



# Cell growth inhibition and apoptosis in breast cancer cells induced by anti-FZD7 scFvs: involvement of bioinformatics-based design of novel epitopes

Neda Zarei<sup>1</sup> · Mehdi Fazeli<sup>1</sup> · Mozafar Mohammadi<sup>2</sup> · Foroogh Nejatollahi<sup>3,4</sup>

Received: 10 October 2017 / Accepted: 23 December 2017 / Published online: 6 February 2018  
© Springer Science+Business Media, LLC, part of Springer Nature 2018

## Abstract

**Background** FZD7 has a critical role as a surface receptor of Wnt/ $\beta$ -catenin signaling in cancer cells. Suppressing Wnt signaling through blocking FZD7 is shown to decrease cell viability, metastasis and invasion. Bioinformatic methods have been a powerful tool in epitope designing studies. Small size, high affinity and human origin of scFv antibodies have provided unique advantages for these recombinant antibodies.

**Methods** Two epitopes from extracellular domain of FZD7 were designed using bioinformatic methods. Specific anti-FZD7 scFvs were selected against these epitopes through panning process. The specificity of the scFvs was assessed by phage ELISA and the ability to bind to FZD7 expressing cell line (MDA-MB-231) was determined by flowcytometry. Antiproliferative and apoptotic effects of the scFvs were evaluated by MTT and Annexin V/PI assays. The effects of selected scFvs on expression level of Survivin, c-Myc and Dvl genes were also evaluated by real-time PCR.

**Results** Results demonstrated selection of two specific scFvs (scFv-I and scFv-II) with frequencies of 35 and 20%. Both antibodies bound to the corresponding peptides and cell surface receptors as shown by phage ELISA and flowcytometry, respectively. The scFvs inhibited cell growth of MDA-MB-231 cells significantly as compared to untreated cells. Growth inhibition of 58.6 and 53.1% were detected for scFv-I and scFv-II, respectively. No significant growth inhibition was detected for SKBR-3 negative control cells. The scFvs induced apoptotic effects in the MDA-MB-231 treated cells after 48 h, which were 81.6 and 74.9% for scFv-I and scFv-II, respectively. Downregulation of Survivin, c-Myc and Dvl genes were also shown after 48h treatment of cells with either of scFvs (59.3–93.8%). ScFv-I showed significant higher antiproliferative and apoptotic effects than scFv-II.

**Conclusions** Bioinformatic methods could effectively select potential epitopes of FZD7 protein and suggest that epitope designing by bioinformatic methods could contribute to the selection of key antigens for cancer immunotherapy. The selected scFvs, especially scFv-I, with high antiproliferative and apoptotic effects could be considered as effective agents for immunotherapy of cancers expressing FZD7 receptor including triple negative breast cancer.

**Keywords** Frizzled 7 · Single chain fragment variable (scFv) · Bioinformatics · Breast cancer · Wnt signaling

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s10549-017-4641-6>) contains supplementary material, which is available to authorized users.

✉ Foroogh Nejatollahi  
nejatollaf@sums.ac.ir

<sup>1</sup> Biotechnology Section, Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

<sup>2</sup> School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>3</sup> Shiraz HIV/AIDS Research Center, Institute of Health, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>4</sup> Recombinant Antibody Laboratory, Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran

## Introduction

Frizzled receptor 7 (FZD7) belong to a 10-member family, all having common structure including a cysteine-rich domain (CRD) and a C-terminal PDZ domain, respectively, exposed in the extracellular and intracellular parts, a seven pass transmembrane domain, and an N-terminal signal peptide [1–4]. Interaction of FZD7 with Wnt ligand occurs through CRD which is then transferred to disheveled (Dvl) in the cytoplasm to activate Wnt signaling. Several studies have shown the critical role of FZD7 as a surface receptor of Wnt/ $\beta$ -catenin signaling in cancer cells. Binding of Wnt ligands to FZD receptors and LRP co-receptor initiate a cascade of signals involved in cell fate and proliferation. It protects  $\beta$ -catenin from phosphorylation and stabilizes its translocation to the nucleus and binding with T cell factor/lymphoid enhancer factor, (TCF/LEF) transcription factors, to activate transcription of tissue-specific oncogenes such as Survivin, c-Myc, and cyclin D1 [2, 5]. Target genes of Wnt signaling are known to be associated with proliferation, invasion and angiogenesis of tumor cells, as well as their resistance to apoptosis. FZD7 has limited expression in normal tissues while is overexpressed on the surface of cancer cells [5, 6], resulting in the over-activation of Wnt target genes and, therefore, initiation and growth of many cancers including triple-negative breast cancer (TNBC) [2, 6–8]. Down regulation of Wnt pathway by targeting FZD7 has led to decrease in vitro cell viability, metastatic and invasion activities [9, 10] and has been recommended as an effective strategy for cancer immunotherapy.

A new approach in suppressing signaling cascades is by applying small molecules to interfere with the interaction of ligand-receptor. Identifying functional epitopes of a receptor, however, is a key step in the effectiveness of receptor targeting. In this field, bioinformatics has provided a powerful and reliable tool in current epitope-designing studies by simulation of spatial structure and molecular prediction of epitope-binding activity [11]. This method has been frequently used in recent vaccine-development studies for scanning protein antigens to select candidate epitopes and has considerably reduced the time and cost of researches [12]. Production of recombinant antibodies, which are of particular interest to both basic and clinical biomedical researchers, are also integrated with bioinformatics improvements. Single chain Fragment variable (scFv) antibodies contain variable regions of heavy (VH) and light (VL) chains connecting together by a short peptide linker [13]. They form necessary parts participating in the recognition of antigens. A variety of advantages including human origin, small size, high affinity and specificity of scFvs have made these antibodies

ideal agents for targeted therapy. Several scFv antibodies are currently in clinical trial stages [14], showing their potential and effectiveness properties. Anti-FZD7 scFv has been selected and their inhibitory effects on breast and colorectal cancer cell lines has been shown [15, 16]. However, the role of epitope designing, its location on the target cell and bioinformatics data in the effectiveness of receptor targeting are not shown.

In the present study, two immunodominant epitopes from the extracellular domain of FZD7 were selected using bioinformatic methods. The epitopes were used for selection of human single chain fragment variable antibodies from a naïve phage library. Antitumor properties of the selected scFvs were investigated on TNBC cell lines.

