cetuximab and scFv molecules (those with Kappa light chain) (Safdari et al. 2013). After another 1 h, the wells were washed again and filled with 100 µL TMB substrate. OD values were registered using spectrophotometer at absorbance wavelengths of 450–630 nm.

Western Blot

A-431 and MCF-7 cells were cultured in RPMI medium and allowed to growth. The cells were precipitated by centrifuging at 5000 g for three min at 5 °C. The cells were suspended in PBS buffer (final concentration of 450000 cells/mL) and sonicated at 80 Amp (15 cycles of 10 s on/off) to prepare cell lysate. The cell lysates were run on a native page (two wells for each cell line, 30 µL cell lysate/well) and the segregated proteins transferred to a PVDF membrane. The membrane was immersed in 5% BSA at 4 °C overnight for blocking non-specific binding. The membrane was excised using a scissor into two parts, one for activity assay of nimotuzumab scFv and the other for cetuximab. Each part contained a lane of A-431 cells and a lane of MCF-7 cells. In native page, the rules of protein migration are different from those of SDS-PAGE, in which proteins are segregated according to their molecular weight. Therefore, on the native page, a well was filled with EGFR synthetic peptide (concentration of 25 µg/mL) as a positive control to show the sites of EGFR (and therefore the expected sites of western blot spots) on the PVDF membrane. The membranes were washed three times (each 5 min) with PBS and separately incubated with 2 mL of cetuximab (25 µg/mL) and nimotuzumab scFv (50 µg/mL). Cetuximab has two binding sites for HRP-Protein L; therefore, for a better comparison we adjusted a concentration of cetuximab to be half the concentration of nimotuzumab. After 2 h, the membranes were washed again (at the same condition) and treated with HRP-Protein L (final concentration of 0.5 µg/mL). After 1.5 h incubation with HRP-Protein L and then three times washing (each 5 min) with wash buffer, freshly prepared DAB substrate was added to visualize spots.

Statistical Analysis

The data obtained from ELISA tests was analyzed using statistical software SigmaPlot 11.

Results

Expression and Purification of Nimotuzumab scFv

Expression of the nimotuzumab scFv gene in Origami™ 2(DE3)pLysS resulted in appearance of a 28.739 KD protein in SDS-PAGE (Fig. 1). Analysis of bacterial pellet and supernatant fraction (after sonication) revealed that nimotuzumab scFv accumulated largely in supernatant fraction. The protein was purified using Ni-TNA resin column by addition of different concentration of imidazole (10, 50, and 250 mM). The maximum amount of the protein was obtained in the first 0.5 mL fraction of 300 mM imidazole solution loaded on Ni-TNA resin column.

3-D Structure of Nimotuzumab scFv

I-TASSER server predicted five models for 3-D structure of nimotuzumab scFv (Fig. 2). In one of the predicted models (Model 3, C-score = −2.24), VH and VL domains do not assemble to form an expected structure of a single chain antibody (Fig. 2). C-score value can be in the range of −5 to 2, the greater c-score value the higher confidence in estimating the quality of predicted models by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/about.html). The best c-scores belonged to models 1 and 4 (0.34 and 0.04, respectively). Superposition

