J Biosci Vol. 43, No. 4, September 2018, pp. 661–671
DOI: 10.1007/s12038-018-9772-3

Construction, expression and functional analysis of anti-B7-H4-scFv-CH3 recombinant antibody

HONGSHUAI JI, JINRUI GUO, YING YANG, CHAOCHAO XU*,† and WEIPING MAO*,†

Jiangsu Province Key Laboratory for Molecular and Medical Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing 210046, People's Republic of China

**Corresponding authors (Email, 141202035@stu.njnu.edu.cn; maoweiping@njnu.edu.cn)*

†These authors contributed equally to this work.

MS received 12 January 2018; accepted 4 May 2018; published online 10 July 2018

The B7-H4 molecule, a unique negative regulator of T lymphocytes which is overexpressed on the surface of various tumor cells, is a particularly important target candidate for tumor therapy because it can be blocked with anti-B7-H4 antibodies to inhibit the B7-H4 signaling pathway. Our previous work established an anti-B7-H4 single-chain variable fragment (scFv) library, so we have now amplified the genes encoding anti-B7-H4-scFv and human IgG1 CH3 and ligated them by overlap extension PCR to obtain a recombinant gene. After sequencing, the gene was cloned into the expression vector pET43.1a and expression was induced in *E. coli* BL21 (DE3) by isopropyl-β-D-1-thiogalactopyranoside (IPTG). The protein was purified on a nickel-nitrilotriacetic acid (Ni-NTA) resin column and its antigen specificity and affinity were examined by ELISA and western blotting. We also established a Lewis lung cancer model in C57BL/6 mice to further identify the biological function of the scFv protein *in vivo*. The results showed that tumor volume, body weight and necrotic tissues in the control group were significantly greater than in the experimental group, indicating that selected scFvs had good biological activity and could inhibit tumor growth in tumor-bearing mice. Our work thus offers a new approach for the development of cancer-targeted therapy.

Keywords. B7-H4; humanization; Lewis lung cancer models; scFv; tumor therapy

1. Introduction

The activation of T lymphocytes requires two unique signaling pathways: the first one is formed by the major histocompatibility complex (MHC) on the antigen-presenting cell (APC) binding to the antigen peptide to constitute an MHC-antigen peptide complex which is recognized by T cell surface receptors; the second pathway is activated when a co-stimulatory molecule on the surface of the APC binds to the T cell surface ligand to produce a complete T cell stimulus signal (Wiendl *et al*. 2003; Miyamoto *et al*. 2005). Without these two pathways, T cells would be incompetent to respond to co-stimulatory signals. Synergistic stimuli can be divided into positive and negative co-stimulatory molecules depending on the effect produced. The B7 family such as B7-1 (CD80), B7-2 (CD86), ICOSL (B7h, B7-H2), B7-H3, PD-L1 B7-H4 (B7x) and other molecules (Van Gool *et al.* 1996) is thought to play a particularly important role as a co-stimulatory molecule that can deliver signals from APCs to T cells and promote or inhibit the proliferation of T cells and the production of cytokines. The B7-H4 molecule is a negative co-stimulatory molecule recently discovered in the B7 family (Lieping 2004). Specific antigens on the surface of tumor cells can induce an immune response, but the strength of this response is very low and cannot inhibit the growth of tumor cells. One possible cause for of the weak immune response is that the surface of tumor cells may express a factor like B7-H4 which can inhibit the immune response. Many studies have shown that the B7-H4 protein in non-lymphoid normal tissue has little or no expression, but is highly expressed in some tumor tissues (Zheng *et al*. 2007). The B7-H4 molecules was inducibled expression on human T cells, B cells, DCs and macrophages (Sica *et al.* 2003; Prasad *et al*. 2003), B7-H4 molecules were constitutively expressed on some tumor cells (Hirotsune *et al*. 2003; Nicosia *et al.* 2003; Salceda *et al.* 2005; Joanne and Woong-Kyung 2013), Such as ovarian cancer, lung cancer, cervical cancer, breast cancer, kidney cancer and endometrial cancer cells.

Studies have also shown that B7-H4 molecules not only help tumor cells escape the killing effect of activated T cells, but also promote the survival of tumor cells. Abnormally high expression of B7-H4 molecules has been detected in many tumor tissues, which was associated with tumor cell evasion of the host immune system (Flies and Chen 2007;

Zou and Chen 2008). Thus, blocking the signaling pathway of B7-H4 molecules with anti-B7-H4 antibody may provide a new method for tumor therapy in which B7-H4 could become a potential target molecule for tumor diagnosis and treatment.

Single-chain variable fragment is a recombinant antibody protein (Blazek and Celer 2003) formed by the heavy-chain variable region (VH) and the light-chain variable region (VL) of the antibody with a short, flexible linker peptide. We ligated the VH and VL genes with a linker and cloned the sequence into a suitable expression vector. The recombinant expression vector was then introduced into a prokaryotic expression system and expressed as antibody protein. Currently, the commonly used linker peptide is a small 15-residue peptide (Takkinen *et al*. 1991; Krebber *et al*. 1997) containing three repeats of four glycines (Gly) and one serine (Ser) residue designated $(Gly_4Ser)_3$. Because the variable region of scFv has with relatively low immunogenicity which is not prone to human anti-heterogeneous protein reactions, and because its molecular mass is small and penetrating power is strong, scFv can be connected with drugs or toxins to create an antibody which can specifically bind to antigen and kill target cells (Kotlan *et al*. 2000; Marty *et al*. 2006). Thus, single-chain antibodies have extensive applications in the treatment of tumors, targeted therapy and anti-tumor drugs.

In our previous work, we prepared scFv against B7-H4 protein. Unfortunately, due to its lack of a crystallizable Fc fragment, the molecular mass of scFv was small and its half-life was short. If used in the clinic, this scFv would be quickly and easily cleared from the body, and secondary antibodies would be unable to link with scFv in the absence of the Fc section, precluding immunological testing. Therefore, the goal of the present study was to generate a modified humanized single-chain antibody to improve the utilization of scFvs, which entailed three aims: First, the construction of the recombinant expression system; second, expression of the recombinant protein and determination of the specificity and affinity of the humanized antibody; and third, *in vivo* verification of the lethality of the recombinant antibodies in tumor cells. We expect that our approach will provide a promising theoretical basis for the application of anti-B7-H4 humanized single-chain antibodies in the treatment of tumors.

