

Production and Evaluation of Specific Single-Chain Antibodies against CTLA-4 for Cancer-Targeted Therapy

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Abstract

Background: Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) molecules are expressed on T-cells and inhibit their function by inhibiting activation of subsequent T-cell molecular pathways. Blocking of CTLA-4 inhibits the growth of malignant tumor cells. Anti-CTLA-4 monoclonal antibodies activate the immune system against cancer. Due to several advantages of single-chain antibodies (scFvs) compared to monoclonal antibodies in cancer immunotherapy, specific anti-CTLA-4 scFvs (single-chain variable fragment) were selected in this study.

Methods: A phage antibody display library of scFvs was analyzed and a panning process was performed against an immunodominant epitope of CTLA-4. PCR and DNA fingerprinting were used to differentiate the specific clones. The specificity of the selected clones was investigated by phage ELISA (Enzyme-linked immunosorbent assay).

Results: Two specific clones with frequencies of 35 and 20% were identified. The clones reacted with the corresponding epitope on ELISA, while no reactivity was observed with an unrelated peptide, M13KO7 helper phage, unrelated scFvs, or no peptide as negative controls.

Conclusions: Targeted therapy against cancer markers is an ideal treatment strategy. Specific human anti-CTLA-4scFvs were selected in this study. These scFvs bound the related epitope. These antibodies have the potential to be used for targeted therapy, where the blocking of CTLA4 receptor is needed. The study suggests further evaluation of the selected scFvs to reveal the effects of the selected antibodies.

Keywords: Cancer immunotherapy, CTLA-4, ScFv antibodies

Introduction

Activation of T-cells for production of immune responses requires two signals from antigen-presenting cells. One signal comes from the major histocompatibility complex (MHC) combined with the antigen, and the other from CD80 (B7.1) or CD86 (B7.2) molecules. T-cell co-stimulation is widely investigated in order to manipulate T-cell reactivity in autoimmune diseases, transplantation, and cancers (1). Activation of T-cells by antigen presenting cells requires co-stimulation between CD28 on the T-cells and B7.1 or B7.2 on the antigen-presenting cells (1). Cytotoxic T lymphocyte-associated antigen 4

(CTLA-4), a second counter receptor for B7.1 and B7.2 on the T-cell surface, binds B7.1 and B7.2 and inhibits T-cell functions by inhibiting activation of subsequent T-cell molecular pathways (2). CTLA-4 has higher affinities for B7.1 and B7.2 than CD28 has, and increases the threshold of signals needed for T-cell activation. T-cell function is decreased by these inhibitory signals from CTLA-4. Blocking CTLA-4 with anti-CTLA-4 antibodies can up-regulate of T-cell function (3). Investigations on CTLA-4-targeted therapy have shown promising strategies for treatment of cancers including melanoma, prostate cancer, renal

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cell carcinoma, non-Hodgkin's lymphoma, colorectal carcinoma, non-small cell lung carcinoma, and breast cancer (4).

Application of monoclonal antibodies against the CTLA-4 antigen is a novel form of cancer immunotherapy (5). Ipilimumab, a human monoclonal antibody against CTLA-4, binds to this marker and blocks the interaction of CTLA-4 with its ligands, B7.1 and B7.2. Blockade of CTLA-4 has been shown to increase T-cell activation and proliferation and has been used to treat late-stage melanoma (6, 7). Abatacept, a fusion protein made from the Fc portion of IgG1 and the extracellular domain of CTLA-4, acts as a selective co-stimulatory modulator and inhibits T-cell activation by binding to B7.1 and B7.2, inhibiting its interaction with CD28 (8). This interaction provides a co-stimulatory signal necessary for T-cell activation (9). Tremelimumab, another humanized monoclonal antibody against CTLA-4, has blocking activity and activates T-cells (10), and is used to treat patients with locally advanced and metastatic melanomas. Treatment with this monoclonal antibody has shown durable objective tumor regression (11).