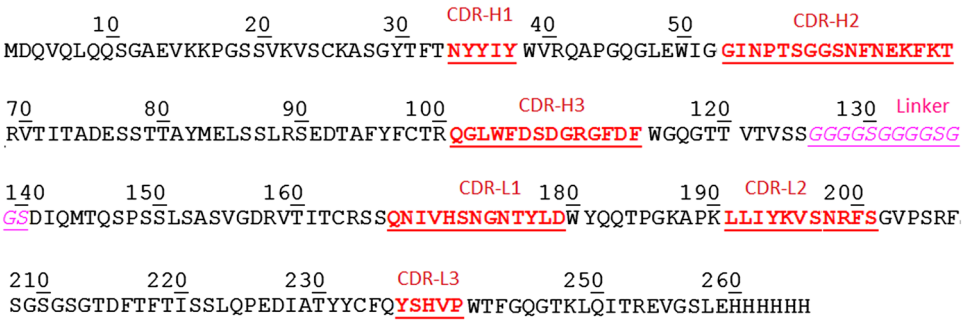

Fig. 1 Amino acid sequence of nimotuzumab scFv. From N-terminal to C-terminal end, the sequence is composed of VH, 15 amino acid glycine-serine linker (*pink-colored italic letters*), VL, and a 6-His tag. *Red-colored underlined letters* indicated the amino acids of complementary determining regions (CDR). The two amino acids resided just before the 6-His tag [(Lucien (L) and Glutamic acid (E)] are the result of XhoI restriction site translation. (Color figure online)

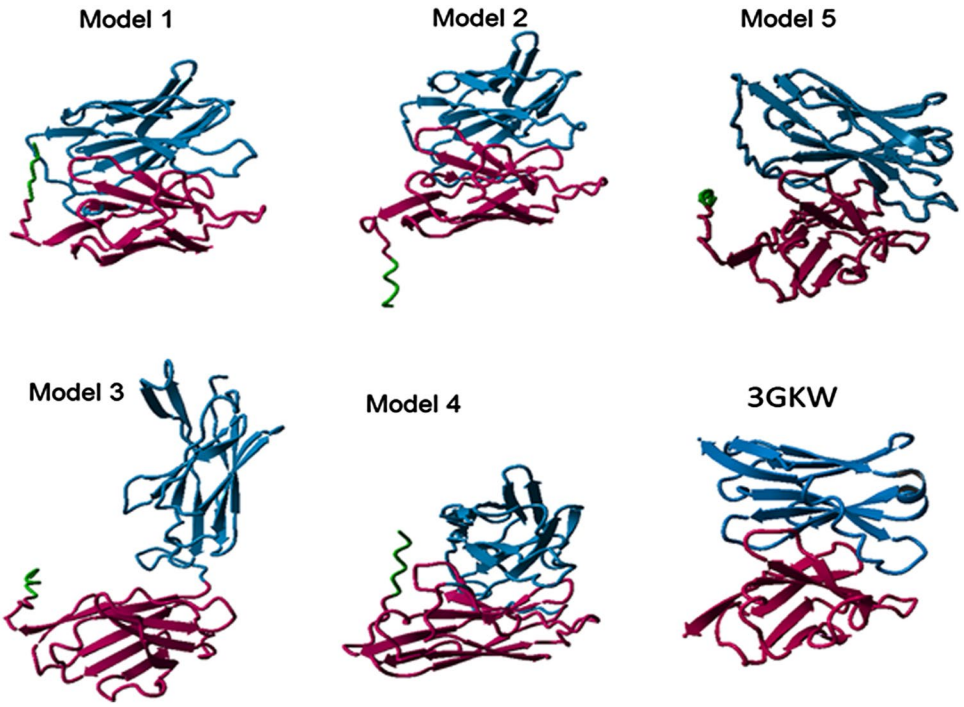

Fig. 2 Prediction of 3-D structure of nimotuzumab scFv using I-TASSER server. Five models were predicted by I-TASSER for 3D structure of nimotuzumab scFv. C-score model 1 = 0.36, C-score model 2 = −1.62, C-score model 3 = −2.24, C-score model 4 = 0.04, and C-score model 5 = −0.91. 3GKW (PDB ID) represents the crystal structure of Fab fragment of nimotuzumab, which has been determined by X-ray crystallography. For better comparison of predicted models, constant domains of 3GKW have been hidden. *Notes* VH and VL domains are shown in *blue* and *pink*, respectively. In each 3-D structure, the green part is C-terminal 6-His tag. (Color figure online)

of nimotuzumab 3-D structure (PDB ID: 3GKW) and the predicted models (using YASARA) was carried out to compare the predicted models. The root-mean-square deviation (RMSD) for model 1, model 2, model 3, model 4 and model 5 were calculated to be 1.587, 1.613, 2.329, 1.609 and 1.601 angstrom, respectively.