## Materials and methods

### Selection of antigenic peptides by bioinformatics methods

Amino acids regarding CRD domain of FZD7 receptor were retrieved from UniProt server [17]. Tertiary structure of CRD domain was created by employing homology modeling using modeler 9.11 software [18]. To select the antigenic epitopes on CRD domain, EpiC, antigenicity and functional prediction server [19] was used. Chimera software [20] was applied for all visualizations.

### Enrichment of anti-FZD7 scFv

A naïve phage antibody library of scFvs was developed as described previously [21, 22]. The library was phage-rescued using M13KO7 helper phage (New England Biolabs Inc., USA) and four rounds of panning process were performed on it to enrich for anti-FZD7 scFvs. Briefly, peptides were coated on immunotubes (Nunc, Denmark) for 16 h at 4 °C. After blocking with skimmed milk 2%, the phage-rescued supernatant ( $10^{11}$  pfu/mL) diluted with blocking solution (1:1) was added and incubated for 1 h at RT. Elution was done using TG1 *E. coli*. Four rounds of panning were followed and colony PCR and DNA fingerprinting with Mva-I restriction enzyme were done to reveal the common patterns. For each peptide antigen, an individual clone with the most frequent pattern was selected for further evaluation.

### Phage ELISA

10  $\mu$ g/mL of peptide was coated on MaxiSorp ELISA plates at 4 °C. Phage-antibodies, anti-FZD7 phages: scFv-I, scFv-II or scFv-III, were added to the plate separately and incubated for 2 h. In a parallel set of experiment, an unrelated peptide, no peptide well, M13KO7 helper phages and an unrelated scFv

were also used as negative controls. After washing, rabbit anti-fd bacteriophage antibody (Sigma, UK) was added and incubated for 1.5 h followed by 1 h incubation with HRP-conjugated anti-rabbit antibody. Bound phages were detected with TMB (3,3',5,5'-tetramethylbenzidine) peroxidase. The test was performed in triplicate and absorbance was detected at 405 nm after 10 min.

### Cell culture

FZD7 high-expressing, MDA-MB-231, and low-expressing, SKBR-3, breast cancer cell lines were purchased from IPI (Iranian Pasteur Institute). The cell lines were grown in RPMI-1640 medium (Gibco™, ThermoFisher Science) supplemented with 10% fetal bovine serum (Biosera, UK) containing 100 µg/mL penicillin and 100 µg/mL streptomycin. Cells were incubated in the humidified incubator with 5% CO<sub>2</sub> at 37 °C.

### Flow cytometry

Cell surface binding capacities of selected scFvs were determined by flow cytometry analysis in comparison with a commercial anti-FZD7 antibody (Abcam, UK). Briefly,  $2 \times 10^5$  cells (MDA-MB-231 or SKBR-3) 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. Cells were harvested with 25% trypsin-0.02% EDTA and washed with ice-cold PBS. Rabbit anti-fd bacteriophage antibody (Sigma-Aldrich, Germany) was added and incubated for 30 min, followed by 30 min incubation with PE-conjugated anti-rabbit antibody. After washing, the amount of bound phage was measured by a FACS Calibur (BD Biosciences, USA) (excitation/emission setting for PE-conjugated antibody was 488/575). The data analysis was performed by FlowJo 7.6 software.

### Cell proliferation assay

Breast cancer cells (MDA-MB-231 or SKBR-3) ( $10 \times 10^4$ ) were seeded per 96-well culture plate well (Nunc, Denmark) and treated with different concentrations of selected phages for 24, 48 and 72 h performed in triplicate. 100 µL of 0.5 mg/mL MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added followed by 4 h incubation at 37 °C. Subsequently, the supernatants were gently removed and the purple crystal products were solubilized by incubating in DMSO. Colorimetric measurement was performed at 570 nm using ELISA reader (BP-800, Biohit, USA). The percentage of cell growth was calculated using the absorbance values of treated and untreated cells as follows:

$$\text{Percentage of cell growth} = \frac{\text{OD}_{570} \text{ treated}}{\text{OD}_{570} \text{ untreated}} \times 100.$$

### Annexin-V assay

Breast cancer cells (MDA-MB-231) ( $4 \times 10^5$ ) were seeded per 6-well culture plate well (Nunc, Denmark) and treated with anti-FZD7-scFvs (2000 scFvs/cell) separately for 24 and 48 h. Harvested cells were stained with Annexin V-FITC (Roche Applied Science, Germany) for 20 min. Cells were then washed and stained with propidium iodide (PI) for 10 min. The untreated cells were gated and analysis was done by FACS Calibur (Becton, USA) (excitation/emission setting for annexin-V-FITC and PI were 488/530 and 535/617, respectively). Data analysis were performed by FlowJo 7.6 software.

### Real-time PCR

Following 48 h treatment of MDA-MB-231 cells with scFv-I and scFv-II separately, real time PCR was performed. Total RNA was isolated using Biozol (Invitrogen) and reverse-transcribed into single-stranded cDNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Quantitative real time PCR was performed in triplicate on an Applied Biosystem 7500 Real-Time PCR System using SYBR Green. GAPDH housekeeping gene transcripts were used as reference for the expression levels of all target genes. The expression level of Survivin, c-Myc and disheveled (Dvl) genes were determined using specific primers. The mean value for expression of each gene was calculated using  $2^{-\Delta\Delta C_T}$  method. Quantitative PCR parameters and the sequence of primers are presented in supplementary information.

### Statistical analysis

Data were analyzed using Mann–Whitney *U* test to compare the means of percentages of cell growth between antibody-treated and untreated cells and maximum effects (antiproliferative and apoptotic) of both anti-FZD7 antibodies. The reduction of gene expression in real time PCR were also compared using Mann–Whitney *U* test. All data are presented as the mean  $\pm$  standard deviation. *P* value < 0.05 was considered statistically significant.

## Results

### Selection of antigenic peptides

The tertiary model for CRD domain of FZD7 receptor was made (Fig. 1A) based on the available crystal structure of FZD8-CRD domain (PDB code 1IJY [23]) as template,

which was retrieved from the protein data bank (RCSB) [24]. Totally, 121 amino acids related to CRD region were submitted in EpiC server and based on the several properties such as solvent accessibility, hydrophilicity, antigenicity, and flexibility of epitopes, two 15 amino acid sequences (peptide I: DAGLEVHQFYPLVKV and peptide II: PVCTVLDQAIP-PCRS) were selected for the experimental studies.

