WITHDRAWN: Reduced tumor volume and increased necrosis of human breast tumor xenograft in mice pretreated by a cocktail of three specific anti-HER2 scFvs: Involvement of stereological methods

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Abstract

Purpose: We assessed the effects of a cocktail of three specific anti HER2-scFvs on breast tumor formation in a xenograft mouse model and evaluated the quantitative changes of the tumor using stereological analysis.

Methods: Three specific anti-HER2 phage-antibody were produced from a scFv-library using phage display technology. Cell binding capacities of the antibodies were assessed by FACS analysis. Soluble forms of antibodies were prepared by infection of HB2151-*Ecoli* and purified using affinity-based spin column purification method. SDS-PAGE-analysis evaluated the purification process. A cocktail containing equal amount/phage of each scFv was prepared. Inbred female BALB/c-mice were pretreated with 5 and 20-mg/kg of soluble-scFv cocktail and 10^{11} phage-cocktail/kg. The mice received 2×10^{6} SKBR-3 human breast cancer cells. Tumor volumes were estimated using Cavalieri principle after preparation of photomicrograph slides. .

Results: Anti-HER2-scFvs bound to SKBR-3 cells significantly higher than isotype control. SDS-PAGE-analysis showed high purification of scFvs. Stereological analysis revealed that the highest reductions in total tumor volume, non-inflammatory and inflammatory volumes, 73%, 78% and 72%, respectively, belonged to the group pretreated with 20-mg/kg of the soluble scFvs-cocktail in comparison with PBS-pretreated mice (*P-value* < 0.0001). The volumetric ratio of the necrotic tissue to the total tumor increased 2.2 and 2 folds in the 20-mg/kg and phage displayed scFvs-cocktail groups in comparison with the PBS-treated mice, respectively (*P-value* <0.05).

Conclusions: Significant reduction of tumor volume and increased necrotic area of human breast cancer cells xenograft by pre-treating with 20-mg/kg anti-HER2-scFv cocktail offers the remarkable anti-tumor effect of the cocktail in vivo.

Introduction

Breast cancer is a heterogeneous disease with distinct histological, clinical, and molecular characteristics. About 25% of breast cancers are HER2-Positive, which tend to grow and spread more aggressively than those without the HER2 receptor (Hart, et al 2020). HER2 belongs to a family of four transmembrane receptor tyrosine kinases that mediates the growth, differentiation, and survival of cells. Overexpression of HER2 is associated with aggressive behavior in the tumor (Gutierrez and Schiff 2011).

The potential targets for immunotherapy could be achieved by identification of tumor specific antigens on the surface of cancer cells (García-Aranda and Redondo 2019). Monoclonal antibodies such as trastuzumab and pertuzumab have been recommended for immunotherapy of HER2-Positive breast cancer (Costa and Czerniecki 2020). However, some advantages of human single chain fragment variables, scFvs, including highest level of consistency and unrivaled reproducibility, confirmed specificity and improved sensitivity and ease of supply also their human origin, small size and high tissue penetration have developed considerable attention for the use of these valuable agents in cancer

immunotargeting approaches (Ehsaei, et al 2017, Moazen, et al 2016, Zarei and Nejatollahi 2017) (Hosseinzadeh, et al 2017, Nejatollahi, et al 2014b, Petrus, et al 2019, Zarei and Nejatollahi 2017).

Until now, recombinant antibody fragments have shown promising anti-cancer activity in vitro as well as phase I and II clinical trials in some types of cancer (Bates and Power 2019, Lu, et al 2020, Zhao, et al 2019). To study the anti-tumor effects of drug candidates against human cancers, several murine models have been developed and used to examine malignant transformation, invasion and metastasis. The human tumor xenograft is one of the most broadly used models, which is applied in preclinical and clinical investigations of anti-cancer therapeutics (Landgraf, et al 2018).

Stereological principles are efficient and reliable geometrical and statistical techniques which are useful for evaluation of the quantitative parameters of tissue structure on the histological sections. Stereology applies mathematics, statistics and a set of rules to estimate parameters such as volumes, dimensions, structures and number of structures in an accurate and precise quantitative form. It allows for analysis of two-dimensional tissue sections in three-dimensional space (Deniz, et al 2018, Eriksen, et al 2017, Matenaers, et al 2018). Stereological methods are widely used in biomedical researches and provided a quick and unbiased tool in the study of cells, tumor volume and tumor regression rate (Kipanyula and Sife 2018, Sarma, et al 2020, Tatar, et al 2018, Zhao 2020).

We have already reported the generation of three specific scFvs against three HER2 epitopes (amino acids: 328–345, 369–384 and 593–603). A library of scFv was performed and three specific scFvs against HER2 epitopes were selected by panning process. The selected scFvs both individually and in combination could down regulate VEGF expression in breast cancer cells (Nejatollahi, et al 2012). A cocktail of three anti-HER2 scFvs induced high anti proliferation effects in breast cancer cells and reduced HER2 expression at both the gene and protein levels individually and in combination (Nejatollahi, et al 2014a). In this study the effects of the anti-HER2 cocktail is investigated in vivo. A xenograft mouse model of HER2-positive breast cancer was applied to assess the anti- cancer effects of three specific anti-HER2 scFvs in a cocktail form.