Despite advantages of monoclonal antibodies, some important problems have been reported; these include high production cost, low tissue penetration, and the human anti-mouse antibody response (HAMA response). Although the synthesized antibodies are humanized, the HAMA response still occurs against the non-human parts of these antibodies (12, 13). Antibody engineering has provided production of small and effective antibodies for cancer immunotherapy. Single-chain antibodies (scFvs), which are composed of variable regions of heavy (VH) and light (VL) chains, are joined by a flexible peptide linker and provide relatively rapid tissue penetration in target tissues when used in high concentrations (14-17). Other benefits of scFvs include high specificities and affinities, low immunogenicities, ease of production, and manipulation possibilities (18-21). Various studies show the effector function of scFvs against the extracellular domain of fibroblast growth factor receptor 3 (FGFR3) (22, 23).

FGFR3 is overexpressed in early stages of bladder cancer and inhibits bladder carcinoma cell line proliferation (23). Inhibition of tumor

angiogenesis by scFvs against vascular endothelial growth factor (anti-VEGF scFvs) has been shown both in vitro and in vivo (24, 25). In this study, specific scFvs against CTLA-4 were selected using a phage display technique, and the specificities of the selected scFvs were evaluated by ELISA.

Materials and Methods

Phage Rescue

An scFv phage antibody library was produced, as described previously (26, 27). The *Escherichia coli* (*E. coli*) -containing phagemid was cultured on 2TYG agar/ampicillin (tryptone, yeast extract, glucose, agar, and ampicillin) (Merck, Germany) plates overnight at 30 °C. The cells were scraped and incubated in 2TYG broth at 37 °C for 1 hr. M13KO7 (kanamycin resistant helper phage) was added and the cells were incubated with shaking at 37 °C for 30 minutes. The cells were centrifuged at 3500 rpm for 20 min. The bacterial pellet was transferred to 2TY broth containing ampicillin and kanamycin and incubated with shaking at 30 °C overnight. The supernatant of the centrifuged culture was passed through 0.2 µm filters and stored at 4 °C.

Panning process

Polystyrene immunotubes were coated with the peptide MHVAQPAVVLA (28) (Nunc, Denmark) and incubated at 4 °C overnight. The tubes were blocked with 2% skimmed milk and incubated at 37 °C for 2 hrs. The tubes were washed four times with phosphate-buffered saline (PBS)/Tween20 and four times with PBS. The phage supernatant was added to the tubes and incubated at room temperature for 1 hr. Logarithmic phase *E. coli* cells were added, incubated at 37 °C for 1 hr, and then centrifuged at 3500 rpm. The bacterial pellet was plated on 2TY agarose/ampicillin plates and incubated at 30 °C overnight. Four rounds of panning were performed to select epitope-specific scFv antibodies.

DNA Fingerprinting of the Selected Clones

The inserts of the selected clones were amplified by PCR. To show the fingerprinting patterns, 17 µl of the PCR products were digested with Mva-I (Roche Applied Science, Germany) and electrophoresed in a 2% agarose gel.

Phage ELISA

Wells of a polystyrene plate were coated with the peptide and incubated overnight at 4 °C. After washing with PBS, wells were blocked with 2% skimmed milk and incubated at 37 °C for 2 hrs. Phage rescue supernatant containing the appropriate scFv, diluted 1:1 with blocking solution, was added to each well and incubated at room temperature for 2 hrs. To remove unbound phage, the wells were washed three times with PBS/Tween20, and three times with PBS. Anti-Fd bacteriophage (Sigma, Germany), an antibody produced in rabbit which is used in ELISA for antiphage immunohistochemistry, was added to each well and incubated for 1 hr. After washing, horseradish peroxidase (HRP) -conjugated anti-rabbit antibody (Sigma, Germany) was added and incubated at room temperature for 1.5 hrs. The wells were washed and citrate buffer containing azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Sigma-Aldrich, Germany) and H₂O₂ were added. Phage ELISA was done for three times and the absorbance was read at 405 nm on an ELISA reader. The wells without peptide, with unrelated

peptide (prostate stem cell antigen peptide), unrelated scFv (scFv to HER2), and M13KO7 helper phage, were included as negative controls.

Results

PCR and DNA Fingerprinting

Figures A and B show PCR and DNA fingerprinting of 20 clones before panning process, respectively. PCR products of 950 bps (VH-linker-VL) were amplified from all the isolated clones before panning (Fig. A). Products of the corresponding Mva-I digests are shown in Figure B.