Figure 1B shows the locations of peptides I and II on FZD7 receptor versus Wnt ligand binding sites, which represents a close contact between the sites of peptides and Wnt binding.

### Isolation of anti-FZD7 scFv antibodies

Fingerprinting patterns of individual library clones and clones obtained after four rounds of panning against FZD7 peptides are shown in Fig. 2. Different fingerprinting patterns of the library represented its diversity while one common pattern with 35% frequency and two common patterns each with 20% frequency were obtained against peptides I and II, respectively.

### Determination of specific binding capacity of scFv to FZD7 peptide by phage ELISA

The reactivity of isolated scFv clones (scFv-I, -II and -III) to the corresponding peptides (peptides I and II) were determined by phage ELISA. The OD detected for reaction of scFv-I, -II and -III with related peptides were 1.7, 1.8 and 1.8, respectively; while the reactivity of scFv-I, -II and -III. In the no peptide wells were 0.5, 0.7 and 1.0, respectively (Fig. 3).

### Flow cytometry analysis for cell binding property of anti-FZD7 scFvs

Flow cytometry assay revealed the cell surface binding of both anti-FZD7-commercial Ab and selected scFvs on MDA-MB-231 and SKBR-3 breast cancer cell line. No significant shift in fluorescent intensity was detected for SKBR-3 cells. The commercial Ab, scFv-I and -II bound to 43.7, 54.4 and 57% of MDA-MB-231 cells and 9.2, 6.4 and 8.3% of SKBR-3 cells, respectively (Fig. 4).

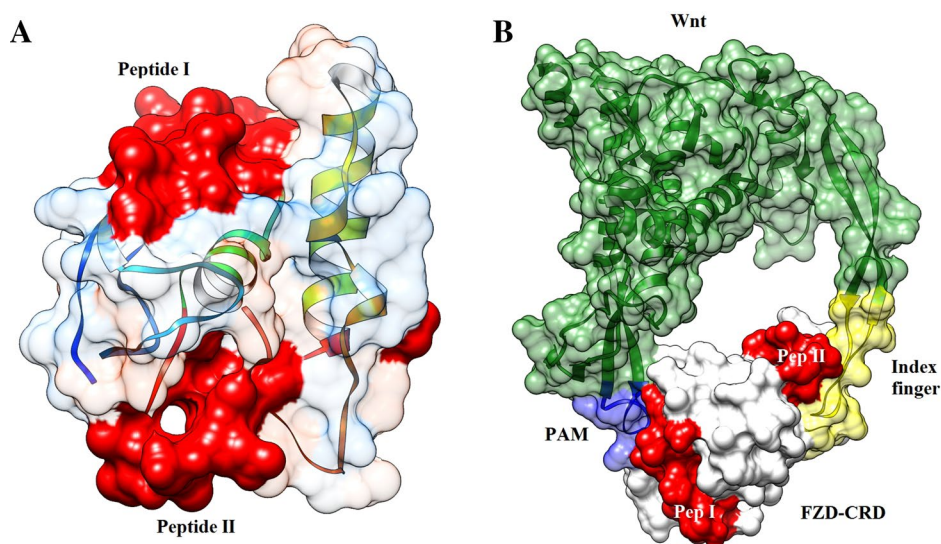
### Anti-proliferative effects of anti-FZD7 scFvs

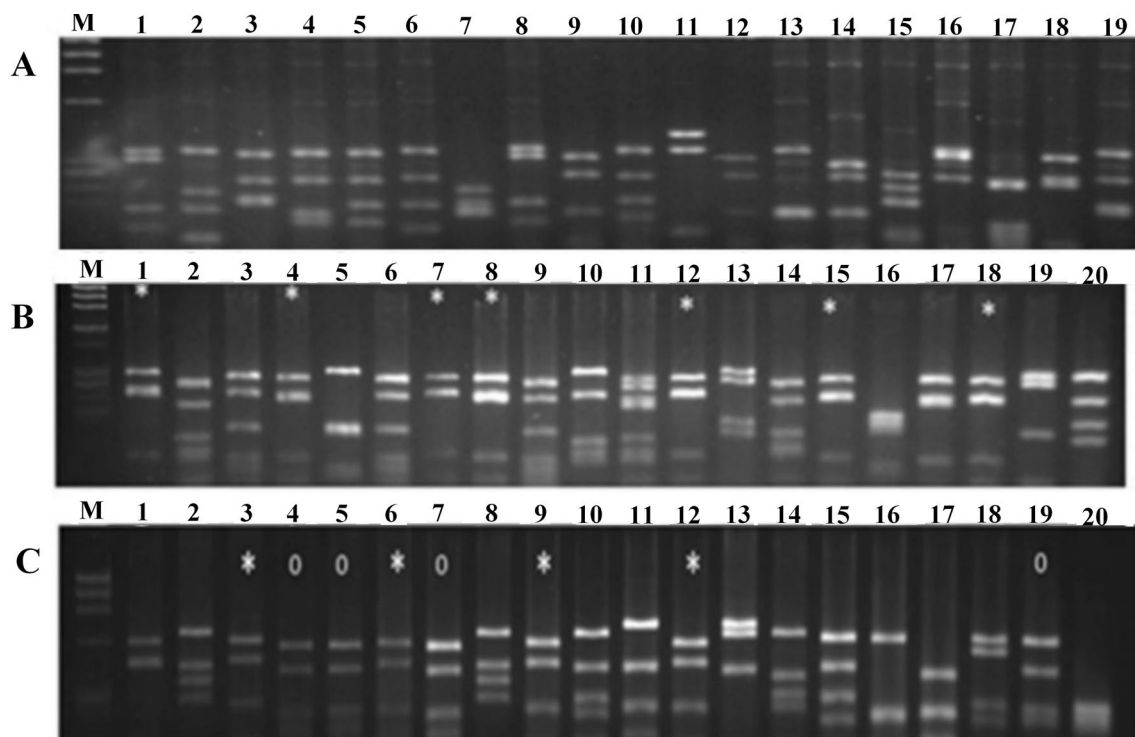
The MDA-MB-231 cell growth was decreased dose-dependently after treatment with both scFv-I and scFv-II. The growth inhibition of 31.8, 45.5 and 58.6% for cells treated with scFv-I (4000 phages/cell) and 33, 44.2 and 53.1% for cells treated with scFv-II (4000 phages/cell) were observed after 24, 48 and 72 h, respectively. ScFv-I inhibited the cell growth of MDA-MB-231 cells significantly higher than scFv-II ( $P < 0.05$ ). No significant inhibitory effect of either of scFvs were observed for SKBR-3 cells ( $P < 0.05$ ) after 24, 48 and 72 h treatments compared to untreated cells; 19.2, 22.4 and 25.3% for scFv-I and 17.3, 21.7 and 24.1% for scFv-II (Fig. 5).