Methods

Anti-HER2 phage antibody preparation

A phage antibody display library of scFv was used to select specific scFvs against three immunodominant epitopes of HER2 (amino acids 328–345, 369–384, 593–603). Helper phage M13KO7 was used to rescue the phage transformed E. coli and prepare phage antibody. Panning process was performed to select the specific clones: Nunc-immunotubes were coated with 100 µg/ml antigenic peptide. The tubes were blocked with 2% skimmed milk, incubated at 37°C for 2 h and washed with PBS/Tween-20. Phage rescue supernatant, 10^{10} to 10^{11} phages in blocking solution were added to the tubes and incubated at room temperature. TG-1 E.coli was added and incubated to allow the infection of bacterial cells by the phages. Centrifugation collected the infected TG1 cells. Four rounds of panning

were used to select high affinity and high specificity scFvs against HER2 epitopes. After preparation of phage antibody from the selected clones, phage concentration was determined. 10 µl of phage antibody supernatant was added to 1 ml of log- phase TG1 E.coli, incubated at 37°C for 1 hr, diluted serially and plated onto 2TY agar / ampicillin medium. Number of colonies were counted and phage concentration was calculated using the following formula.

CFU/ml (Pfu/ml) = Number of colonies × dilution factor / volume of culture plate.

Cell culture

The SKBR-3 breast cancer cell line was purchased from IBRC (Iranian Biological Resource Center, Tehran, Iran) and cultured in RPMI-1640 medium (Biosera, UK) supplemented with 10% fetal bovine serum (Biosera, UK) containing 100 Unit/ml penicillin and 100 mg/ml streptomycin, incubated in the humidified incubator with 5% CO2 at 37°C.

Flow cytometry binding assay

Cell surface binding capacities of phage-antibodies were determined by FACS analysis. Briefly, 2×10^5 SKBR-3 cells were treated with 200 µl of selected-scFv antibody (10^{11}Pfu/ml) and incubated for 60 min at 4°C. Cells treated with M13KO7 helper phage was used as isotype control. After washing with ice-cold PBS buffer, rabbit anti-fd bacteriophage antibody (Sigma-Aldrich, Germany) was added and incubated at room temperature for 30 min, followed by 30 min incubation with PE-conjugated anti-rabbit antibody. The amount of bound phage was measured by a FACS Calibur (BD Biosciences, USA) following washing.

Production of soluble single chain antibody fragments

Recombinant phages of the selected clones against HER2 epitopes, peptide I (amino acids 328–345), Peptide II (amino acids 369–384) and peptide III (amino acids 593–603) obtain from our previous research (Nejatollahi, et al 2012, Nejatollahi, et al 2014a), were used to infect HB2151 *E.coli* bacteria. In order to induce expression of scFvs, 1mM of IPTG was added to the infected HB2151 *E.coli* and incubated at 30^{oC} overnight. Following centrifugation, the pellet was resuspended in 500µl of TES buffer (with PH:8) on ice and immediately 750µl of distilled water added and well mixed. The tube incubated at $4^{\circ}C$ for 1 hr and occasionally inverted. After centrifuged at 14000 RPM and $4^{\circ}C$ for 20 min, supernatant contained the soluble scFvs was filtered and stored at $-20^{\circ}C$.

Purification of scFvs

Purification of soluble scFvs was done using the affinity-based spin column purification device. The 10 kDa ultra centrifugal filter (Amicon, USA) inserted into the provided tubes. 15 ml of soluble scFvs was added to the filter and centrifuged at 4°C and 9000 rpm for 1 hr. The supernatant containing proteins with molecular weight higher than 10 kDa was transferred to 30 kDa ultra centrifugal filter (Amicon, USA). After spanning at 14000 rpm for 30 min, the soluble scFv was purified in the microcentrifuge tube. The concentrations of the purified scFvs were determined using Nano-drop 2000 spectrophotometer (Thermo

Fisher Scientific). A cocktail containing the equal amount of the three scFvs was prepared and the concentration of the cocktail was also determined by the Nano-drop 2000 spectrophotometer.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving gel (10%) was submerged in migration buffer (1X Tris-glycine buffer). 15µl of the three bacterial protein-extracts containing the soluble scFvs before purification and three purified scFvs were loaded in stacking gel wells. Separated proteins were detected by Commassie Blue R-250 buffer and de-stained with destaining solution (200ml methanol, 100ml glacial acetic acid, 700ml ddH_2O). The SDS-PAGE gels were examined for expected ~ 25 KD size of the scFv.

PEGylation of phage-scFvs

1ml of 2.5M PEG/NaCl (PEG-8000 20%, NaCl 2.5M) was added to 4ml of each recombinant phage antibody supernatant, shaken and placed on ice for 1 hr. After centrifugation, the pellet was re-suspended in 500µl of PBS. The concentration of the obtained phages was determined using Nano-drop 2000 spectrophotometer. A cocktail of the three scFvs was prepared using equal phages of each scFvs. The final phage concentration of the cocktail was also determined by Nano-drop 2000 spectrophotometer.