2. Materials and methods

2.1 *RNA extraction, amplification and ligation of genes*

Fresh human peripheral blood obtained from the JiangsSu Provincial Blood Center was diluted 1:1 with RPMI-1640 cell culture medium (Invitrogen, USA). We then added 4 mL of human lymphocyte fraction and the diluted blood was transferred to a 15 mL sterile centrifuge tube and centrifuged at 3,500*g* for 20 min at 37°C. After centrifugation, the mononuclear cells were removed and placed in a new Petri dish. Total RNA was extracted from the peripheral blood mononuclear cells using a Trizol kit (Invitrogen, USA) according to the manufacturer's instructions and the absorbance at 260 nm/280 nm was determined. The extracted total RNA was reverse transcribed into cDNA using the cDNA First-strand Synthesis Kit (Takara, Japan). Primers P1 and P2 (table 1) were designed on the basis of the third constant region gene sequence of the human immunoglobulin IgG1 heavy chain obtained from NCBI (Genbank ID: 9857752). The PCR reaction was carried out using DNA polymerase (Vazyme, China) and the cDNA as a template in order to amplify the CH3 gene. The cycle conditions were as follows: 94°C for 5 min, 35 cycles of amplification (94°C for 30 s, 58°C for 30 s, and 72°C for 40 s) and a final extension at 72°C for 5 min. Collection and purification of the target fragment was carried out using an Agarose Gel DNA Extraction Kit (Takara, Japan). The target gene was cloned into the pMD19-T Vector (Vazyme, China) for T-A clones, and the positive strain was identified by colony PCR. The anti-B7-H4-scFv gene was amplified by PCR from the DH5α strain containing the anti-B7H4-scFv/pET43.1a recombinant plasmid as a template with primers P3 and P4 (table 1). The reaction conditions were as stated above.

The humanized CH3 gene was linked to the anti-B7-H4-scFv gene by overlap extension PCR. At the time of primer design, the operation of overlap extension PCR had been taken into account in advance so that primer P1 and P4 both contained a flexible linker. First, the genetic sequence of CH3 and anti-B7-H4-scFv were mixed with PCR Mixture (Vazyme, China) without primers for 5 cycles in order to ensure the two genes had self-ligated. The P2 and P3 primers were then added to the reaction system under the following cycle conditions: 94°C for 2 min, 5 cycles of amplification

Table 1. Primers used for PCR

Primer	Sequence (5′ ~ 3′)	Target
P1	GCTGCAGGCGGTAGCGGTGGCAGCCCCGAGAACCAC	CH3
P2	GAGTCATTCTGCGGCCGCTCATTTACCCGGGGACAG	CH3
P3	CCGGAATTCATGGCCAAGGTGCAGCTCGAGGAGTCTGG	anti-B7-H4-scFv
P4	TCGGGGCTGCCACCGCTACCGCCTGCAGCATCAGCCCGTTT	anti-B7-H4-scFv

The linker sequence is underlined in P1 and P4; the digestion site is underlined in P2 and P3.

(94°C for 30 s, 58°C for 30 s and 72°C for 40 s) and a final extension at 72°C for 5 min. To amplify the anti-B7-H4-scFv-CH3 gene, the number of cycles was increased to 35, while the other conditions were unchanged. The target fragment was purified with Agarose Gel DNA Extraction Kit (Takara, Japan) and stored at −20°C.

2.2 *Construction, expression and purification of the recombinant expression vector*

The selected target scFv DNAs were digested with *EcoR* I and *Not* I (Takara, Japan) for 2 h and ligated to the pET43.1a plasmid at the corresponding restriction sites. The ligation mixture was transformed into *E. coli* DH5α competent cells for recombinant plasmid. After sequencing to verify the correct target strain, the recombinant plasmid anti-B7H4-scFv-CH3/pET43.1a was extracted and transformed into *E. coli* BL21 (DE3) competent cells. *E. coli* cells containing the recombinant anti-B7-H4-scFv-CH3-pET43.1a vector plasmid were cultured and stored at −20°C.

E. coli cells containing the recombinant gene were transferred into new Luria broth medium containing ampicillin (50 mg/mL) and incubated at 37°C on a rotary shaker at 200 rpm until the OD_{600} was approximately 0.6; isopropyl-β-D-1-thiogalactopyr-anoside (IPTG) was then added to induce expression at 37°C. After different hours of induction, the cultures were collected and centrifuged at 8,000*g* for 15 min at 4°C, after which the supernatants were discarded and the precipitates resuspended in PBS. An Ultrasonic Crusher (Branson, USA) was used to disrupt the bacterial cell solution on ice, which was then centrifuged; the supernatant was collected, precipitated and stored at −20°C. The expressed protein containing a His-tag was purified on a nickel-nitrilotriacetic acid (Ni-NTA) resin column. The protein was eluted with different concentrations of imidazole and the eluent was collected and loaded into a dialysis bag for dialysis in physiological saline for 24 h. After dialysis, the protein was concentrated with sodium nitroprusside. A small amount of protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after concentration and the remaining protein was stored at −80°C.

2.3 *Analysis of affinity by ELISA*

The affinity of purified anti-B7H4 antibody for B7-H4 protein was verified by ELISA. First, the B7-H4 protein and inducible T-cell costimulator (ICOS) protein with a His-tag as positive control was diluted to 2.5 μg/mL in 50 mM bicarbonate buffer. The wells of 96-well plates (Corning Costar, USA) were coated with 120 μL of sample and incubated with 5% skim milk for 2 h at 37°C. Second, 100 μL of humanized scFv was added to each well with PBS as a blank control for 40 min at 37°C. After washing the plates with PBS containing 0.05% Tween-20 (PBST) 4 times, binding was detected by horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibody (Vazyme, China). Color was obtained by use of the tetramethyl benzidine (TMB) chemiluminescence system (Sigma, USA). After the reaction was terminated, the OD_{450} was read using a microplate reader (Biotek, USA). The OD value of the blank control was N, the measured value of the sample was P and P/N≥2.1 was considered positive.

2.4 *Analysis of antigen-binding specificity by Western blotting*

The protein binding activity of anti-B7H4 scFvs against B7-H4 protein was analyzed by western blot. The purified B7-H4 extracellular domain protein was subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). After transfer, the membrane was incubated with 5% skim milk in PBS, PH 7.4 for 2 h. After washing four times for 10 min with PBST, the membrane was incubated separately with anti-B7-H4-scFv, anti-B7-H4-scFv-CH3, anti-ICOSL-scFv or anti-ICOSL-scFv-CH3 as the primary antibody overnight at 4°C, followed by incubation with HRP-labeled goat anti-mouse IgG antibody as the secondary antibody for 8 h at 4°C. Finally, the blot was visualized by ECL chemiluminescence (Takara, Japan).

2.5 *Cell and animal culture*

LLC cells (Meixuan Biology, China) were cultured in RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum and 100 U/mL streptomycin and penicillin, and were maintained in a humidified incubator with 5% CO2 at 37°C. Experiments were performed on cells passaged for at least three generations with a confluency of 80%. Twenty 6-week-old female C57BL/6 mice were purchased from Carvins Experimental Animals Center of Changzhou with weights of approximately 16–18 g, and injection of scFvs was initiated when the transplanted tumor size was suitable for experimental treatment. The mice were housed for 5 days to adapt to the new environment.