### Apoptotic effect of anti-FZD7 scFvs

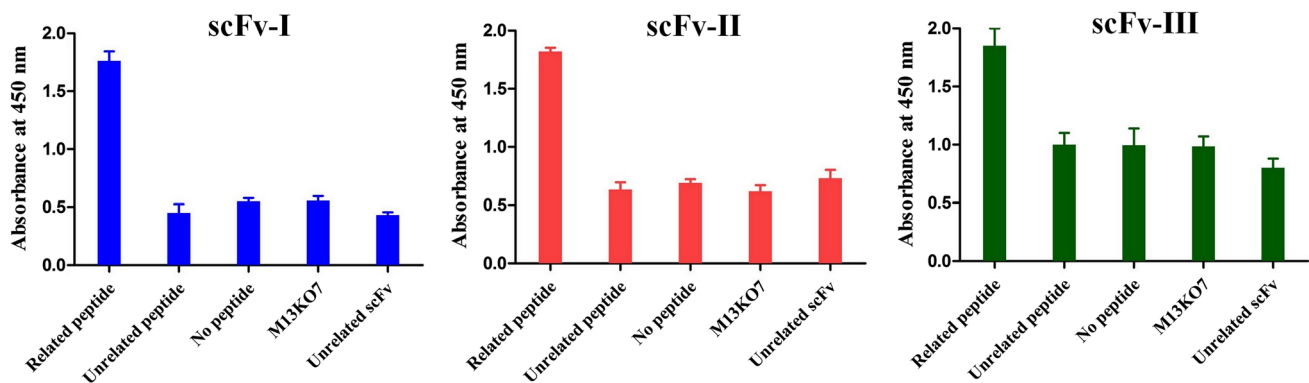
After 24 h treatment with scFv-I and scFv-II, MDA-MB-231 cells showed 48.7 and 37.3% apoptotic cell death which increased to 81.6 and 74.9%, respectively, after 48 h (Fig. 6). Apoptotic effects detected for scFv-I was significantly higher than scFv-II ( $P < 0.05$ ).

**Fig. 1** Surface style of FZD7-CRD domain with elements of secondary structure. Two selected antigenic peptides, peptide I and II, are shown in red (A). Surface representation of Wnt in complex with FZD-CRD. The extended palmitoleic acid (PAM) group and index finger are shown in blue and yellow colors, respectively (B). Modeller 9.11 comparative modeling software was used to create tertiary structure





**Fig. 2** Mva-I DNA fingerprinting of scFv clones. Fingerprinting patterns of un-panned library (a). Common patterns against peptide I (lanes 1, 4, 7, 8, 12, 15, 18) (b). Peptide II (lanes 3, 6, 9, 12) and (lanes 4, 5, 7, 19) (c). The common patterns are shown by asterisk or superscript 0



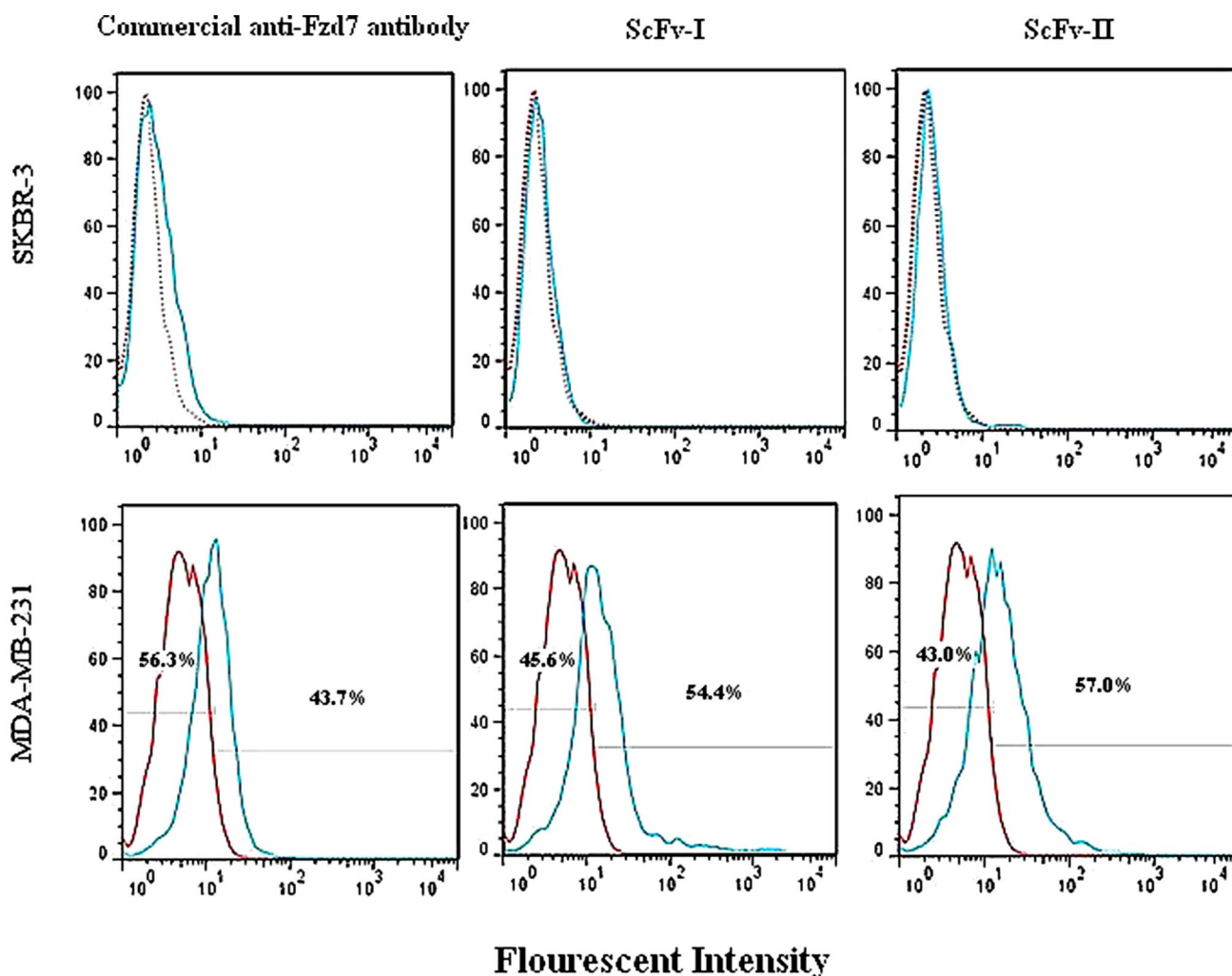
**Fig. 3** Phage ELISA results of selected scFvs against FZD7 peptides. ScFv-I and -II bound to the corresponding peptides higher than no peptide, unrelated peptide, unrelated scFv and M13KO7 wells. There