In vivo assay

The effects of scFv cocktail on the inhibition of breast tumor xenograft formation was investigated. Thirty-two inbred 6–8 week old female BALB/c mice (weights 20–25 gr) (purchased from Iran Pasteur Institute) were used for the study. The mice were divided into four groups, eight mice in each group. All mice were exposed to whole-body radiation, 4.50 GY, (day 0) under ketamine (100 mg/kg, i.p) and xylazine (10 mg/kg, i.p) anesthesia. In day1, the first group as the control group, administrated PBS, the second and third groups received anti- HER2-scFvs cocktail dose at 5 and 20 mg/kg of soluble scFvs, respectively and the fourth group administrated phage scFvs cocktail dose at 1×10^{11} phage/kg through peritoneal injection. The same injections were also administered at the intervals of three days (days 4, 7, 10, 13). All mice were again exposed to radiation, 2.25 GY, at day 12 under ketamine and xylazine anesthesia and received 2×10^{6} SKBR-3 human breast cancer cells at day 13. After centrifugation, the cells were re-suspended in 100µl serum-free medium and subcutaneously injected into the right inguinal flank of the female mice using a 21-gauge needle. Animals were sacrificed at day 21. The tumor tissues were transferred into 10% fixative formalin buffer. The schedule used for administration of scFvs and radiation and SKBR3 cells injections is shown in Table 1.

Table 1
Schedule of in vivo assay. Cocktail administration, radiation, cell injection and sacrifice times.

Day	0	1	4	7	10	12	13	21
Scfvs Cocktail Administration		*	*	*	*		*	
Radiation	*					*		
Skbr-3 Cells Injection							*	
Sacrifice								*

Tissue preparation

At the end of the 21st day, the tumors were dissected out, processed and embedded in the paraffin blocks, and the whole tissue was wholly sectioned. Using Systematic Random Sampling (SRS) method, 11–13 sections were sampled and stained with Hematoxylin and Eosin (H&E) and used to estimate tumor volumes.

Estimation of Tumor Volume by Stereology

The main quantitative parameters of tumor volumes were estimated using point counting based on the Cavalieri's principle, using an Eclipse microscope (Nikon, E200, Japan) linked to a camera (Nikon, DS-Fi, Japan). Systematic random sampling was applied to samples which serially sectioned (11–13 sections in each solid tumor). The image of each section was assessed at a final magnification of 110X using the stereological software (Stereo Lite, SUMS, Shiraz, Iran). The total volume of each tumor was estimated, using the following formula:

$$\left(V\left(tumor\right) = \sum A\left(tumor\right) \times d\right)$$

Where "∑A (tumor)" is the sum area of sections and "d" is the distance between sampled sections.

Furthermore, the region of interest including inflammatory, non-inflammatory and necrotic areas were identified and the volume density was estimated based on point counting, using the software and the following formula:

$$\left(V_V\left(regionofinterest\right) = \sum P\left(regionofinterest\right) / \sum p\left(totaltumormass\right)\right)$$

Where "V_V" is volume fraction and "$\sum P$" is sum of the points land on the region of interest or reference space.

Estimation of the coefficient of error (CE)

The calculation of coefficient of error (CE) defines how much effort should be done to reach a suitable variance in the estimates. The CE (V) for the estimate of the volume was calculated using the following formula (Foldager, et al 2015, Gundersen and Jensen 1987):

$$CE\ (V)\ =\ (\frac{\mathbf{1}}{\sum \boldsymbol{P}}) \times [\frac{\mathbf{1}}{\mathbf{240}}(\mathbf{3} \times \sum \boldsymbol{P_i P_i} + \sum \boldsymbol{P_i P_{i+2}} - \mathbf{4}\sum \boldsymbol{P_i P_{i+1}}) + \mathbf{0.0724} \times \frac{\boldsymbol{b}^{\frac{1}{2}}}{\boldsymbol{a}} \times (\boldsymbol{n}\sum \boldsymbol{P_i})^{\frac{1}{2}}]^{\mathbf{1/2}}$$

Where "b" and "a" demonstrate the mean section boundary length and mean sectional area, respectively and "$\sum P$" was the sum of the point count.

Statistical analysis

Quantitative stereological data were analyzed using Mann–Whitney U test and P-value < 0.05 was considered statistically significant.

Results

Cell binding analysis by flowcytometry

Flow cytometry assay revealed the cell surface binding ability of selected anti-HER-2 phage-antibodies on SKBR-3 breast cancer cell line. scFv I, II and III bound to 87.8%, 85.1% and 91.6% of SKBR-3 cells while M13KO7 helper phage as isotype control bound to 12.2%, 14.9% and 8.4% of the cells, respectively (Fig. 1).