PCR products of 950 bps were also amplified from all the isolated clones after panning (Fig. C). The corresponding Mva-I digests of those PCR products are shown in Figure D. Two dominant fingerprint patterns (specific repeated designs) were obtained after panning. Clones with pattern 1, scFv1 (Lanes 1, 4, 7, 12, 13, 14, and 20), with a frequency of 35%, and pattern 2, scFv2 (Lanes 5, 9, 11, and 16), with a frequency of 20%, were selected.

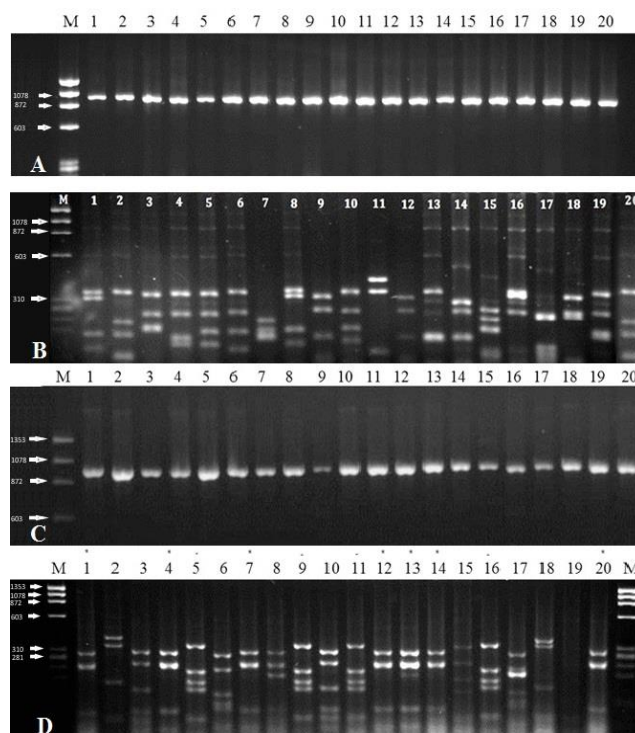


Fig. 1. (A). Agarose gel electrophoresis of the PCR products of the selected clones before panning. A 950 bp band was amplified from each clone. M: Φ X174DNA marker. (B). Agarose gel electrophoresis of Mva-I digests of the PCR products before panning. (C) Agarose gel electrophoresis of the PCR products of the selected clones after panning. A 950 bp band was amplified from each clone. M: Φ X174DNA marker. (D) Agarose gel electrophoresis of Mva-I digests of the PCR products after panning. Pattern 1, scFv1, is marked with a star (Lanes 1, 4, 7, 12, 13, 14, and 20), and pattern 2, scFv2, is marked with negative sign (Lanes 5, 9, 11, and 16).

Phage Enzyme Linked Immunosorbent Assay (Phage ELISA)

Phage ELISA was used to demonstrate the specific binding of the selected scFv antibodies to the corresponding peptide. The absorbances of the wells coated with the corresponding peptide for the two

selected scFvs were greater than those of the wells containing no peptide. Optical densities (ODs) of 0.66 and 0.59 were obtained for the reactions of scFv1 and scFv2 with their corresponding epitopes, while ODs less than 0.2 were obtained with all the negative controls. (Fig. 2).