is not a great (twofold) difference between the reactivity of scFv-III with related peptide and no peptide wells

### Real-time PCR

Significant decrease in the mRNA expression levels of Survivin, c-Myc and Dvl genes in MDA-MB-231 cell line treated with anti-FZD7-scFvs compared to untreated cells were detected ( $P < 0.05$ ). After 48 h treatment of MDA-MB-231 cells with scFv-I and scFv-II, the level

of Survivin gene expression showed 93.8 and 92.5% decrease, respectively (Fig. 7). In addition, 84.7 and 81.8% reduction in c-Myc gene expression level and 75.4 and 59.3% reduction in Dvl gene expression level were found after 48 h treatment with scFv-I and scFv-II, respectively. No significant difference in the gene expressions induced by scFv-I and -II was detected ( $P > 0.05$ ).



**Fig. 4** Flow cytometry binding analysis of selected scFvs on the breast cancer cell line. A shift in fluorescence value for the MDA-MB-231 cells treated with commercial anti-FZD7 Ab, scFv-I and

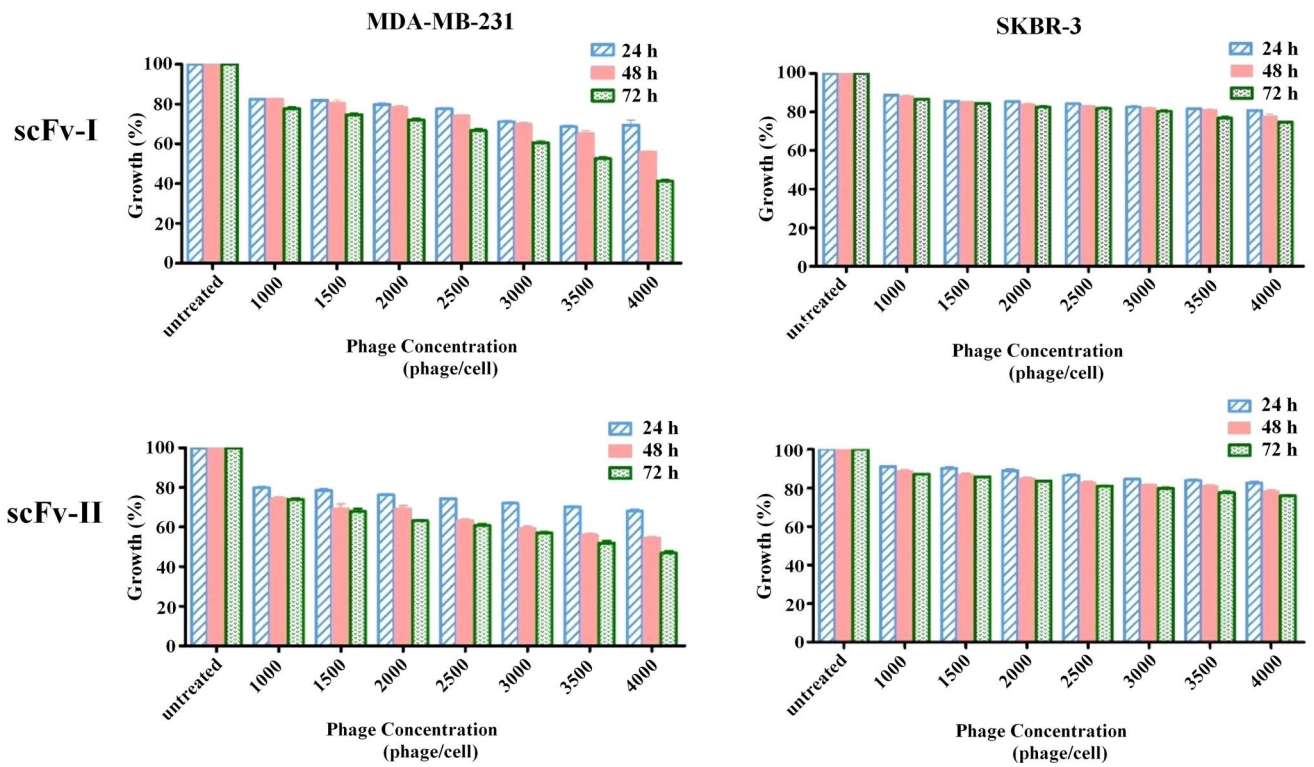
scFv-II compared to treatment with M13KO7 helper phage, isotype control, was detected. No shift in fluorescent intensity was observed in SKBR-3 treated cells. Flow Jo 7.6 was used for data analysis

## Discussion

Targeting Wnt pathway through interruption of ligand-receptor is an efficient therapeutic strategy in cancers over-expressing FZD7. The development of recombinant single chain antibodies along with the progresses in bioinformatic techniques broadens the spectrum of challenging strategies toward targeted therapy of cancers. In this study, two immunodominant epitopes of FZD7 were selected using bioinformatic methods. Homology modeling is commonly used to interpret protein structure and function and to highlight the correlation between the 3D conformation of an antibody and its binding activity [25–27]. There are two domains in FZD cysteine-rich domain which have critical roles in the interaction with Wnt molecules, a palmitoleic acid lipid (PAM) group projecting and ‘index finger’ region. Conservation analysis of these interactions revealed that the majority of