Results of ELISA with EGFR Peptide

We considered three different concentrations of EGFR peptide (62.5, 125, and 250 ng/100 µL) in ELISA assay. At peptide concentration of 62.5 ng, the lowest concentration of nimotuzumab scFv (3.125 µg/mL) caused an OD value of 0.653. OD values did not increase further when the antibody concentration increased to the higher levels (6.25, 25, and 50 µg/mL), indicating that EGFR molecules coated on ELISA wells have been saturated by the antibody molecules. In fact, peptide concentration needs to be increased to higher levels to gain higher OD values. Similar trend was observed when EGFR peptide interacted with cetuximab (Fig. 2). These data indicate that peptide concentration is a limiting factor that prevents OD values from being increased. When EGFR concentration increased to 5 µg/mL, OD values increased strikingly (Fig. 3); the lowest level of cetuximab (3.125 µg/mL) produced an OD value as high as 2.63 (data not shown).

Results of ELISA with A-431 and MCF-7

Nimotuzumab scFv could recognize EGFR in A-431 cell lysate. The plots of OD values versus the logarithm of antibody concentrations indicated that nimotuzumab scFv binds to A-431 cell in a specific manner. At each antigen level, OD value increased by increasing the antibody concentration and created a sigmoid curve (Fig. 4a). Antibody concentrations at $OD_{50\%}$ points for cellular levels of 40,000, 20,000, and 10,000 cells/well were calculated to be 5.888, 4.897 and 5.370 µg/mL, respectively. Using the formula optimized for single chain antibodies, the affinity of nimotuzumab scFv for A-431 cell was calculated to be 6.93×10^{10}/Mol. Antibody concentrations at $OD_{50\%}$ points of sigmoid curves are used to calculate antibody affinity (Safdari et al. 2014). There were significant differences between OD_{100} values caused by nimotuzumab scFv and cetuximab at A-431 cell levels of 40,000 and 20,000, but there was no significant difference at level 10,000 (Table 1). At cellular level of 40,000 cells/well, cetuximab concentrations of 50, 25, 12.5, 6.25 and 3.125 µg/mL produced OD values of 1.73, 1.689, 1.646 and 1.392, respectively. By increasing the cetuximab concentration from 6.25 to 50 µg/mL, no significant increment occurred in OD values, indicating that higher concentrations of A-431 cells should be coated to have higher OD values. At cellular level of 20,000 cells/well, the OD value caused by 3.125 µg/mL of