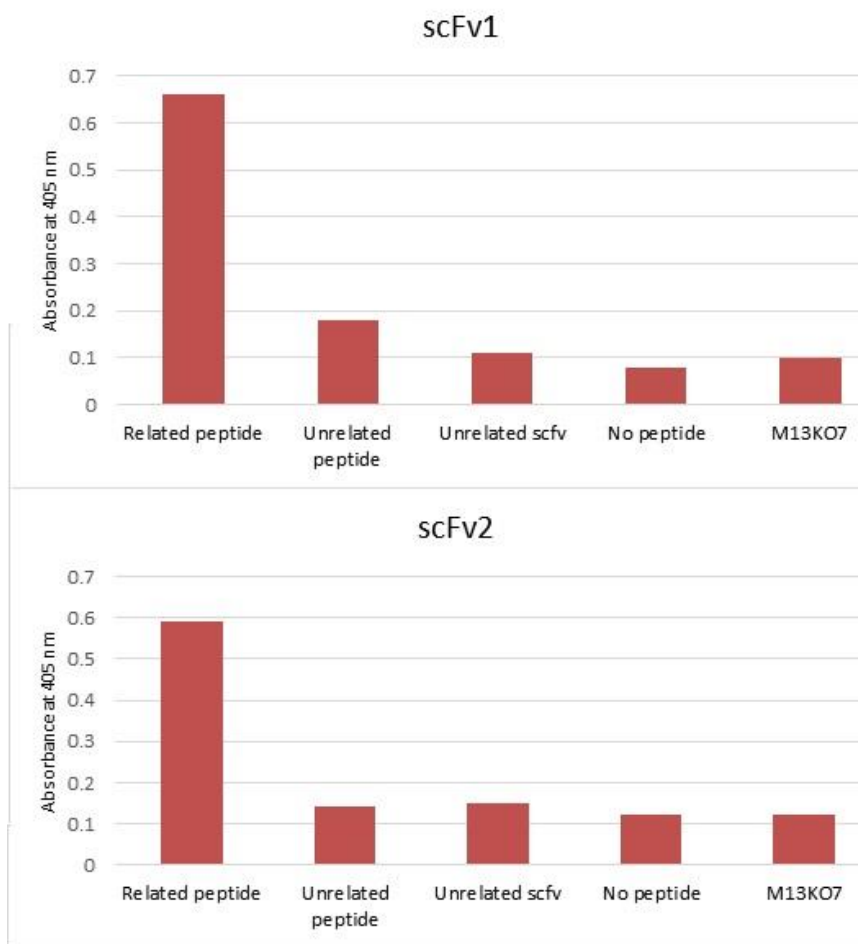


Fig. 2. ELISA results of scFv1 and scFv2.

As Thathaisong showed (29), if the absorbance of selected scFv is more than 2 times greater than the absorbance of the negative controls there is significant difference between them, so there is no need for further statistical analysis. In this study the absorbance of the wells coated with mentioned peptide for the selected scFv1 and scFv2 was 8.25 and 4.9 folds higher than that of the negative control wells, respectively.

Discussion

Although cancer treatment has progressed greatly in recent decades, most treatments have disadvantages. Radiotherapy and chemotherapy

are not targeted and affect both normal and malignant cells. These methods also have numerous side effects including sore skin, nausea, loss of appetite, diarrhea, infertility, hair loss, lymphedema, and blood and nervous system disorders, many of which are irreversible. In addition, chemotherapy drugs have limited efficacy (30). Monoclonal antibody treatment offers improved results compared to previous methods, but some disadvantages and rejection in humans has limited their clinical use (31, 32). ScFv treatment has been introduced as one of the best cancer immunotherapies due to the human origin

of the antibodies and their small sizes and tissue penetration abilities (32).

Because inhibition of CTLA-4 to B7 binding is critical in cancer treatment, blocking CTLA-4 may be an effective immunotherapy (33). In this study, specific scFvs were selected against CTLA-4. The amino acid sequence MHVAQPAVVLA, located in the N-terminus of CTLA-4, was used as the epitope. This epitope has been reported as an immunodominant CTLA-4 epitope, and a human monoclonal antibody produced against it inhibited tumor growth in a murine fibrosarcoma *in vivo* (28, 34).

A panning process was applied to select specific scFvs against CTLA-4. DNA fingerprinting of the library identified two specific scFvs with frequencies of 35% and 20%. The panning process has been used to select specific scFvs against different targets. To select specific scFvs against the extracellular domain of FGF3, which has a significant role in bladder carcinoma cell line proliferation (22), a library was panned against the receptor and the antibodies were selected (23).

Five specific antibodies against MUC1, an antigen overexpressed in ovary adenocarcinoma, were selected, and their relative reactivities analyzed by phage ELISA (35). ScFvs against a specific epitope of P185, a phosphoglycoprotein that is overexpressed in most cancers of epithelial origin and increases tumor aggressiveness, were isolated and their specificities evaluated by phage ELISA (27).