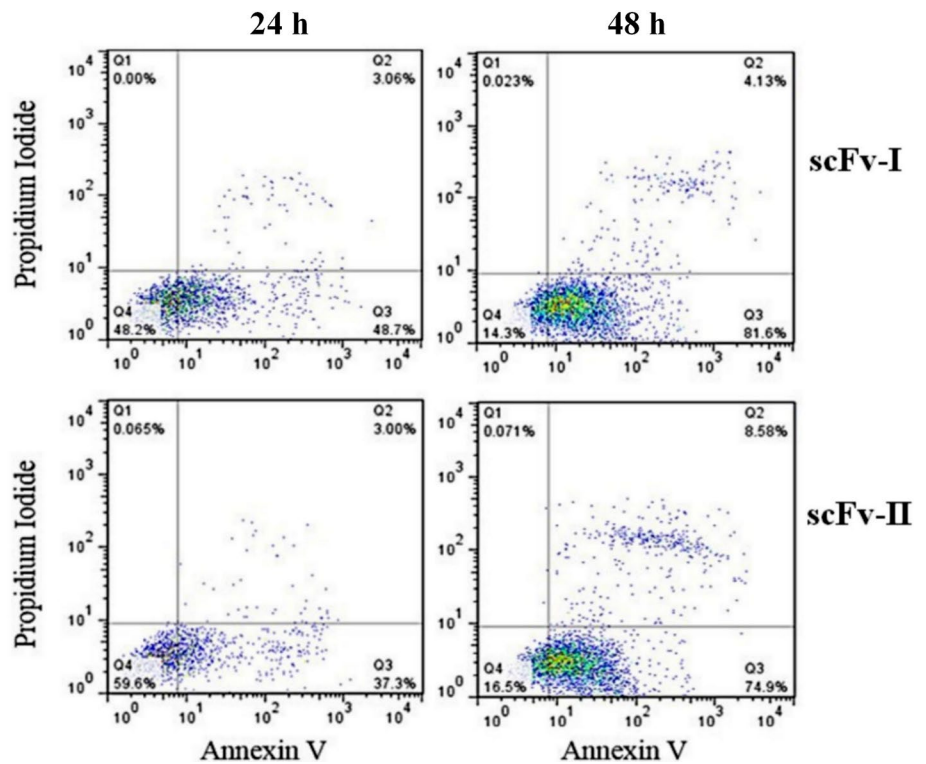
side-chain specific interactions are conserved in all frizzled-CRD molecules [28]. As shown in Fig. 1B, two selected epitopes (peptide I and peptide II) are located very close to the areas where Wnt interacts with FZD-CRD. Therefore, by causing steric hindrance, our scFvs can inhibit the interaction of ligand-receptor and prevent triggering of the tumorigenesis signal cascades into the cells.

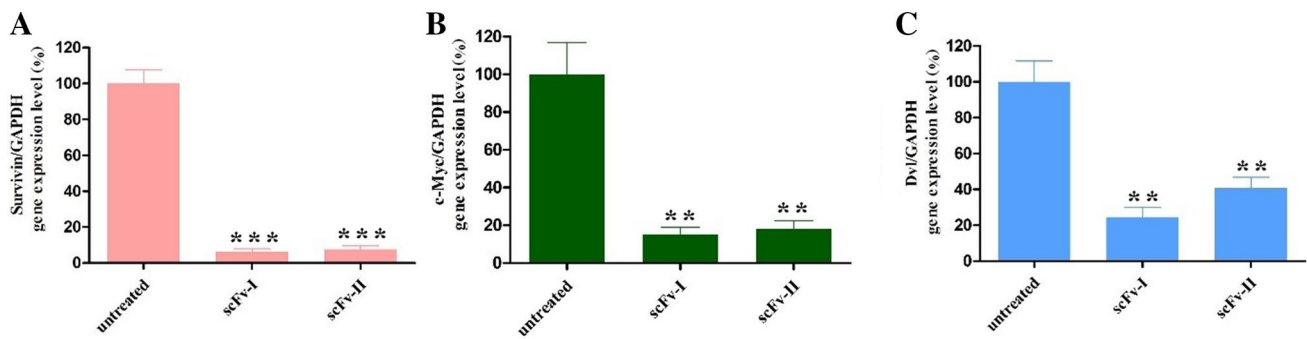
Following four rounds of panning, fingerprinting results showed a common pattern with 35% frequency for scFv-I, and two common patterns for scFv-II, each with 20% frequency. Isolating specific scFv antibodies by panning technique has been applied for different proteins including MUC18, P185 and Nogo receptor-1 [22, 25, 29]. Existence of common patterns confirm the enrichment of these specific clones against peptides [30, 31]. As examined by phage ELISA, two of the isolated scFvs against selected epitopes, scFv-I and scFv-II, specifically bound to the corresponding



**Fig. 5** Cell growth of MDA-MB-231 and SKBR-3 breast cancer cells treated with different concentrations of phage scFv-I and -II compared to untreated cells after 24, 48 and 72 h

**Fig. 6** Annexin V/PI apoptosis assay in MDA-MB-231 cell line treated with scFv-I and -II after 24, 48 and 72 h. The highest apoptosis cell death was detected for scFv-I after 48 h treatment





**Fig. 7** Downregulation of Survivin (a), c-Myc (b) and Dvl (c) genes after 48 h treatment of MDA-MB-231 cells with anti-FZD7 scFv-I and scFv-II antibodies. Untreated cells were used as negative control. The values are compare to untreated cells, the negative control

peptides compared to the control peptide. When the average of OD values is at least two-fold greater than wells without peptide the phage ELISA is regarded as a positive reaction [32]. Low reactivity was observed in negative control wells, while scFv-I and scFv-II showed strong reactions against the corresponding peptides. The reactivity of scFv-III with related peptide in comparison with no peptide as well as unrelated peptide did not show a twofold difference. Thus, scFv-III was not considered in further experiments.