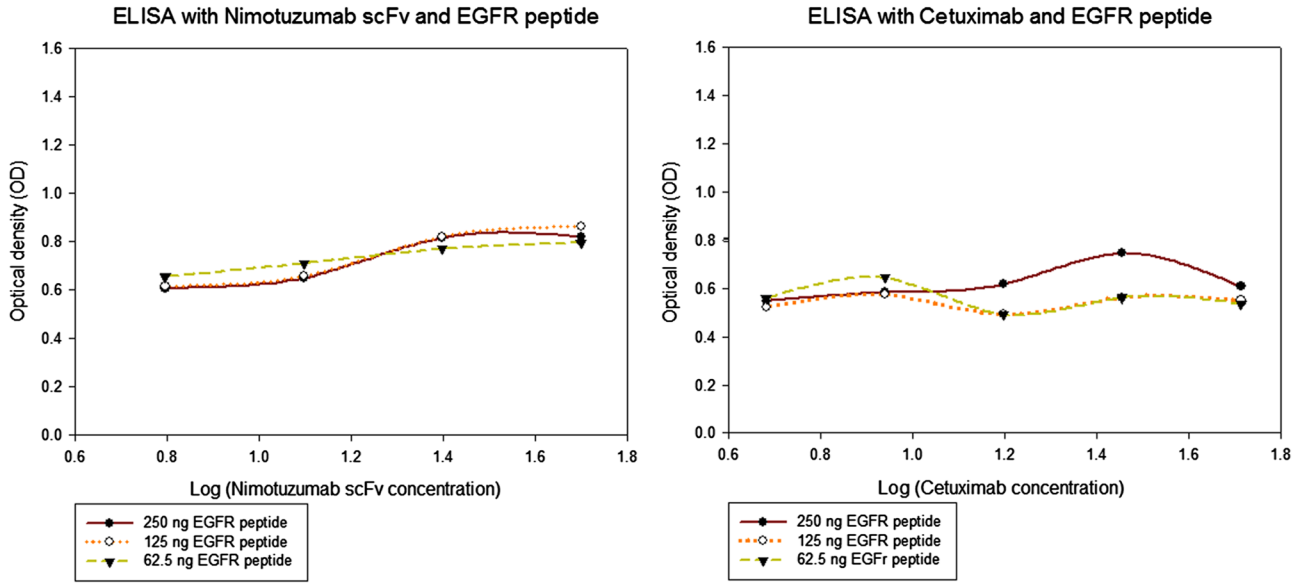

Fig. 3 Activity of nimotuzumab scFv and cetuximab in recognizing EGFR peptide. EGFR peptide has been coated at three levels, including 250, 125, and 62.5 ng/mL. For concentrations of nimotuzumab scFv (50, 25, 6.25, and 3.125 µg/mL) and five concentrations of cetuximab (50, 25, 12.5, 6.25, and 3.125 µg/mL) have been tested. In the case of nimotuzumab scFv (the *left panel*), all OD values are in the range of 0.6–0.85. OD values produced by cetuximab range from 0.549 to 0.746 (the *right panel*)

Table 1 Comparison of OD100 values caused by nimotuzumab scFv and cetuximab

Number of cells/well	Antibody	OD100%	SD	SEM	Result
40,000	scFv	1.353	0.0870	0.0502	There is a statistically significant difference between the input groups (P = 0.013)
	Cetuximab	1.720	0.120	0.0694	
20,000	scFv	1.060	0.0605	0.0349	There is a statistically significant difference between the input groups (P = 0.001)
	Cetuximab	1.600	0.0954	0.0551	
10,000	scFv	0.780	0.0533	0.0308	There is not a statistically significant difference between the input groups (P = 0.093)
	Cetuximab	0.957	0.129	0.0742	

SD Standard deviation, *SEM* standard error of the mean, *OD* Optical density

cetuximab was 0.903, a little more than expected $OD_{50\%}$ point (OD = 0.8). Using SigmaPlot software, antibody concentration at $OD_{50\%}$ point was found to be 2.366 µg/mL (Anti Log 0.374 = 2.366). At the lowest level of A-431 cells (10,000 cells), OD values increased in a linear fashion by increase of cetuximab concentration (Fig. 4b). This indicates that cetuximab concentration should be increased to reach upper plateau of sigmoid curve.

When the lysate of MCF-7 cells was coated as antigen, nimotuzumab scFv could not produce any significant OD value. No significant increase was observed in OD values by increasing the concentration of nimotuzumab scFv or antigen level (Fig. 4c). In the higher level of MCF-7 cells (40000cells/well), cetuximab caused OD values ranging from 0.268 to 0.918. The higher concentration of cetuximab, the higher OD values were observed. OD values increased in a linear fashion, and did not form upper plateau of sigmoid curve. Surprisingly, cetuximab (at all concentrations, except for 50 µg/mL) caused higher OD values at lower concentration of MCF-7 cells (20,000 cells). OD values ranged from 0.509 to 0.87, indicating that no significant increase occurred in OD values by increasing cetuximab concentration. At MCF-7 level of 10,000 cells/well, OD values were relatively higher than those observed in 20,000 and 40,000 cell/well (Fig. 4d). We repeated the experiment with MCF-7 cells but observed the same trend of OD values (relatively higher OD values in lower MCF-7 concentrations).