In the current study, the panning results were confirmed by ELISA. The ELISA results showed that the two selected scFvs bound to the corresponding peptide with greater affinities than the negative controls. Moreover, the epitope was not detected by the antibody controls, unrelated scFv, or M13KO7. The unrelated scFv to HER2 did not react with the CTLA-4 peptide. The absorbances of the wells coated with the CTLA-4 peptide for scFv1 and scFv2 were 8.25 and 4.9 - fold higher than that of the wells containing no peptide, respectively. The specificity of the selected scFvs to the corresponding peptide has been shown in various phage ELISA assessments. The optical density (OD) of specific scFvs against influenza-A virus H5N1 subtype at 405 nm were shown by Thathaisong et al. to be two-fold greater

than the negative controls in a positive phage ELISA (29). There was no reaction with an unrelated peptide and the result represented the specific reaction of the selected scFvs with the corresponding peptide.

CTLA-4 is expressed on many cancerous cells and is introduced as a potential marker for immunotherapy (33, 36). Several antibodies against CTLA-4 are under study in clinical trials. Tremelimumab is currently in phase II of a clinical trial and being investigated for melanoma and malignant mesothelioma treatments (37). Ipilimumab is in phase III of a clinical trial and has shown promise against melanoma and prostate and lung cancers (37, 38). Due to several advantages of scFvs over full-length antibodies, these small and high-affinity human antibodies have been applied for cancer-targeted therapy. scFvs against EpCAM, HER2, IL-25, and CEA have been reported as effective against cancer cells (39-41). Also, immune-conjugated forms of specific scFvs, including immunocytokines against ganglioside (GD2) (42), anti-tumor associated antigen (TAA) photodynamic or sonodynamic (PS) -conjugated antibody (43), and anti-VEGFR-2scFv conjugated with As₂O₃-stealth nanoparticles (44), have shown promise against tumor growth and angiogenesis. The specific anti-CTLA-4 scFvs selected in this study are new agents with promise for targeted therapy of cancers expressing CTLA-4. The antibodies could interfere with cellular process by binding to the extracellular portion of CTLA-4. These antibodies originate from human immunoglobulin and do not induce a HAMA response. In addition, the small sizes and high affinities of the selected scFvs contribute to efficient penetration and effective functions than routine monoclonal antibodies. Also, conjugated antibodies can be produced to exert additional anti-cancer effects. Further investigations are needed to evaluate the anti-tumor effects of the selected antibodies *in vitro* and *in vivo*.