2.6 *Tumor xenograft model*

When the LLC cells reached the desired confluency, they were digested with 0.25% trypsin for about 30 s, followed by the addition of 3 mL RPMI 1640 medium to terminate the reaction and centrifugation at 1,000*g* for 3 min. The cells were resuspended in serum-free RPMI 1640 medium and the

concentration was adjusted to 5×10^6 cells/mL. After shaving the right forelimbs of mice and cleaning the forelimb armpits with alcohol-soaked cotton balls, the LLC cells were subcutaneously injected. When the tumor size reached 80–100 mm^3, the tumor-bearing mice were randomly divided into model and test groups. The test group was subcutaneously injected with 20 mg/kg anti-B7-H4-scFv-CH3 protein in 100 μL PBS and the model group was subcutaneously injected with physiological saline. Tumor diameters were measured using a vernier caliper (Southwest Jiahua Precision Measuring Tool, Taiwan) and the weights of the mice were recorded from the beginning of antibody administration. At the end of the experiment, the mice were euthanatized and the tumors were excised, weighed and embedded in paraffin blocks as described below. The tumor volume (TV) was calculated using the following formula: $TV = 1/2\times a\times b^2$, where a and b represented tumor length and width, respectively.

2.7 *Pathological observation of subcutaneous tumor tissue*

Three sections from each tumor were randomly selected for hematoxylin-eosin staining. Tumor blocks were immersed in 4% paraformaldehyde for 16 h and rinsed with tap water. Dehydration was carried out using 50% ethanol, 75% ethanol, 85% ethanol, 95% ethanol × 2 and 100% ethanol × 2. The dehydrated tumor block was immersed in wax and paraffin embedded, then sectioned (5 μm), dried and dewaxed twice, each for 5 min. The sections were rehydrated using anhydrous ethanol, 95% ethanol, 90% ethanol, 80% ethanol, 70% ethanol and distilled water for 5 min per soak. After dyeing and dehydration, the slices were soaked for 5 min each in two changes of xylene, then sealed using neutral gum. Finally, tumor tissue was observed under a microscope.

3. Results

3.1 *Amplification and ligation of the target gene*

Total RNA was extracted from human peripheral blood B cells with an OD_{260}/OD_{280} ratio of 1.917, indicating that the RNA had been sufficiently purified. Agarose gel electrophoresis showed a clear band between 250 and 500 bp (figure 1, lane 1), consistent with the size of the CH3 gene reported by NCBI at 323 bp. The anti-B7-H4-scFv gene was amplified by PCR from the DH5α strain expressing the anti-B7-H4-scFv gene as a template. Agarose gel electrophoresis of the PCR product showed a length of approximately 750 bp (figure 1, lane 2), which was consistent with the size of 744 bp previously determined in our laboratory.

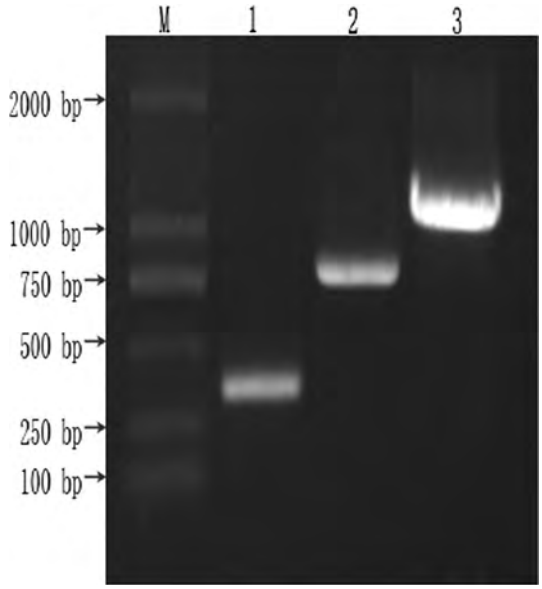

Figure 1. Agarose gel electrophoresis of PCR-amplified products from the B7-H4 gene, CH3 gene and the target gene encoding anti-B7-H4-scFv-CH3. Lane M: DL 2000 DNA marker; lane 1: amplified CH3 gene (323 bp); lane 2: amplified anti-ICOSL-scFv gene (744 bp); lane 3: amplified anti-B7-H4-scFv-CH3 gene (1079 bp).

The CH3 sequence was ligated to the anti-B7-H4-scFv gene by overlap extension PCR and the PCR product was identified by 1% agarose gel electrophoresis as a band of about 1,000 bp (figure 1, lane 3). This was consistent with the sum of 1,079 bp for the combined lengths of the linked and linker genes. The genes were then cloned into the pMD19-T vector and identified by colony PCR. Positive clones were sequenced, and the sequencing results were consistent with the expected analysis. The obtained sequence of the anti-B7-H4-scFv-CH3 gene wss shown as follows (table 2).

3.2 *Construction, expression and purification of the recombinant expression vector*

The recombinant plasmid encoding the anti-B7-H4-scFv-CH3 gene was transformed into DH5α and was identified by colony PCR. The results showed that the amplified fragment was consistent with the size of the target gene fragment. Subsequently, the colonies were placed under positive selection and positive colonies were picked and sequenced. The recombinant plasmids were then transfected into *E. coli* BL21 (DE3) and induced by IPTG. A protein was obtained with a molecular mass between 100 kDa and 135 kDa, consistent with the expected protein size. We determined that optimal recombinant protein expression was obtained at an IPTG concentration of 0.6 mM for 10 h and that the target protein was expressed in the supernatant. Since the

Table 2. The obtained sequence of the anti-B7-H4-scFv-CH3 gene

Sequence (5′ ~ 3′)
AAGGTGCAGCTCGAGGAGTCTGGAGATGAGCTGGTAAAGCCTGGGGCC
TCAGTGAAGCTGTCCTGCAAGGCTTCTGGCTACACCT
TCACCAGCTACTGGATTAACTGGATAAAACAGAGGCCTGGACAGGGCCTTGAGT
GGATAGGACGTATCGCTCCTGGAAGTGGCAGTACTTTCTACATTGAAATGTTCA
AGGCCAAGGCAACACTGACTGTAGACACATCCTCCAGCACAGCCTACATTCACC
TCAGCAGCCTGTCATCTGAGGACTCTGCTGTCTATTTCTGTGCAAGAGGATATT
ACTACGGTAGTGGTTACGGGGGGTTTGTTTACTGGGGCCAAGGGACCACGGTCA
CCGTCTCCTCAGGTGGTGGTGGTAGCGGCGGCGGCGGCTCTGGTGGTGGTGGAT
CCGACATTGAGCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGA
AGGTCACCATATCCTGCAGTGCCAGCTCAAGTGTAAGTTACATGTACTGGTACC
AGCAGAAGCCAGGATCCTCCCCCAAACTCTGGATTTATAGCACATCCGACCTGG
CTTCTGGAGTCCCTGTTCGCTTCAGTGGCAGTGGATCTGGGACCTCTTACTCTC
TCACAATCAGCAGAGTGGAGGCTGAAGATGCTGCCACTTATTATTGCCAGCAGT
GGAATAGTAACCCACGGACGTTCGGTGGAGGCACCGAGCTGGAAATCAAACGGG
CTGATGCTGCAGGCGGT
AGCGGTGGCAGCCCCGAGAACCACAGGTGTACACC
CTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTG
GTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAG
CCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTC
TTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTC
TTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGC
CTCTCCCTGTCCCCGGGTAAATGA