Specificity of binding of selected clones to the FZD7 receptor expressed on the surface of breast cancer cell lines were evaluated by flow cytometry. Yang et al. reported that FZD7 is overexpressed on the surface of MDA-MB-231 cells while it has a low expression level in SKBR-3 cells [6]. As these two cell lines were used for further in vitro analysis, the presence of FZD7 was primarily confirmed by flow cytometry using a commercial anti-FZD7 antibody. Obtained results showed 43.7 and 9.2% binding of commercial anti-FZD7 antibody with MDA-MB-231 and SKBR-3 cell lines, respectively. Selected scFv-I and -II bound to 54.4 and 57% of MDA-MB-231 cancer cells while their bindings to SKBR-3 were only 6.4 and 8.3%. We also evaluated the antiproliferative potential of the obtained anti-FZD7 scFvs as a therapeutic target for breast cancer treatment. The anti-FZD7 phage-antibodies decreased cell growth in MDA-MB-231 cells, dose-dependently compared to untreated cells, while no significant effects were observed on cell growth of SKBR-3 negative cell line. This is in accordance with the expression level of FZD7 receptor on these two cell lines which was demonstrated in flow cytometry analysis. Moreover, comparison of the antiproliferative results of scFv-I and scFv-II on MDA-MB-231 cells demonstrated a significant higher effect of scFv-I ( $P < 0.05$ ). Apoptotic analysis also indicated that 48 h after exposure to selected antibodies the majority of MDA-MB-231 cells enter the early apoptotic phase. Utilization of FZD7 shRNA to suppress Wnt signaling cascade resulted in a similar significant inhibitory effects in cell proliferation and tumor

growth in TNBC cells [6]. When Wnt signaling is activated in cancer cells, through the interaction of ligand-receptor,  $\beta$ -catenin accumulates in the cytoplasm and translocate into the nucleus. Cooperation of  $\beta$ -catenin with transcription factors in the nucleus activates proto-oncogenes such as Survivin and c-Myc [33]. Overexpression of Survivin has been shown in several studies [34–36]. Survivin is related to apoptosis inhibition and chemoresistance of hepatocellular carcinoma (HCC) [36–38]. It induces proliferation of cancer cells by initiating cell cycle, a decrease in the G0/G1 phase and an increase in the S phase [35]. Furthermore, overexpression of c-Myc, a common oncoprotein involved in the pathogenesis of HCC, results in tumor growth [34] and transcription of c-Myc gene is under the control of Wnt signaling [39]. Therefore, it is not surprising that inhibition of Wnt signaling in cancer cells is associated with downregulation of c-Myc. When translated, c-Myc protein binds to enhancer sequence of some target genes, including cyclins, that involves in cell cycle control [3].

Expression level of Survivin and c-Myc mRNA decreased significantly after 48 h treatment with each of the selected scFv antibodies. These results indicated that the antiproliferative and apoptotic effects were transduced through Wnt pathway and are consistent with a previous studies showing that treatment of tumor tissues with anti-Wnt-1 antibody reduced Survivin, cyclin D1 and c-Myc expressions, supporting that over-activation of Wnt signaling occurs through interaction of receptor-ligand [34]. Dvl expression was also downregulated following treatment with selected anti-FZD7 scFvs. Inhibition of Wnt signaling using a monoclonal antibody against Wnt-1 also leads to decrease of Dvl mRNA [40]. Although Dvl gene is not directly controlled by Wnt pathway, it is possible that transcription factors regulating Dvl expression are under the control of Wnt pathway [40].

The antiproliferative and apoptotic effects of scFv-I was significantly higher than scFv-II. However, no significant difference between gene expression reductions induced by these two antibodies was detected. As shown in Fig. 1B,



scFv-I was selected against a peptide designed from an extracellular domain near PAM group, an important domain participating in the interaction of Wnt ligands and FZD receptors. This is confirmed by conservation analysis showing that almost all parts of this sequence is conserved [28]. It is reported that Wnt signaling pathway is deactivated by eliminating PAM moiety [41]. Therefore, scFv-I which is selected against a sequence near this group might interfere with interaction of FZD-Wnt more efficiently than scFv-II.

Modern immunotherapy of cancer relies on direct suppressing of over-activated signaling pathways, most specifically those that are tightly related to uncontrolled proliferation of cells. Bioinformatic techniques has greatly advanced the process of targeting specific sites of signaling molecules through providing a comprehensive insight into the structure of targets [42]. In this study, specific anti-FZD7 scFvs were selected by applying immunodominant epitopes of FZD7 designed by molecular modeling. The results suggest that inhibition of Wnt signaling is an effective strategy to inhibit proliferation and induce apoptosis of cancer cells. Among different approaches that can be used for this purpose, single chain antibodies have many advantages. No HAMA (human antimouse antibody) reaction, deeply penetration in tumor cells and ability for genetic manipulation provides additional effector functions that make these recombinant antibodies attractive for clinical use. The inhibitory effects of the selected antibodies confirm that bioinformatic approach was able to effectively screen potential epitopes of FZD7 protein and suggest that epitope designing by bioinformatic methods could contribute to the selection of key antigens for cancer immunotherapy. The anti-FZD7 scFv-I with high antiproliferative and apoptotic effects offer a new promising treatment strategy for cancers overexpressing FZD7 receptor including triple negative breast cancer.

**Acknowledgements** This study was financially supported by Shiraz University (Grant No 94GCU4M1286) and Shiraz University of Medical Sciences (Grant No 9667). The present article was extracted from the thesis written by Neda Zarei.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