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Reference

1. Vanhove B, Laflamme G, Coulon F, Mouglin M, Vusio P, Haspot F, et al. Selective blockade of CD28 and not CTLA-4 with a single-chain Fv-alpha1-antitrypsin fusion antibody. *Blood*. 2003;102(2):564-70.
2. Griffin MD, Hong DK, Holman PO, Lee KM, Whitters MJ, O'Herrin SM, et al. Blockade of T cell activation using a surface-linked single-chain antibody to CTLA-4 (CD152). *Journal of immunology*. 2000;164(9):4433-42.
3. Egen JG, Kuhns MS, Allison JP. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol*. 2002;3(7):611-8.
4. Kapadia D, Fong L. CTLA-4 blockade: autoimmunity as treatment. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2005;23(35):8926-8.
5. Crnkic Kapetanovic M, Saxne T, Jonsson G, Truedsson L, Geborek P. Rituximab and abatacept but not tocilizumab impair antibody response to pneumococcal conjugate vaccine in patients with rheumatoid arthritis. *Arthritis research & therapy*. 2013;15(5):R171.
6. De Petris G, Gatius Caldero S, Chen L, Xiao SY, Dhungel BM, Wendel Spizcka AJ, et al. Histopathological changes in the gastrointestinal tract due to drugs: an update for the surgical pathologist (part I of II). *International journal of surgical pathology*. 2014;22(2):120-8.
7. Simeone E, Grimaldi AM, Esposito A, Curvietto M, Palla M, Paone M, et al. Serious haematological toxicity during and after ipilimumab treatment: a case series. *Journal of medical case reports*. 2014;8:240.
8. Kremer JM, Genant HK, Moreland LW, Russell AS, Emery P, Abud-Mendoza C, et al. Results of a two-year followup study of patients with rheumatoid arthritis who received a combination of abatacept and methotrexate. *Arthritis and rheumatism*. 2008;58(4):953-63.
9. Herrero-Beaumont G, Martinez Calatrava MJ, Castaneda S. Abatacept mechanism of action: concordance with its clinical profile. *Reumatologia clinica*. 2012;8(2):78-83.
10. Ribas A, Hanson DC, Noe DA, Millham R, Guyot DJ, Bernstein SH, et al. Tremelimumab (CP-675,206), a cytotoxic T lymphocyte associated antigen 4 blocking monoclonal antibody in clinical development for patients with cancer. *The oncologist*. 2007;12(7):873-83.
11. Robert C, Thomas L, Bondarenko I, O'Day S, M DJ, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *The New England journal of medicine*. 2011;364(26):2517-26.
12. Watkins NA, Ouwehand WH. Introduction to antibody engineering and phage display. *Vox sanguinis*. 2000;78(2):72-9.
13. Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NB, Hamid M. scFv antibody: principles and clinical application. *Clinical & developmental immunology*. 2012;2012:980250.
14. Younesi V, Nejatollahi F. Induction of anti-proliferative and apoptotic effects by anti-IL-25 receptor single chain antibodies in breast cancer cells. *International immunopharmacology*. 2014;23(2):624-32.
15. Luo Y, Pang H, Li S, Cao H, Peng Z, Fan C, et al. Production and radioimmunoimaging of novel fully human phage display recombinant antibodies and growth inhibition of lung adenocarcinoma cell line overexpressing Prx I. *Cancer biology & therapy*. 2009;8(14):1369-77.
16. Moazen B, Ebrahimi E, Nejatollahi F. Single Chain Antibodies Against gp55 of Human Cytomegalovirus (HCMV) for Prophylaxis and Treatment of HCMV Infections. *Jundishapur journal of microbiology*. 2016;9(3):e16241.
17. MOHAMMADI M, NEJATOLLAHI F. 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*. 2014;5(4):835-47.
18. Weiner LM, Dhodapkar MV, Ferrone S. Monoclonal antibodies for cancer immunotherapy. *Lancet*. 2009;373(9668):1033-40.
19. Nejatollahi F, Abdi S, Asgharpour M. Antiproliferative and apoptotic effects of a specific antiprostata stem cell single chain antibody on human prostate cancer cells. *Journal of oncology*. 2013;2013:839831.
20. Nejatollahi F, Jaberipour M, Asgharpour M. Triple blockade of HER2 by a cocktail of anti-HER2 scFv antibodies induces high antiproliferative effects in breast cancer cells. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2014;35(8):7887-95.
21. Nejatollahi F, Silakhori S, Moazen B. Isolation and Evaluation of Specific Human Recombinant Antibodies from a Phage Display Library against HER3 Cancer Signaling Antigen. *Middle East Journal of Cancer*. 2014;5(3):137-44.
22. Martinez-Torrecedrada J, Cifuentes G, Lopez-Serra P, Saenz P, Martinez A, Casal JI. Targeting the extracellular domain of fibroblast growth factor receptor 3 with human single-chain Fv antibodies

inhibits bladder carcinoma cell line proliferation. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2005;11(17):6280-90.

23. Liu Z, Zhang J, Fan H, Yin R, Zheng Z, Xu Q, et al. Expression and purification of soluble single-chain Fv against human fibroblast growth factor receptor 3 fused with Sumo tag in *Escherichia coli*. *Electronic Journal of Biotechnology*. 2015;18(4):302-6.

24. Vitaliti A, Wittmer M, Steiner R, Wyder L, Neri D, Klemenz R. Inhibition of tumor angiogenesis by a single-chain antibody directed against vascular endothelial growth factor. *Cancer research*. 2000;60(16):4311-4.

25. Ranjbar R, Nejatollahi F, Ahmadi ASN, Hafezi H, Safaie A. Expression of Vascular Endothelial Growth Factor (VEGF) and Epidermal Growth Factor Receptor (EGFR) in Patients With Serous Ovarian Carcinoma and Their Clinical Significance. *Iranian journal of cancer prevention*. 2015;8(4).