The linker sequence is underlined, the forward sequence is scFv and the followed is CH3 sequence

pET-43.1a plasmid carried a His tag, the recombinant protein expressed the same tag, allowing it to be bound to a Ni-NTA column and eluted with 10–50 mM imidazole. Finally, SDS-PAGE was used to isolate a single target protein between 100 and 135 kDa (figure 2C, lane 4), which was confirmed by immunoblotting to be the target protein containing the His-tag (figure 2C, lane 5).

3.3 *Analysis of protein affinity by ELISA*

To determine the binding capacity of the purified scFvs, indirect ELISA was used to analyze the affinity of the anti-B7-H4-scFv-CH3 antibody protein for the B7-H4 extracellular domain. The results showed that anti-B7-H4-scFv-CH3 antibody protein had high affinity for the B7-H4 protein antigen in comparison to its affinity for the ICOS protein antigen, which was close to that of the blank PBS control (figure 3). In addition, the increasing OD values as peptide concentration increased demonstrated the obvious capacity of anti-B7-H4-scFv-CH3 to bind high concentrations of antigen.

3.4 *Detection of antigen-binding specificity by Western blot analysis*

The binding activity of anti-B7-H4-scFv-CH3 antibody protein to the B7-H4 extracellular domain was examined by western blot. The results showed the presence of a 27 kDa protein (figure 4A, lane 3) after the nitrocellulose membrane was incubated with anti-B7-H4-scFv-CH3 antibody (figure 4B, lane 2). No protein was detected when anti-B7-H4-scFv, anti-ICOSL-scFv or anti-ICOSL-scFv-CH3 antibody was used (figure 4B, lanes 1, 3 and 4). We also detected the binding of anti-B7-H4-scFv-CH3 antibody to B7-H4 protein from HepG2, Hela, A549, MCF-7, LLC and 293 cells. The results showed that the antibody protein could bind with B7-H4 protein expressed in these five tumor cell lines, although the amount of B7-H4 protein expressed in each tumor cell line was different (figure 4C).

3.5 *Characterization of the tumor transplantation model*

3.5.1 *Tumor volume changes:* When subcutaneous tumors in the mice reached 80–100 mm^3 in size, the tumor-bearing mice were injected subcutaneously with scFv proteins or saline for 15 days. TV was calculated according to formula $TV = 1/2 \times a \times b^2$, where a and b represented tumor length and width, respectively. The effect of anti-B7-H4-scFv-CH3 antibody protein injections on the growth of Lewis lung cancer xenografts in C57BL/6 mice is shown in figure 5A and changes in tumor volume is shown in figure 5B.

3.5.2 *Relative tumor proliferation rate:* Relative tumor volume (RTV) was calculated by the formula $RTV = V_t/V_0$, where V_0 was the measured tumor volume at the very