### References

- King TD, Zhang W, Suto MJ, Li Y (2012) Frizzled7 as an emerging target for cancer therapy. *Cell Signal* 24(4):846–851
- Anastas JN, Moon RT (2013) WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* 13(1):11–26
- Sherwood V (2015) WNT signaling: an emerging mediator of cancer cell metabolism? *Mol Cell Biol* 35(1):2–10
- Peifer M, Polakis P (2000) Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. *Science* 287(5458):1606–1609
- Yang L, Kim CC, Yen Y (2011) FZD7 in triple negative breast cancer cells. INTECH Open Access Publisher, Rijeka
- Yang L, Wu X, Wang Y, Zhang K, Wu J, Yuan Y et al (2011) FZD7 has a critical role in cell proliferation in triple negative breast cancer. *Oncogene* 30(43):4437–4446
- Howe LR, Brown AM (2004) Wnt signaling and breast cancer. *Cancer Biol Ther* 3(1):36–41
- Benhaj K, Akcali KC, Ozturk M (2006) Redundant expression of canonical Wnt ligands in human breast cancer cell lines. *Oncol Rep* 15(3):701–707
- Deng B, Zhang S, Miao Y, Zhang Y, Wen F, Guo K (2015) Down-regulation of Frizzled-7 expression inhibits migration, invasion, and epithelial–mesenchymal transition of cervical cancer cell lines. *Med Oncol* 32(4):1–9
- Ueno K, Hazama S, Mitomori S, Nishioka M, Suehiro Y, Hirata H et al (2009) Down-regulation of frizzled-7 expression decreases survival, invasion and metastatic capabilities of colon cancer cells. *Br J Cancer* 101(8):1374–1381
- Shang G-G, Zhang J-H, Lü Y-G, Yun J (2011) Bioinformatics-led design of single-chain antibody molecules targeting DNA sequences for retinoblastoma. *Int J Ophthalmol* 4(1):8
- Sirskyj D, Diaz-Mitoma F, Golshani A, Kumar A, Azizi A (2011) Innovative bioinformatic approaches for developing peptide-based vaccines against hypervariable viruses. *Immunol Cell Biol* 89(1):81–89
- Mohammadi M, Nejatollahi F, Sakhteman A, Zarei N (2016) In-silico analysis of three different tag polypeptides with dual roles in scFv antibodies. *J Theor Biol* 402:100–106
- Thie H, Meyer T, Schirrmann T, Hust M, Dubel S (2008) Phage display derived therapeutic antibodies. *Curr Pharm Biotechnol* 9(6):439–446
- Fazeli M, Zarei N, Moazen B, Nejatollahi F (2017) Anti-proliferative effects of human anti-FZD7 single chain antibodies on colorectal cancer cells. *Shiraz E-Med J* 18(3):e59936
- Nickho H, Younesi V, Aghebati-Maleki L, Motallebnezhad M, Majidi Zolbanin J, Movassagh Pour A et al (2017) Developing and characterization of single chain variable fragment (scFv) antibody against frizzled 7 (Fzd7) receptor. *Bioengineered* 8(5):501–510
- UniProt Consortium (2017) UniProt: the universal protein knowledgebase. *Nucleic Acids Res* 45(D1):D158–D169
- Fiser A, Sali A (2003) Modeller: generation and refinement of homology-based protein structure models. *Methods Enzymol* 374:461–491
- Haslam NJ, Gibson TJ (2010) EpiC: an open resource for exploring epitopes to aid antibody-based experiments. *J Proteome Res* 9(7):3759–3763
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC et al (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* 25(13):1605–1612
- Nejatollahi F, Silakhori S, Moazen B (2014) Isolation and evaluation of specific human recombinant antibodies from a phage display library against HER3 cancer signaling antigen. *Middle East J Cancer* 5(3):137–144
- Nejatollahi F, Malek-Hosseini Z, Mehrabani D (2008) Development of single chain antibodies to P185 tumor antigen. *Iran Red Crescent Med J* 2008(4):298–302
- Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J, Leahy DJ (2001) Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. *Nature* 412(6842):86–90

24. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H et al (2000) The protein data bank. *Nucleic Acids Res* 28(1):235–242
25. Mohammadi M, Nejatollahi F, Ghasemi Y, Faraji SN (2016) Antimetastatic and anti-invasion effects of a specific anti-MUC18 scFv antibody on breast cancer cells. *Appl Biochem Biotechnol* 181:379–390
26. Barker N, Clevers H (2006) Mining the Wnt pathway for cancer therapeutics. *Nat Rev Drug Discovery* 5(12):997–1014
27. Le Gall F, Reusch U, Little M, Kipriyanov SM (2004) Effect of linker sequences between the antibody variable domains on the formation, stability and biological activity of a bispecific tandem diabody. *Protein Eng Des Sel* 17(4):357–366
28. Janda CY, Waghray D, Levin AM, Thomas C, Garcia KC (2012) Structural basis of Wnt recognition by Frizzled. *Science* 337(6090):59–64
29. Ehsaei B, Nejatollahi F, Mohammadi M (2017) Specific single chain antibodies against a neuronal growth inhibitor receptor, nogo receptor 1: promising new antibodies for the immunotherapy of multiple sclerosis. *Shiraz E-Med J* 18(1):e45358
30. Sotelo PH, Collazo N, Zúñiga R, Gutiérrez-González M, Catalán D, Ribeiro CH et al (eds) (2012) An efficient method for variable region assembly in the construction of scFv phage display libraries using independent strand amplification. Taylor & Francis, Abingdon
31. Bagheri V, Nejatollahi F, Esmaeili SA, Momtazi AA, Motamedifar M, Sahebkar A (2017) Neutralizing human recombinant antibodies against herpes simplex virus type 1 glycoproteins B from a phage-displayed scFv antibody library. *Life Sci* 169:1–5
32. Thathaisong U, Maneewatch S, Kulkeaw K, Thueng-In K, Pongpair O, Srimanote P et al (2008) Human monoclonal single chain anti-bodies (HuScFv) that bind to the polymerase proteins of influenza A virus. *Asian Pac J Allergy Immunol* 26(1):23
33. Polakis P (2012) Wnt signaling in cancer. *Cold Spring Harbor Perspect Biol* 4(5):a008052
34. Wei W, Chua M-S, Grepper S, So SK (2009) Blockade of Wnt-1 signaling leads to anti-tumor effects in hepatocellular carcinoma cells. *Mol Cancer* 8(1):76
35. Ito T, Shiraki K, Sugimoto K, Yamanaka T, Fujikawa K, Ito M et al (2000) Survivin promotes cell proliferation in human hepatocellular carcinoma. *Hepatology* 31(5):1080–1085
36. Altieri DC (2008) Survivin, cancer networks and pathway-directed drug discovery. *Nat Rev Cancer* 8(1):61–70
37. Wei W, Chua M-S, Grepper S, So SK (2011) Soluble Frizzled-7 receptor inhibits Wnt signaling and sensitizes hepatocellular carcinoma cells towards doxorubicin. *Mol Cancer* 10(1):1
38. Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC et al (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 396(6711):580–584
39. He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, Da Costa LT et al (1998) Identification of c-MYC as a target of the APC pathway. *Science* 281(5382):1509–1512
40. He B, You L, Uematsu K, Xu Z, Lee AY, Matsangou M et al (2004) A monoclonal antibody against Wnt-1 induces apoptosis in human cancer cells. *Neoplasia* 6(1):7–14
41. Zhang X, Cheong S-M, Amado NG, Reis AH, MacDonald BT, Zebisch M et al (2015) Notum is required for neural and head induction via Wnt deacylation, oxidation, and inactivation. *Dev Cell* 32(6):719–730
42. Mohammadi M, Nejatollahi F (2014) 3D structural modeling of neutralizing scFv against glycoprotein-D of HSV-1 and evaluation of antigen–antibody interactions by bioinformatic methods. *Int J Pharm Biol Sci* 5(4):835–847