SDS-PAGE analysis of expressed scFvs proteins

SDS-PAGE showed the expression of soluble scFvs. The presence of 25 KD scFvs-I, scFv-II, and scFv-III bands in soluble extracts was confirmed (Fig. 2a). Comparison between purified and non-purified scFvs in SDS-PAGE analysis represented a high quality purification using affinity-based spin column purification (Fig. 2b).

ScFv concentration

The concentrations of soluble scFv-I, scFv-II and scFv-III after purification by ultra-certification were 9.01, 6.30 and 5.94 mg/ml as determined by nanodrop 2000, respectively and the concentration of the soluble scFvs cocktail containing equal amount of each scFvs was 6.25 mg/ml.

After PEGylation, clones of each dilution were counted and phage concentration per milliliter was determined for scFv-I, scFv-II and scFv-III which were 8.8×10^{13}, 4.03×10^{14} and 4.8×10^{13} phage particles/ml respectively. The phage cocktail concentration, which was contained equal phage of each phage antibody was 1.56×10^{14} phage/ml.

Stereological evaluation

The quantitative parameters are shown in Fig. 3. In comparison with PBS-pretreated mice, the groups pretreated with doses of 5 mg/kg, 20 mg/kg of scFv soluble cocktail and 10^{11} phage/ kg of phage displayed scFvs cocktail represented following significant results. The mean value of the total tumor volume reduced 51%, 73% and 58%, respectively (*P value* < 0.0001) (Fig. 3a). The mean value of the non-inflammatory area volumes showed decrease 53%, 78% and 56%, respectively (*P value* < 0.001) (Fig. 3b) and the mean value of the inflammatory area volume containing tumor cells plus inflammatory cells, reduced 52%, 72%, 65%, respectively (*P value* < 0.0001) (Fig. 3c). Pre-treating mice with dose of 20 mg/kg scFv cocktail, demonstrated significant reductions of total tumor, non-inflammatory and inflammatory volumes compared to pre-treating with dose of 5 mg/kg scFv (*P value* < 0.05).

The volumetric ratio of the necrotic tissue to the total tumor increased 2.2 and 2 folds in the 20 mg/kg and phage displayed scFvs cocktail groups in comparison with the PBS-treated mice, respectively *(P value* < 0.05) (Fig. 3d). However, no significant changes in the ratio of necrotic tissue to total tumor was observed for 5 mg/kg concentration of scFvs cocktail compared to PBS-treated mice **(***P value* > 0.05) (Fig. 3d).

The necrotic region of the tumor after treating with 5mg/kg, 20mg/kg of soluble scFv cocktail, and 1×1011 Phage-scFv cocktail/ kg has been shown in comparison with the tumor section of PBS treated mice (Fig. 4). Large necrotic parts were found in tumor which was treated with both 20mg/kg of soluble scFv cocktail and 1×10^{11} Phage-scFv cocktail/kg which is demonstrated by eosinophilia. The calculations of CE for different stereological estimations revealed that CE (V) was 0.02–0.05.

Discussion

Immunotherapy of breast cancer demonstrated improved outcomes including prevention of recurrence and increase of survival rate in human (Park, et al 2018). HER2 is a strong prognostic factor for relapse and poor survival of breast cancers and high HER2 expression links to increase risks of death (Kipanyula and Sife 2018). Trastuzumab is widely used to treat patients with HER2-positive breast cancer. Although increased response rate to the antibody and improved survival have been reported, a number of HER2-positive breast cancer patients, 50–66%, fail to respond to trastuzumab due to primary and acquired resistance to the antibody (Criscitiello and Curigliano 2015, Petrelli and Barni 2012). In addition, cardiotoxicity has been reported in patients following long-term trastuzumab (Bouwer, et al 2021).

Cancer immunotargeting using scFvs have shown promising results (Zhang, et al 2017) constructed an anti-HER2 scFv and conjugated the antibody to DM1. High and rapid tumor distribution and penetration

compared to trustuzumab was demonstrated.

In this study, the anti-tumor effects of three specific anti-HER2 scFvs were investigated in vivo. The antibodies were previously selected in our laboratory against three immunodominant epitopes of HER2 including peptide I (amino acid 328–384), peptide II (amino acid 369–384) and peptide III (amino acid 593–603). The anti-proliferative effects of a cocktail containing the three scFvs on BT-474 and SKBR-3 breast cancer cells were 77.4% and 76.5%, respectively (Nejatollahi, et al 2014a).

In the current study, the binding ability of the three scFvs on SKBR-3 cells were evaluated by FACS analysis. Based on the shift in the fluorescence intensities, the binding capacity of scFv-III (91.6%) was found to be more than scFvs-I (87.8%) and scFv-II (85.1%). SDS PAGE gel electrophoresis following solubilization, confirmed the presence of 25 KD soluble scFvs. SDS PAGE analysis using unpurified and purified scFvs revealed a high yield purification after ultracentrifugation method.