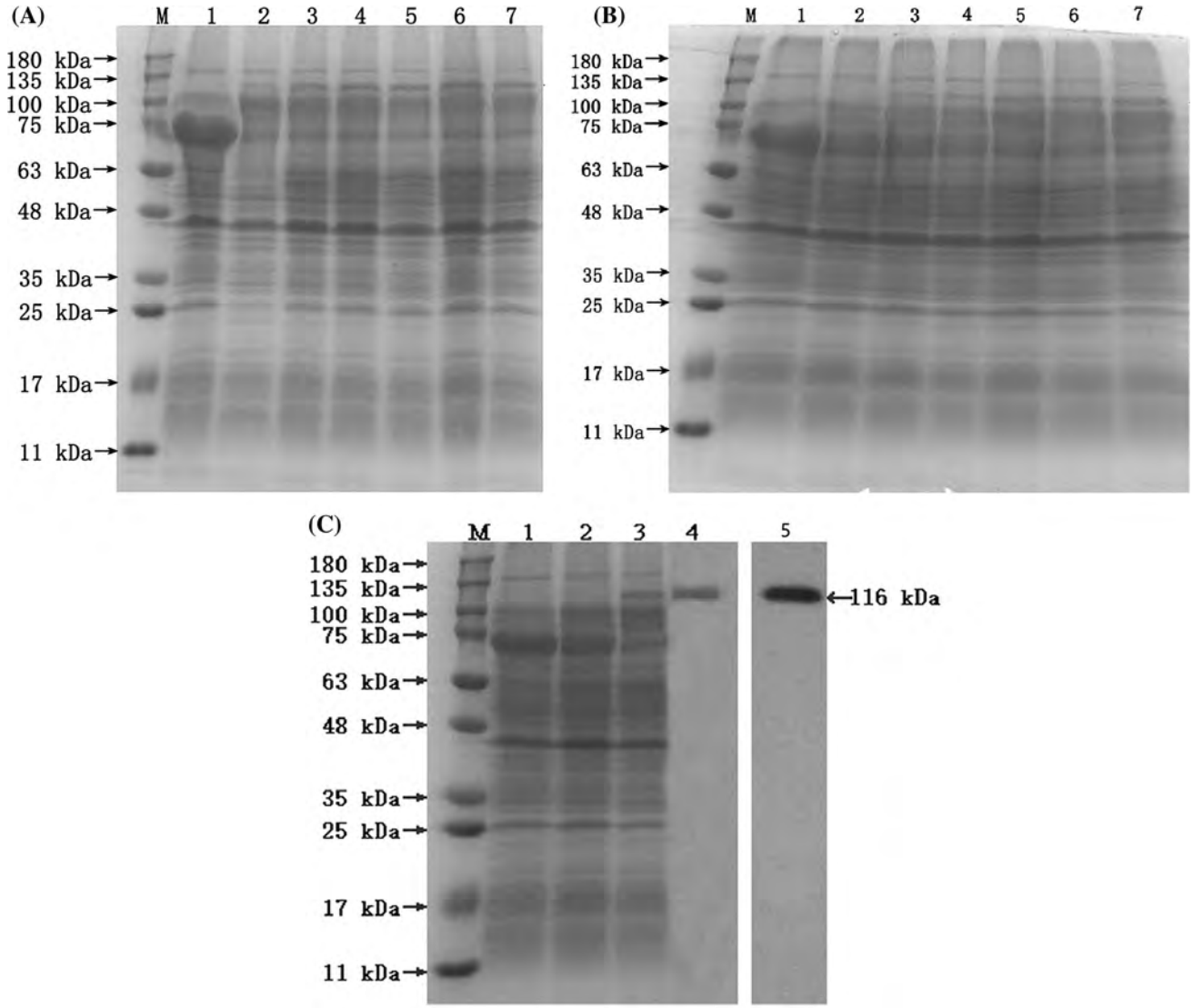

Figure 2. Constrution and Purification of the soluble scFv protein analyzed by SDS-PAGE with staining by Coomassie Brilliant Blue. Lane M: 180 kDa protein marker; lane 1: lytic of BL 21 (pET-43.1a). (**A**) Lanes 2–7: Recombinant strain pET43.1a-anti-B7-H4-scFv-CH3-BL21 induced by IPTG in 0.5 mM, 0.6 mM, 0.8 mM, 1 mM. (**B**) Lanes 2–7: The recombinant strain pET43.1a-anti-B7-H4-scFv-CH3-BL21 was harvested at 4 h, 6 h, 8 h, 10 h and 12 h. (**C**) Lane 2: lytic of BL 21 (pET43.1a-Anti-B7-H4-ScFv-CH3-BL21) without IPTG; lane 3: lytic of BL 21 (pET43.1a-Anti-B7-H4-ScFv-CH3-BL21) induced by IPTG; lane 4: recombinant protein purified by Ni^{2+} column; lane 5: Western blot of the purified protein.

beginning of administration, V_t was the tumor volume at each measurement. We used the RTV to calculate the anti-tumor effect of anti-B7-H4-scFv-CH3 protein on the relative tumor proliferation rate *T/C* (%) as follows: $T/C\ (\%) = T_{RTV}/C_{RTV} \times 100$, where T_{RTV} is the test group RTV and C_{RTV} is the model group RTV. The antibody protein was administered subcutaneously at a dose of 100 μL and the resulting *T/C* (%) of the Lewis lung cancer xenografts in C57BL/6 mice was 41.7% (figure 5C).

3.5.3 *Tumor weight and tumor inhibition rate:* At the end of the experiment, the average tumor weight in the test group was 0.898 ± 0.115 g, which was significantly different from the model group value of 2.029 ± 0.195 g ($p<0.01$). The tumor inhibition rate produced by the anti-B7-H4-scFv-CH3 antibody was 55.7%. The experimental results of the inhibitory effect of antibody protein on transplanted Lewis lung cancer tumors in C57BL/6 mice were shown as follows (table 3 and figure 5D–E).

3.5.4 *Histopathological observations: Model Group:* Tumor morphology was large and irregular, and tumor tissue necrosis was rare after incision. Tumor cells were arranged in group or nests which included interstitial blood vessels

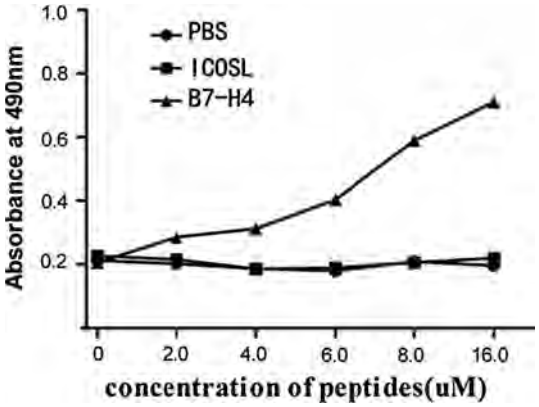

Figure 3. Indirect ELISA binding assay. Relative antigen-binding curves were plotted for PBS, ICOSL and B7H4 antigen. PBS and ICOSL were used as a blank control and negative control, respectively.

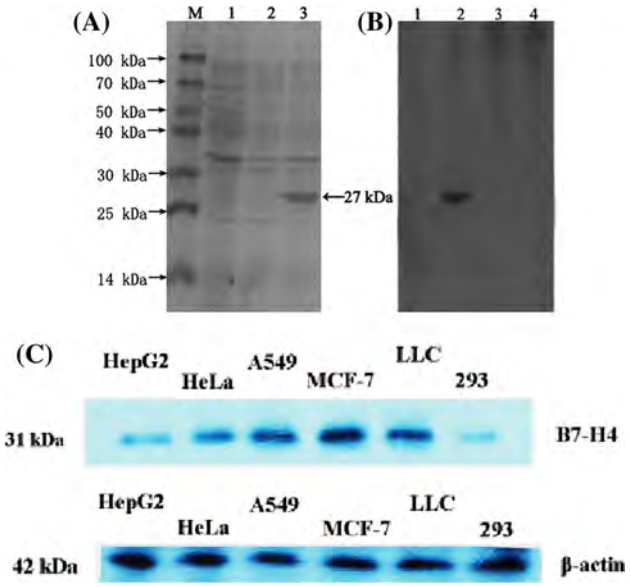

Figure 4. SDS-PAGE (**A**) Lane M: Protein marker with molecular weights up to 100 kDa; lane 1: E.coli BL21 (DE3) containing pET-28a (+) plasmid; lane 2: recombinant strain pET28a-B7-H4-BL21; lane 3: IPTG-induced recombinant strain pET28a-B7-H4-BL21. Western blot analysis. (**B**) Lanes 1–4: anti-B7-H4-scFv, anti-B7-H4-scFv-CH3, anti-ICOSL-scFv and anti-ICOSL-scFv-CH3 were used as primary antibodies to examine binding to the B7-H4 region protein. (**C**) Western blot assay of B7-H4-ScFv-CH3 protein binding to B7-H4 from HepG2, Hela, A549, MCF-7, LLC and 293 cells.

and poorly differentiated cells of different sizes undergoing active growth, without significant necrosis (figure 6A).

Treatment Group: Tumor size and volume were significantly lower than in the model group, and tumor necrosis was present. Tumor cell morphology was similar to the model group, but tumor cell density was lower than in the model group and focal necrosis was found in the tumor tissue (figure 6B).