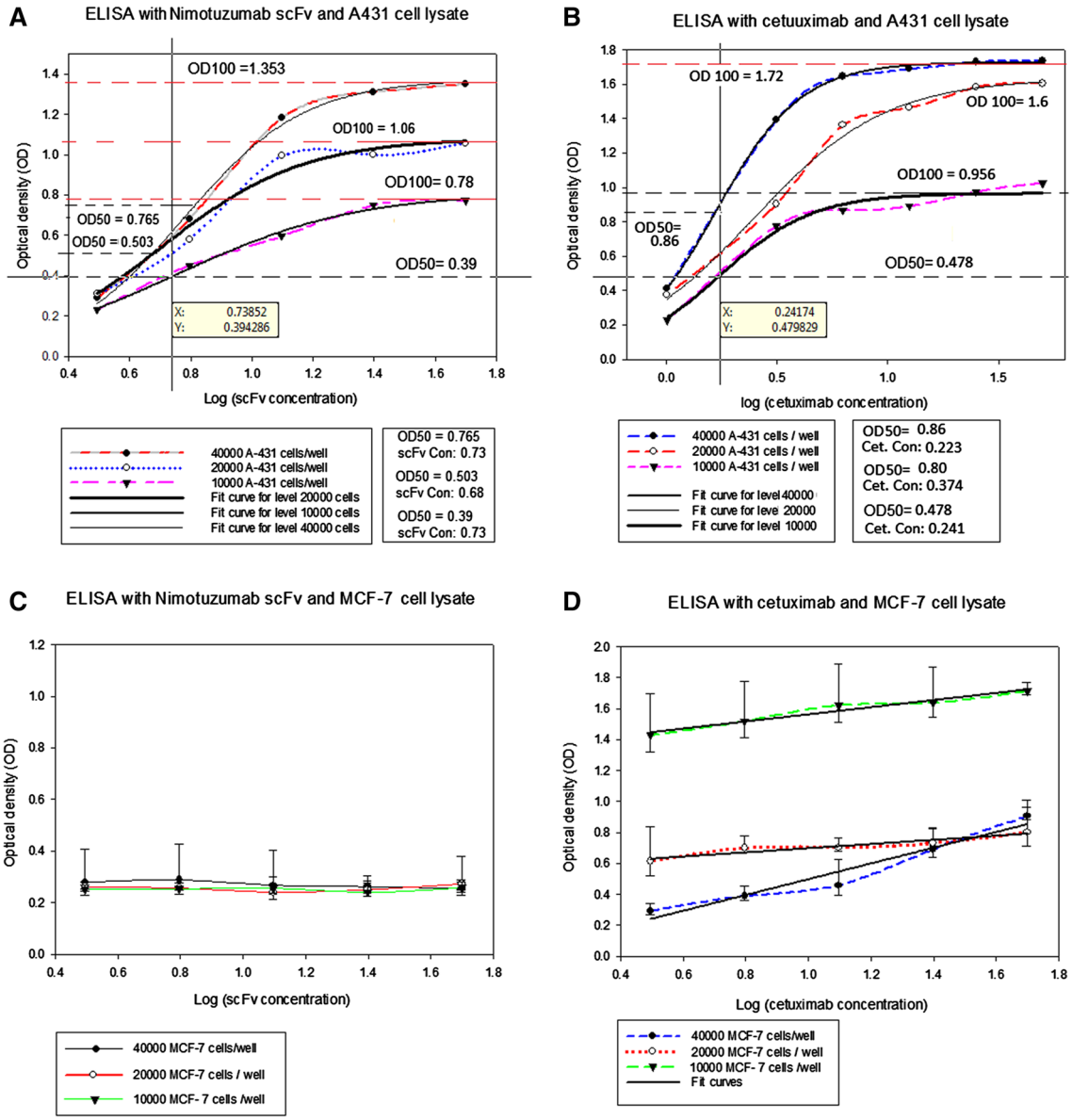

Fig. 4 Activity of nimotuzumab scFv and cetuximab in recognizing A-431 and MCF-7 cells in ELISA. **a** Activity of nimotuzumab scFv in recognizing A-431 cells (EGFR-overexpressing cells). At each cellular level, the curve of "antibody concentrations" versus "OD values" is sigmoidal. **b** Activity of cetuximab in recognizing A-431 cells. Cetuximab can efficiently recognize A-431 cells. **c** Activity of Nimotuzumab scFv in recognizing MCF-7 cells. Nimotuzumab scFv seems to be unable to recognize MCF-7 cells. These cells express low levels of EGFR molecules. **d** Activity of cetuximab in recognizing MCF-7 cells. OD values increase in a linear manner by increasing antibody concentration but do not form sigmoidal curves. *Note* The X axes indicate the logarithms of antibody concentrations. For both antibodies, five concentrations have been used, including 50, 25, 12.5, 6.25 and 3.25 μg/mL. Log 50 = 1.699, Log 25 = 1.3979, Log 12.5 = 1.0969, Log 6.25 = 0.7959, and Log 3.125 = 0.4949

Western Blotting

As predicted, cetuximab (at concentration of 25 μg/mL) was able to efficiently recognize EGFR synthetic peptide transferred to PVDF membrane. Cetuximab was also able to recognize EGFR-overexpressing cancer cells (A-431 cells), but it could not recognize MCF-7 cells, which usually express low levels of EGFR. Nimotuzumab scFv exhibited the same interaction trend in recognizing A-431 and MCF-7 cells. 