The Xenografts of treated and untreated experimental mice groups were investigated using stereological analysis. The results of using Cavalieri's principle demonstrated that the groups of mice which received four injections 5mg/kg or 20mg/kg of soluble anti-HER2 scFv cocktail or 10^{11} phage/kg displayed anti-HER2 scFv cocktail reduced tumor volume, non- inflammatory and inflammatory volumes. The highest reductions in total tumor volume, non-inflammatory and inflammatory volumes were obtained in the group pretreated with dose at 20 mg/kg of the soluble scFvs cocktail, 73%, 78% and 72%, respectively in comparison with PBS-pretreated mice. The reductions were significantly higher than those obtained for 5 mg/kg concentration of scFv cocktail, 51%, 53% and 52% respectively. These results represent the anti-tumor effect of anti-HER2 scFv cocktail in a dose-dependent manner. A number of investigations show the important role of stereological methods in the quantitative evaluation of tumor cells and tumor microenvironment. The effect of captopril and irbesartan in the volume of colorectal cancer liver metastases was evaluated. Analysis of results by stereological methods represented a marked reduction in the volume of colorectal liver metastasis and changes in tumor microvasculature, which demonstrated important results in the assessment of drug effectiveness in a mouse model of colorectal cancer (Neo, et al 2007). The use of Cavalieri's principle as a valid stereological method for obtaining quantitative information about tumor status were shown in total lung volume measurement following NK cell mediated lysis in lung cancer (von Hörsten, et al 2000) and breast tumor malignancy grading determination after estimation of tumor volume and the epithelium volume density by stereological analysis (Santamaría, et al 2022).

Down-regulation of HER2 gene in HER2 positive breast cancer cells had led to tumor regression (Wilson, et al 2002). In the current study, in vivo evaluation of the cocktail showed 2.2 and 2 folds' increase in the volumetric ratio of the necrotic tissue to the total tumor in the groups pretreated with 20 mg/kg soluble scFvs and phage scFvs cocktails in comparison with the PBS-treated mice, respectively. However, no significant increase of volumetric ratio of necrotic tissue to total tumor was observed for 5 mg/kg of soluble scFv cocktail, although the increase in the ratio can be observed compared to PBS-pretreated mice. The experiment demonstrated that 5mg/kg antibody concentration is not enough to cause a

significant increase in the necrotic area as well as remarkable decrease in total volume while 20 mg/kg concentration of antibody was sufficient for demonstrating the significant effects of the scFv cocktail. Also, 1×10^{11} phage antibody/ kg was enough for representing the antibody effects. The results show that the effects of anti- HER2 scFvs are due to increasing of necrotic tissue as well as reducing of tumor cells crowded with inflammatory cells. The necrotic part can be observed by an accumulation of eosinophilic substances, which are inside or outside of the tumor. Necrotic areas were characterized by increased affinity to eosin during histological staining. It could be attributed to the loss of the normal basophilic substances of the cells due to necrosis process (Miller and Zachary 2017, Rosenberg, et al 2013).

Reduced tumor volume and increased necrotic area of human breast cancer cell xenograft by pre-treating with anti-HER2 scFv cocktails represents a new preventive strategy for HER2 positive breast cancers that has the potential to prevent recurrence of the disease following surgery.

Conclusions

Immunotherapy of breast cancer and preventing recurrence has shown improved outcomes. Due to several advantages of single chain antibodies, these agents are used as unique agents for cancer immunotherapy. Here we demonstrated the effects of a cocktail containing three specific anti-HER2 scFvs on breast tumor xenograft and evaluated the quantitative changes of the tumor using stereological analysis. It was found that when 20mg/kg of soluble anti-HER2 scFv cocktail was used before Xenograft formation, the highest total tumor volume, non-inflammatory and inflammatory volumes was obtained and also the volumetric ratio of the necrotic tissue to the total tumor increased 2.2 folds. The significant reduction of tumor volume and increased necrotic area on breast tumor xenograft following treatment with the anti-HER2 cocktail provides a proof-of-concept of its potential application in clinic as a new preventive strategy against HER2 positive breast cancers.

Declarations

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Contributions

Foroogh Nejatollahi and Elham Nadimi (co-first author) conceived of the study and its design, participated in the study conception and helped draft and revise the manuscript for intellectual content. Elham Nadimi performed data collection and analyzed data. Ali Noorafshan helped in the stereology study and analysis. Setareh Moazen drafted the manuscript. Ali mohammad Alizadeh participated in xenograft formation, Solmaz khalighfard helped in animal study.

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical approval

Animal study was conducted according to relevant national and international guideline and Institutional Animal Care and Use Committee (IACUC) of Tehran University of Medical Sciences, Iran.