4. Discussion

In recent years, tumor immunotherapy has attracted serious attention by enhancing the body's own immune response to attack and kill the tumor cells. The B7-H4 molecule is a novel target for tumor immunotherapy, and B7-H4 mRNA is commonly found in various human tissues and organs including kidney, ovary, lung, spleen, stomach, pancreas and others. However, the B7-H4 protein is only expressed in macrophages, mature dendritic cells, B cells and full-time antigen-presenting cells (He *et al.* 2013), suggesting that it may be involved in immune regulation of T lymphocytes. Choi *et al*. (Choi *et al*. 2003) used immunohistochemistry to detect the expression of B7-H4 protein in various tissues and found high expression in lung and ovarian cancer tissues but little in normal tissue. B7-H4 protein was reported to be highly expressed on the surface of breast cancer (Salceda *et al*. 2005), lung cancer (Tringler *et al*. 2006), ovarian cancer (Sun *et al*. 2006) and other tumor cells, suggesting that it may help tumor cells escape host immune surveillance by inhibiting the activation of T cells. The underlying mechanism for this effect is the binding of B7-H4 protein to its corresponding receptors on the surface of activated T cells, which inhibits the secretion of cytokines such as IL-2, IL-10 and IFN-γ as well as T cell proliferation, and cannot be relieved by the activation of molecules such as CD2. B7-H4 protein can also activate the Fas/FasL signaling pathway to promote the apoptosis of T cells and inhibit their division, so that T cell division is suspended in the G0/G1 phase of the cell cycle and negative regulation of the T cell immune response is affected (Sica *et al*. 2003; Song *et al*. 2008; Yi and Chen 2009). In a mouse model of experimental autoimmune encephalomyelitis, mice treated with B7-H4 monoclonal antibody were more likely to develop the disease (Podojil *et al*. 2013). A laboratory has developed a monoclonal antibody against B7-H4 5G3, which binds to B7-H4 to block the B7-H4 signaling pathway, thereby enhancing T cell immune response. Some scientists constructed a human B7-H4 variable region expression vector and successfully expressed the B7-H4 variable region protein. They immunized mice with the fused B7-H4 variable region protein, and then SP2/0 Myeloma cells were transplanted subcutaneously in mice. The experimental results showed that the growth of the transplanted tumor in mice in the immunized group was somewhat inhibited compared with the control group (Mu *et al.* 2014). These findings illustrate that B7-H4 plays a negative role in the activation of T cells.

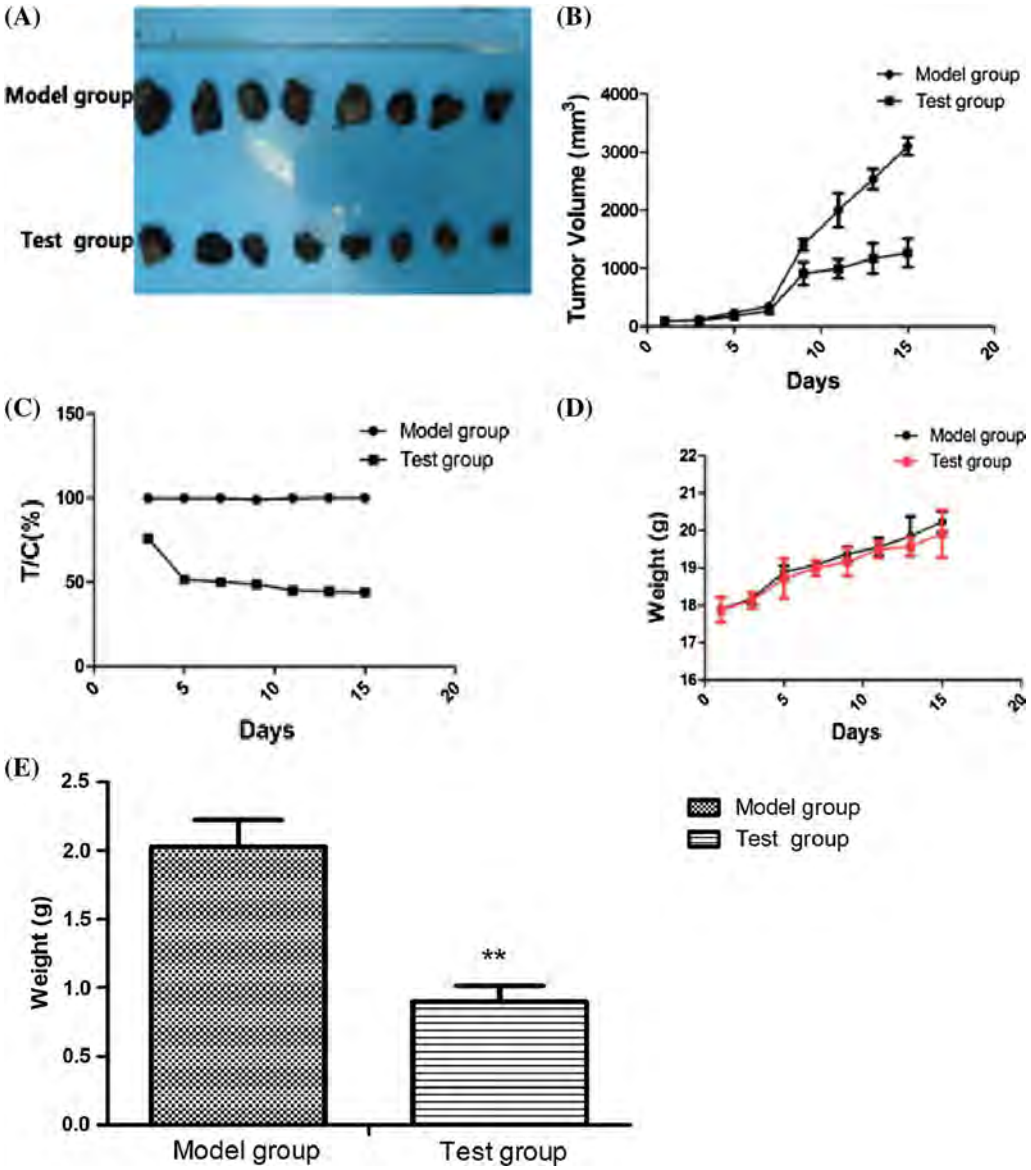

Figure 5. Anti-B7-H4-scFv-CH3 antibody treatment inhibits the growth of tumors. (**A**) Comparison of tumor size between the test group and model group. (**B**) The effect of anti-B7-H4-scFv-CH3 protein on tumor growth in C57BL/6 tumor-bearing mice. (C) The effect of anti-B7-H4-scFv-CH3 protein on the RTV of C57BL/6 mouse Lewis lung cancer-transplanted tumors. (**D**) The effect of anti-B7-H4-scFv-CH3 protein on the weight of C57BL/6 tumor-bearing mice. (**E**) The effect of anti-B7-H4-scFv-CH3 protein on tumor weight in C57 BL/6 tumor-bearing mice. **p<0.01 compared with the model group.