50 μg/mL of nimotuzumab scFv could recognize A-431 cells and produced a brown spot as intense as that produced by 25 μg/mL of cetuximab. No band was formed in the lane of MCF-7 lysate (Fig. 5). These results indicate that nimotuzumab scFv have folded correctly and is able to recognize its conformational epitope on EGFR molecules.

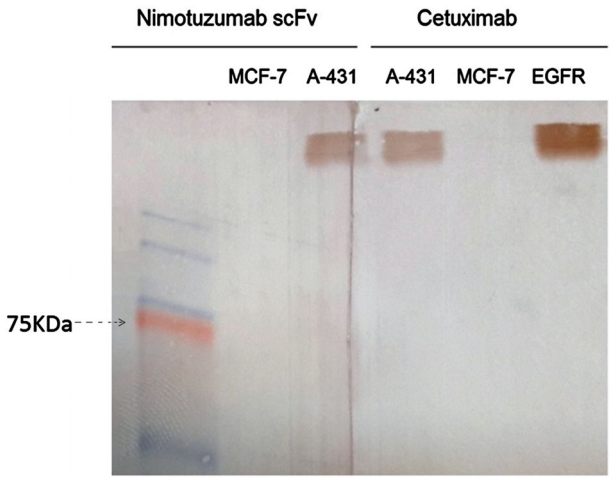

Fig. 5 Activity assay of nimotuzumab scFv by Western blotting. Since the epitope recognized by nimotuzumab is a conformational epitope, native-page was used (instead of SDS-PAGE) for separation of cell proteins. Like cetuximab, nimotuzumab scFv can recognize A-431 cells, which express high level of EGFR. Number of A-431 cells: 15,000, number of MCF-7 cells: 15,000, concentration of EGFR peptide 25 µg/ml, concentration of coated cetuximab 25 µg/ml

Discussion

I-TASSER server predicted five models for 3-D structure of nimotuzumab scFv. C-score values for model 1, model 2, model 3, model 4, and model 5 were calculated to be 0.36, −1.62, −2.24, 0.04, and −0.91, respectively. Based on these values, Model 1 was the most likely 3-D structure of nimotuzumab scFv. Superposition of nimotuzumab Fab fragment (PDB ID: 3GKW) and the five predicted models confirmed that model 1 (with RMSD of 1.587) was the most likely 3-D structure of nimotuzumab scFv. RMSD is the average distance of the atoms in two superposed molecules. The lower RMSD value the more similarity in 3-D structure(Coutsias et al. 2004).