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Figures

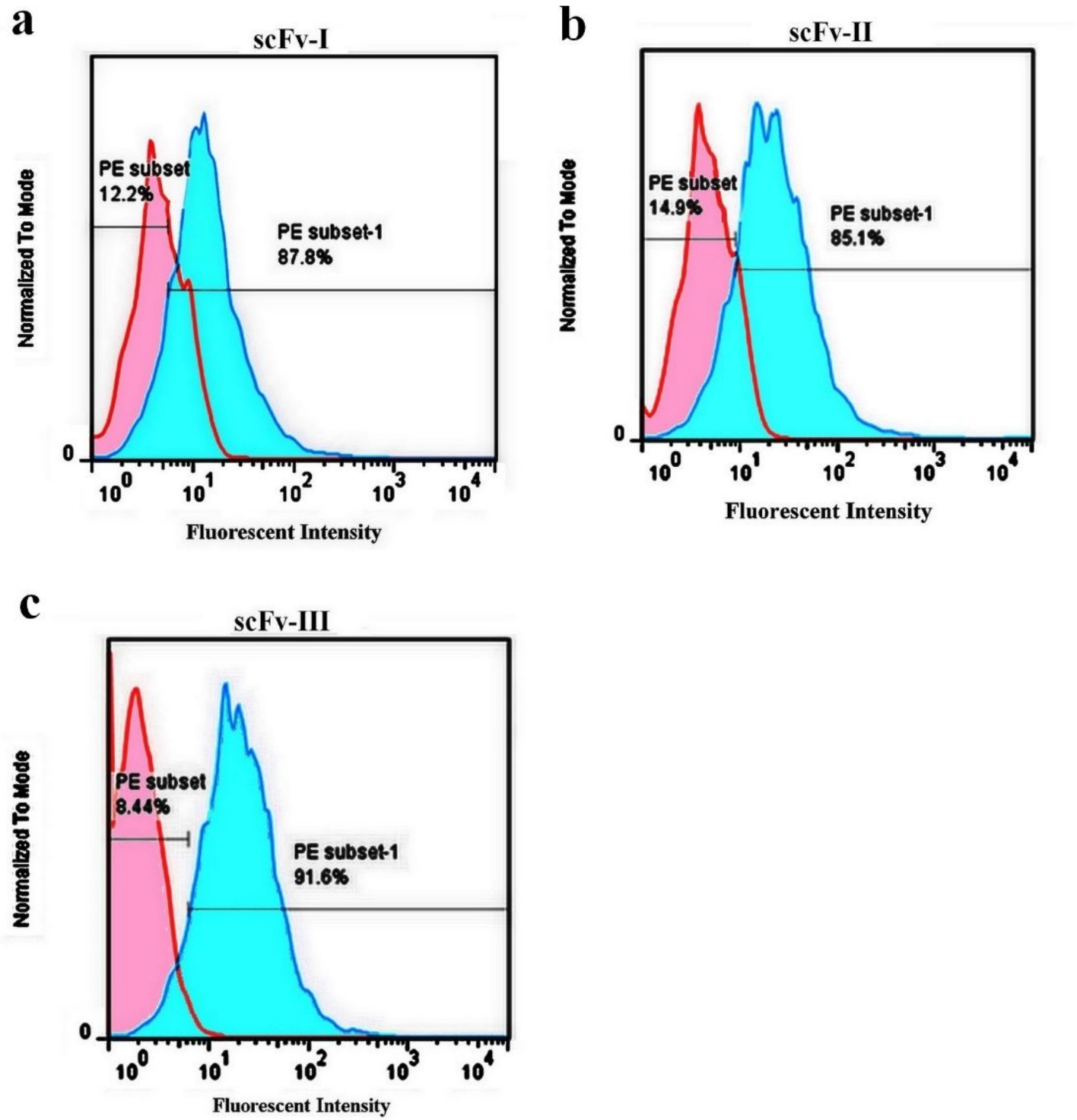

Figure 1

Flow cytometry histograms showing the binding analysis of anti-HER2 scFvs on SKBR3 cells. The scFv I (a), II (b) and III (c) bound to SKBR3 cells as shown by the shift in fluorescence intensity compared to isotype control containing cells treated with M13KO7 helper phage. Isotype and scFvs-treated cells represented red and blue respectively.