The aim of the present work was to evaluate the effectiveness of anti-B7-H4-scFv-CH3 against tumors *in vitro* and *in vivo*. ScFv can inhibit signaling pathways to improve immunity by binding with the T lymphocyte negative co-stimulatory factor B7-H4. B7-H4 antibodies have been developed by many laboratories to block the B7-H4 signaling pathway, but most of them are mouse monoclonal or single-chain antibodies. Mouse monoclonal antibodies can easily produce a human anti-mouse antibody response. As for single-chain antibodies, while their development has produced good results, there are still some limitations or shortcomings in their practical application. Some of these problems have been solved; for example, the short half-life of single-chain antibodies has been extended by modifying the antibody into a single-chain antibody polymer. In this study, two human anti-CEA-scFv-CH3 antibody molecules were prepared by ligating human IgG1 CH3 to anti-CEA-scFv using short peptides of two and ten amino acid residues. Both antibodies displayed good biological activity and were more efficient in targeting tumors than traditional

Table 3. The effect of anti-B7-H4-scFv-CH3 protein on the weight of C57BL/6 mouse Lewis lung cancer-transplanted tumors and the metastatic rate ($\bar{X} \pm SD$, n = 8, weight in g)

Group	Dose (μL)	Number of starting animals	Number of final animals	Tumor weight (g)	Tumor inhibition rate (%)
Model	—	8	8	2.029±0.195	—
Test	100	8	8	0.898±0.115**	55.7

Compared with the model group, * $p<0.05$, ** $p<0.01$.

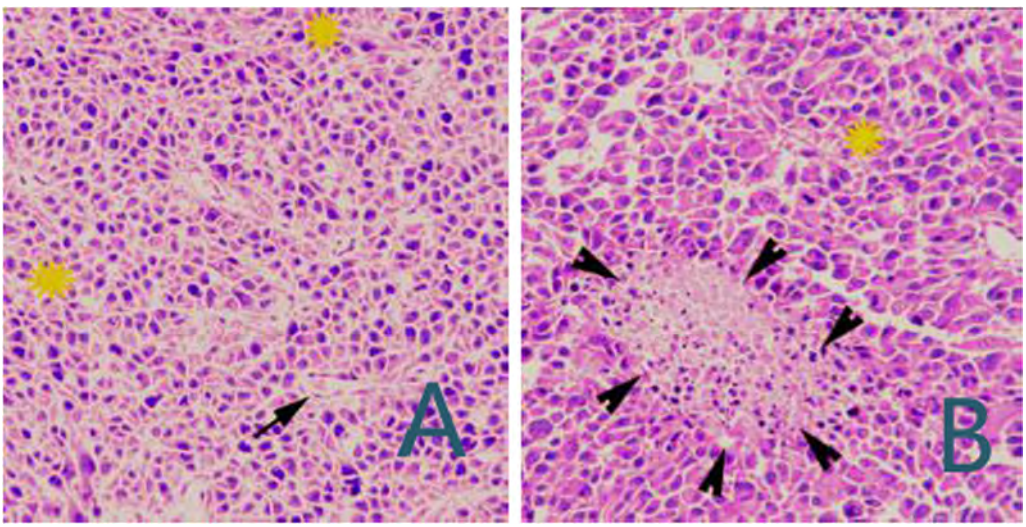

Figure 6. Light micrographs of tumor tissue from the model and test groups of mice. (**A**) Light micrograph of tumor tissue in the model group (×200). Tumor cell growth was active (yellow stars) and cells were poorly differentiated, round to oval in shape and of different sizes. Nuclear division was obvious. The arrangement of tumor cells was lamellar and not nested, with rich, interstitial blood vessels (black arrow) and no obvious necrosis. (**B**) Light micrograph of tumor tissue in the test group (×200). The morphological structure of tumor cells was similar to the model group (yellow stars) and mitotic. Tumor cell arrangement was less dense than the model group, and sites of focal necrosis were present (region surrounded by black arrowheads).

antibody molecules. In the present report, human IgG1 CH3 was connected to anti-B7-H4 scFv using the flexible $(Gly_4Ser)_3$ linker. Based on the humanized fragments, the fusion protein can be expected to have a longer serum half-life and thus result in more exposure to tumors and T cells. Furthermore, due to the incorporation of the human IgG1 CH3 domain, the engineered protein will likely show reduced immunogenicity when administered in a clinical setting. Finally, given the absence of the Fc domain, secondary peroxidase-conjugated antibodies will be unable to bind to the scFv, making detection of the human IgG1 CH3 segment more convenient if scFv attach a humanized fragment.

The anti-B7-H4-scFv gene and CH3 gene were ligated by overlap extension PCR. The desired protein was a fusion protein constructed by connecting the recombinant gene to the pET43.1a expression vector containing a soluble Nus-tag. The recombinant vector was then transformed into *E. coli* BL21 (DE3) and induced by IPTG under conditions we determined to be optimal at 0.6 mM IPTG for 10 h (figure 2A–B). The recombinant protein size was 116 kDa by SDS-PAGE, in agreement with our theoretical calculations. Since the protein expressed by the pET-43.1a plasmid contained a His-tag, the target protein carried the same tag, allowing the protein to be purified on a Ni-NTA column. The target protein was eluted with 10–50 mM imidazole. Finally, analysis by SDS-PAGE showed a single band between 100 and 135 kDa, and we further confirmed by immunoblotting that the target protein contained a His-tag. The binding activity of the scFv protein and B7-H4 extracellular domain protein was examined by ELISA and western blot. With increasing scFv protein concentration, the binding of antigen and antibody also increased. Subsequently, western blot was used to detect the binding capacity of various tumor cell B7-H4 proteins, one of which was mouse lung cancer cells (figure 5C, lane 5). In previous experiments, the amino acid sequences of mB7-H4 and hB7-H4 were compared and found to have a homology as high as 87.63%, which suggested that hB7-H4 and mB7-H4 may be cross-linked to the receptor (Choi *et al*. 2003). In fact, western blot results

showed that the scFv protein could not only bind to the B7-H4 protein from human tumor cells, but also to the mouse B7-H4 protein, which we used to examine the expression of antibody protein in tumor-growth biological function experiments.

Previous research has shown that overexpression of B7-H4 can inhibit tumor cell growth in tumor-bearing mice (Salceda *et al*. 2005). Researchers used rhB7-H4IgV vaccine to immunize tumor-bearing mice and showed that the vaccine could inhibit the growth of SP2/0 transplanted tumors (Zhu *et al*. 2008). Therefore, we used B7-H4 scFv to interfere with the B7-H4 signaling pathway for inhibiting tumor growth, and established a mouse tumor-bearing model to analyze the effectiveness of scFv protein *in vivo*. In order to ensure the effectiveness of the experiment, we used healthy mice with normal immune function to establish the test model, and to ensure the success of modeling, we used the same transplant tumor. In a previous experiment, we found that the scFv protein could bind to B7-H4 in Lewis lung cancer cells. Lewis lung cancer is derived from a lung epithelium-like carcinoma in a C57BL/6 pure-strain mouse, is prone to metastasis and is the most successful transplanted tumor in mice (Matthys *et al*. 1991). For this reason, mouse Lewis lung cancer is a common model for the study of tumors.