Both nimotuzumab scFv and cetuximab (control antibody) were able to recognize EGFR peptide. We used three peptide concentrations, including 62.5, 125, and 250 ng/mL. At peptide concentration of 62.5 ng/mL, nimotuzumab scFv and cetuximab (both at concentration of 3.125 µg/mL) produced OD values of 0.653 and 0.645, respectively. No significant increment in OD values was observed when antibody concentration raised to higher levels (up to 50 µg/mL), indicating that all the antigens (peptide molecules) have been saturated by antibody molecules. To demonstrate this hypothesis, we increased the peptide concentration to 5 µg/mL and applied the same concentrations of cetuximab. We observed that OD values improved strikingly, ranging from 2.63 (at antibody concentration of 3.125 µg/mL) to ≥3 [at the higher antibody concentrations (6.25, 12.5, 25, and 50 µg/mL](data not shown). In ELISA test we observed that nimotuzumab scFv could recognize A-431 cells only; OD values increased in a dose dependent manner and created a sigmoid curve. Formation of upper plateau in the curve of "OD" versus "logarithms of antibody concentration" indicates the specificity of antibody-antigen interaction (Laidley and Thomas 1994; Taylor and Cooper 1989). Compared to nimotuzumab scFv, Cetuximab produced higher OD values with the lysate of A-431 cells. Cetuximab also produced relatively higher OD values with MCF-7 cells; however, the values were out of standard range for making sigmoid curves of "OD" versus "antibody concentration". At each antigen (cell lysate) concentration, OD values increased in a linear fashion without forming upper plateau. Failing to reach upper plateau upon increasing antigen and antibody concentration indicate that MCF-7 cells remain unsaturated and therefore are unspecific targets for cetuximab. In western blot test, we observed that nimotuzumab scFv could recognize A-431 cells while it was unable to bind to MCF-7 cells. In the lanes of A-431 cell lysate on the PVDF membrane, nimotuzumab produced western blot spots as dense as those produced by cetuximab (Fig. 5). However, it does not necessary mean that these antibodies have equal affinity for A-431 cells. Color density in western blot tests depends on the number of antibody molecules that bind to antigen molecules. Color density also depends on the number of HRP- conjugated molecules bound to antibody molecules. Cetuximab have two light chains, and as a result, two binding sites for HRP-Protein L. Therefore, to make a precise comparison between cetuximab and nimotuzumab scFv, we adjusted the concentration of cetuximab to be half the concentration of nimotuzuman scFv. We used 25 µg/mL of cetuximab and 50 µg/mL of nimotuzumab scFv for comparison in western blot. Molecular weights of these antibodies are different. 2.5 µg of nimotuzumab (concentration of 25 µg/mL, equal to 2.5 µg/100 µL in each ELISA well) contains nearly 5.238×10^{13} molecules while 5 µg of cetuximab (50 µg/mL, equal to 5 µg/100µL in each ELISA well) contains nearly 2.065×10^{13} molecules. By considering the color intensity and the number of the antibody molecules taken part in western blot test, it can be deduced that cetuximab is stronger than nimotuzumab scFv. Comparison of cetuximab and nimotuzumab scFv in ELISA tests confirms that cetuximab has a higher affinity for A-431 cells and therefore for EGFR.

Conclusion

Nimotuzumab scFv can recognize EGFR synthetic peptide and EGFR-overexpressing cells in an efficient manner. The affinity of this antibody for EGFR is lower than that

of cetuximab, but is strong enough to target EGFR-overexpressing cells.

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Compliance with Ethical Standards

Conflict of interest There are no conflicts of interests to declare.

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