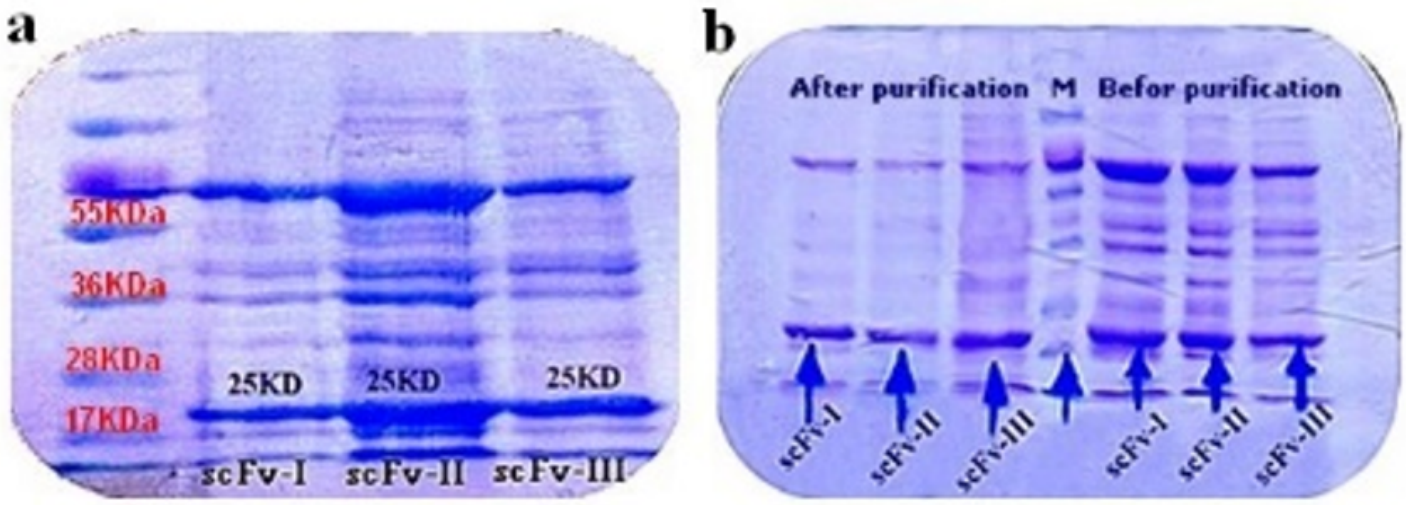

Figure 2

SDS PAGE result of soluble scFvs. 25 KDa band for each scFv was obtained (a). SDS-PAGE analysis before and after purification of the three scFvs, showing the purified scFvs (b).

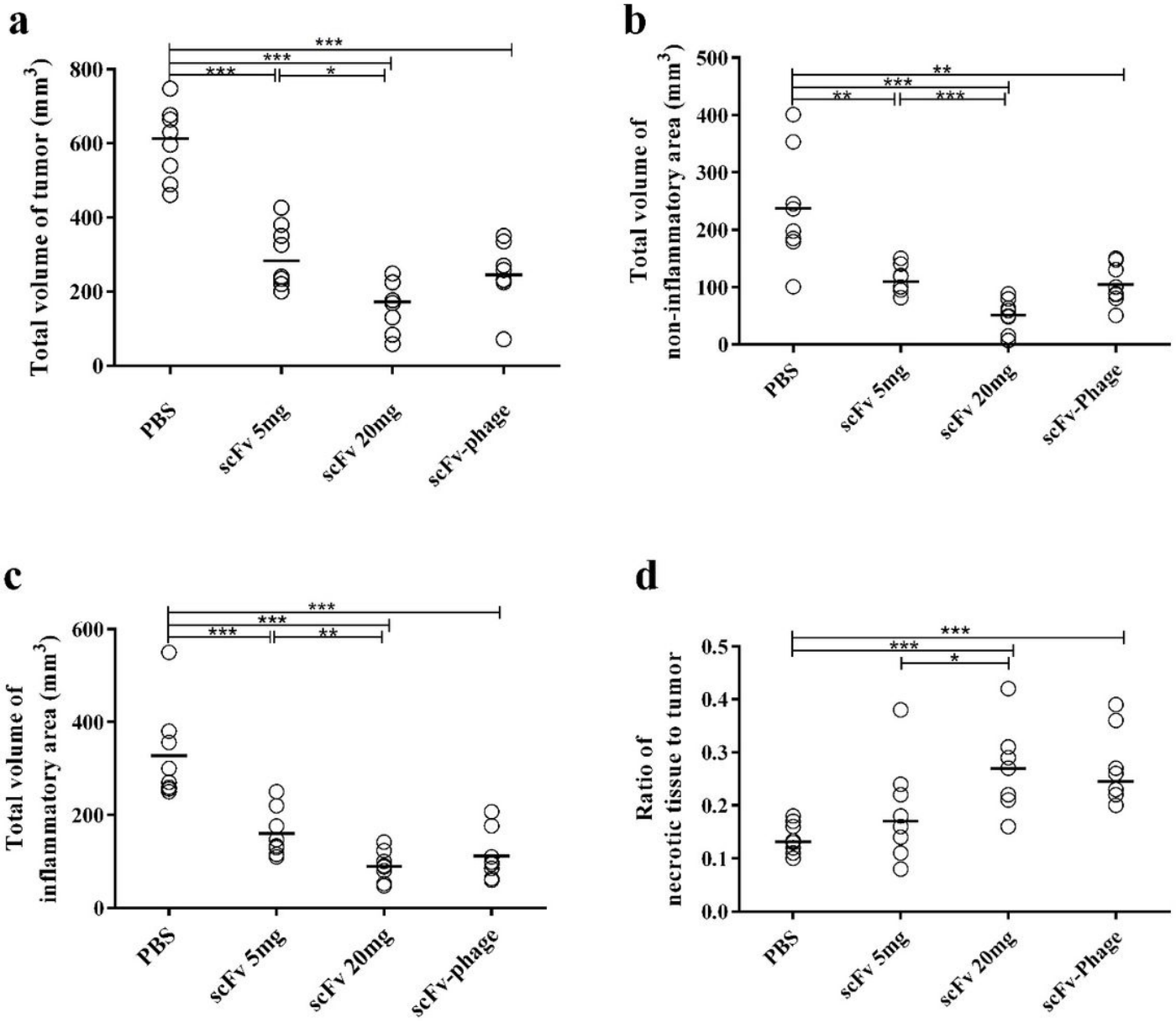

Figure 3

The aligned dot plot representing the quantitative parameters of total volume of the tumor (a), non-inflammatory (b), inflammatory (c) areas, and ratio of the necrotic tissue to the total tumor in the different groups (d). Each dot represents an animal and the horizontal bar is the median of the mentioned parameters in the experimental groups. *: P<0.01, **: P<0.001, ***: P<0.0001