26. Nejatollahi F, Hodgetts SJ, Valley PJ, Burnie JP. Neutralising human recombinant antibodies to human cytomegalovirus glycoproteins gB and gH. *FEMS immunology and medical microbiology*. 2002;34(3):237-44.

27. Nejatollahi F, Malek-Hosseini Z, Mehrabani D. Development of Single Chain Antibodies to P185 Tumor Antigen. *Iran Red Crescent Med J*. 2008;10(4):298-302.

28. Hanson DC, Neveu MJ, Mueller EE, Hanke JH, Gilman SC, Davis CG, et al. Human monoclonal antibodies to ctla-4. *Google Patents*; 2012.

29. Thathaisong U, Maneewatch S, Kulkeaw K, Thueng-In K, Pongpair O, Srimanote P, et al. Human monoclonal single chain antibodies (HuScFv) that bind to the polymerase proteins of influenza A virus. *Asian Pacific journal of allergy and immunology / launched by the Allergy and Immunology Society of Thailand*. 2008;26(1):23-35.

30. Arora A, Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. *The Journal of pharmacology and experimental therapeutics*. 2005;315(3):971-9.

31. Barjaktarevic IZ, Qadir N, Suri A, Santamauro JT, Stover D. Organizing pneumonia as a side effect of ipilimumab treatment of melanoma. *Chest*. 2013;143(3):858-61.

32. Nejatollahi F, Ranjbar R, Younesi V, Asgharpour M. Deregulation of HER2 downstream signaling in breast cancer cells by a cocktail of anti-HER2 scFvs. *Oncology research*. 2013;20(8):333-40.

33. Mocellin S, Nitti D. CTLA-4 blockade and the renaissance of cancer immunotherapy. *Biochimica et biophysica acta*. 2013;1836(2):187-96.

34. Grosso JF, Jure-Kunkel MN. CTLA-4 blockade in tumor models: an overview of preclinical and translational research. *Cancer immunity*. 2013;13:5.

35. Henderikx P, Kandilogiannaki M, Petrarca C, von Mensdorff-Pouilly S, Hilgers JH, Krambovitis E, et al. Human single-chain Fv antibodies to MUC1 core peptide selected from phage display libraries recognize unique epitopes and predominantly bind adenocarcinoma. *Cancer research*. 1998;58(19):4324-32.

36. Matikas A, Mavroudis D. Beyond CTLA-4: novel immunotherapy strategies for metastatic melanoma. *Future oncology*. 2015;11(6):997-1009.

37. Ribas A. Anti-CTLA4 Antibody Clinical Trials in Melanoma. *Update on cancer therapeutics*. 2007;2(3):133-9.

38. Weber J. Review: anti-CTLA-4 antibody ipilimumab: case studies of clinical response and immune-related adverse events. *The oncologist*. 2007;12(7):864-72.

39. Simon M, Stefan N, Pluckthun A, Zangemeister-Wittke U. Epithelial cell adhesion molecule-targeted drug delivery for cancer therapy. *Expert opinion on drug delivery*. 2013;10(4):451-68.

40. Ayat H, Burrone OR, Sadghizadeh M, Jahanzad E, Rastgou N, Moghadasi S, et al. Isolation of scFv antibody fragments against HER2 and CEA tumor antigens from combinatorial antibody libraries derived from cancer patients. *Biologicals : journal of the International Association of Biological Standardization*. 2013;41(6):345-54.

41. Nejatollahi F, Asgharpour M, Jaberipour M. Down-regulation of vascular endothelial growth factor expression by anti-Her2/neu single chain antibodies. *Medical oncology*. 2012;29(1):378-83.

42. Ahmed M, Cheung NK. Engineering anti-GD2 monoclonal antibodies for cancer immunotherapy. *FEBS letters*. 2014;588(2):288-97.

43. Kuroki M, Shirasu N. Novel treatment strategies for cancer and their tumor-targeting approaches using antibodies against tumor-associated antigens. *Anticancer research*. 2014;34(8):4481-8.

44. Xiangbao Y, Linqun W, Mingwen H, Fan Z, Kai W, Xin Y, et al. Humanized anti-VEGFR-2 ScFv-As2O3-stealth nanoparticles, an antibody conjugate with potent and selective anti-hepatocellular carcinoma activity. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*. 2014;68(5):597-602.