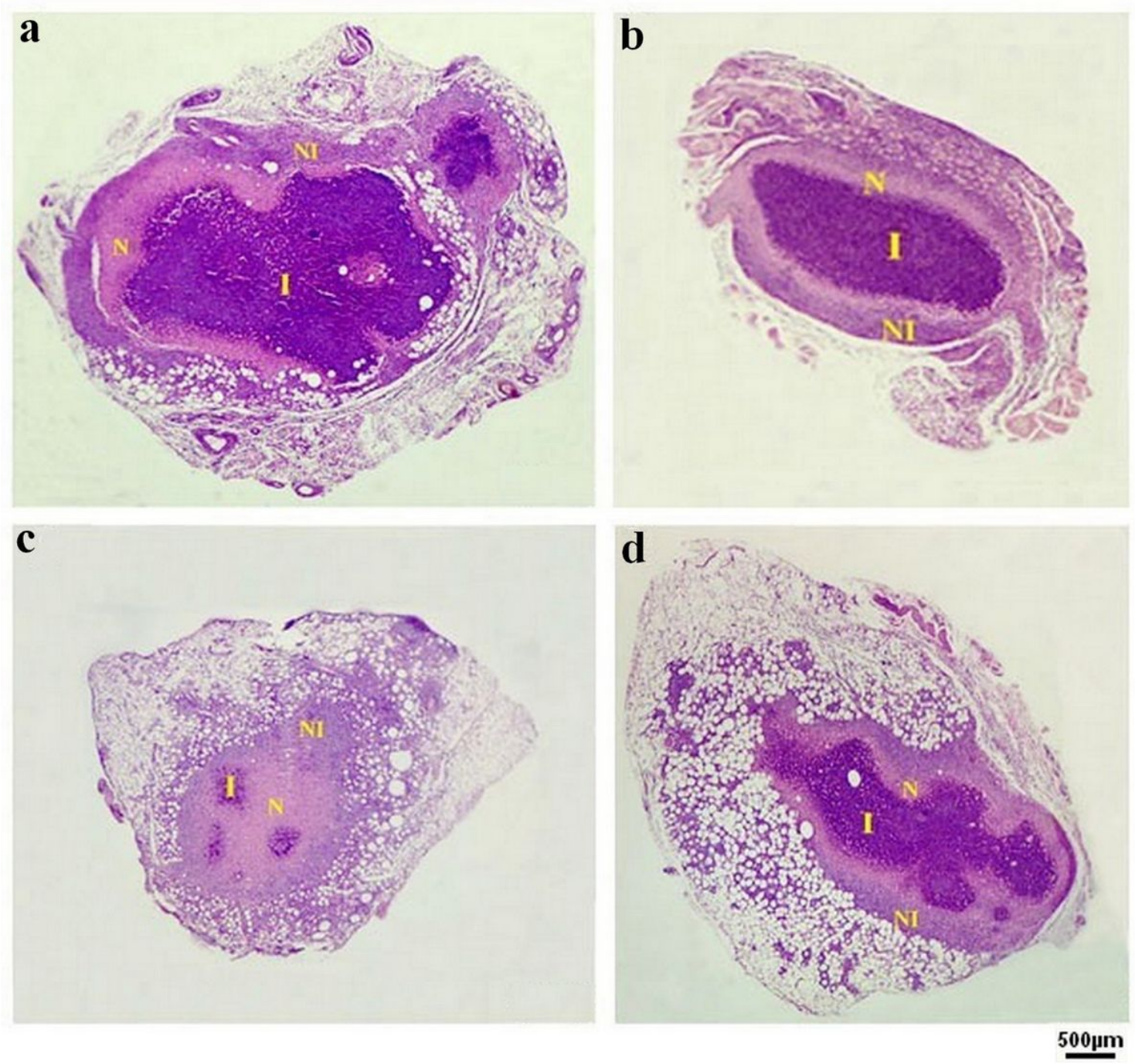

Figure 4

The photomicrographs of the xenograft human breast cancer (SKBR-3) in the experimental mice groups. The mice pretreated with PBS (a), 5mg/kg of scFv cocktail (b), 20mg/kg of scFv cocktail (c), and 1×10^{11} Phage-scFv cocktail (d). *N:* The necrotic parts of the tumor, *I:* The areas of tumor cells crowded with inflammatory cells, *NI:* The areas of non-inflammatory cells of tumor.