In the present experiment, we adjusted the concentration of scFv protein to a dose of 20 mg/kg and observed that the *T/C* (%) of Lewis lung cancer was 41.7% and the tumor inhibition rate was 55.7%. At the end of the experiment, the average tumor weight in the test group was 0.898 ± 0.115 g, which was significantly less than in the model group 2.029 ± 0.195 g, $^{**}p<0.01$ (table 3). There was no significant difference in body weight between the test group and model group. We also used hematoxylin-eosin to stain the tumor tissue, which showed that the tumor cells were in nested lamellar arrangements containing interstitial blood vessels, and that the shapes of tumor cells were round to oval, which indicated that modeling was successful. Analysis of antibody protein inhibition of the growth of tumor cells was performed in tumor-bearing mice (Miyamoto *et al*. 2005). On the one hand, the presence of antibody protein blocked the binding of B7-H4 to its receptor on the surface of active T cells, relieving the inhibition of T cell growth; on the other hand, antibody binding to B7-H4 protein on the surface of tumor cells blocked B7-H4 signaling molecules, modulating immune surveillance as a result, and increasing the immune response of T cells. To confirm these results, it will be necessary perform additional experiments on the biological functions of antibody proteins *in vivo* and *in vitro*.

In conclusion, evaluation of anti-B7-H4-scFv-CH3 revealed that the recombinant antibody had a high affinity for B7-H4 protein and was effective in killing B7-H4-expressing tumor cells *in vivo*. At a dose of 20 mg/kg, anti-B7-H4-scFv-CH3 resulted in significant tumor regression in mice. These results make clear that anti-B7-H4-scFv-CH3 may be a potential candidate for further development as an anti-tumor treatment for B7-H4 protein-expressing tumor cells *in vivo*.

References

Blazek D and Celer V 2003 The production and application of single-chain antibody fragments. *Folia Microbiol*. **48** 687–698

Choi IH, Zhu G, Sica GL, Strome SE, Cheville JC, Lau JS, Zhu Y, Flies DB, Tamada K and Chen L 2003 Genomic organization and expression analysis of B7-H4, an immune inhibitory molecule of the B7 family. *J. Immunol*. **171** 4650–4654

Flies DB and Chen L 2007 The new B7s: playing a pivotal role in tumor immunity. *J. Immunother.* **30** 251–260

He C, Qiao H, Jiang H and Sun X 2013 The inhibitory role of B7-H4 in antitumor immunity: association with cancer progression and survival. *Clin. Dev. Immunol*. **2011** 3330–3334

Hirotsune S, Yoshida N, Chen A, Garrett L, Sugiyama F, Takahashi S, Yagami K, Wynshaw-Boris A and Yoshiki A 2003 An expressed pseudogene regulates the messenger-RNA stability of its homologous coding gene. *Nature* **423** 91–96

Joanne L and Woong-Kyung S 2013 Host B7-H4 regulates antitumor T cell responses through inhibition of myeloid-derived suppressor cells in a 4T1 tumor transplantation model. *J. Immunol*. **190** 6651–6661

Kotlan B, Simsa P, Gruel N, Foldi J, Fridman WH, Petranyi G and Teillaud JL 2000 A scFv phage display mini library generated from the immunoglobulin repertoire of breast medullary carcinoma infiltrating B lymphocytes. *Dis. Markers* **16** 25–27

Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR and Pluckthun A 1997 Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J. Immunol. Methods* **201** 35–55

Lieping C 2004 Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat. Rev. Immunol*. **4** 336–347

Marty C, Langer Z, Sigrist S, Schott H, Schwendener R and Ballmer-Hofer K 2006 Isolation and characterization of a scFv antibody specific for tumor endothelial marker 1 (TEM1), a new reagent for targeted tumor therapy. *Cancer Lett*. **235** 298–308

Matthys P, Heremans H, Opdenakker G and Billiau A 1991 Anti-interferon-gamma antibody treatment, growth of Lewis lung tumours in mice and tumour-associated cachexia. *Eur. J. Cancer Clin. Oncol*. **27** 182–187

Miyamoto K, Kingsley CI, Zhang X, Jabs C, Izikson L, Sobel RA, Weiner HL, Kuchroo VK and Sharpe AH 2005 The ICOS molecule plays a crucial role in the development of mucosal tolerance. *J. Immunol*. **175** 7341–7347

Mu N, Liu N, Hao Q, Xu Y, Li J, Li W, Wu S, Zhang C and Su H 2014 Inhibition of mouse SP2/0 myeloma cell growth by the B7-H4 protein vaccine. *BMB Rep.* **47** 399–404

Nicosia SV, Bai W, Cheng JQ, Coppola D and Kruk PA 2003 Oncogenic pathways implicated in ovarian epithelial cancer. *Hematol. Oncol. Clin. North Am.* **17** 927–943

Podojil JR, Liu LN, Marshall SA, Chiang MY, Goings GE, Chen LP, Langermann S and Miller SD 2013 B7-H4Ig inhibits mouse and human T-cell function and treats EAE via IL-10/Treg-dependent mechanisms. *J. Autoimmunity* **44** 71–81

Prasad DV, Richards S, Mai XM and Dong C 2003 B7S1, a novel B7 family member that negatively regulates T cell activation. *Immunity* **18** 863–873

Salceda S, Tang T, Kmet M, Munteanu A, Ghosh M, Macina R, Pilington G and Papkoff J 2005 The immunomodulatory protein B7-H4 is overexpressed in breast and ovarian cancers and promotes epithelial cell transformation. *Exp. Cell Res.* **306** 128–141

Sica GL, Choi IH, Zhu G, Tamada K, Wang SD, Tamura H, Chapoval AI, Flies DB, Bajorath J and Chen L 2003 B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* **18** 849–861

Song H, Park G, Kim YS, Hur I, Kim H, Ryu JW, Lee HK, Cho DH, Choi IH, Lee WJ and Hur DY 2008 B7-H4 reverse signaling induces the apoptosis of EBV-transformed B cells through Fas ligand up-regulation. *Cancer Lett*. **266** 227–237

Sun Y, Wang Y, Zhao J, Gu M, Giscombe R, Lefert AK and Wang X 2006 B7-H3 and B7-H4 expression in non-small-cell lung cancer. *Lung Cancer* **53** 143–151

Takkinen K, Laukkanen ML, Sizmann D, Alfthan K, Imminen T, Vanne L, Kaartinen M, Knowles JK and Teeri TT 1991 An active single-chain antibody containing a cellulase linker domain is secreted by *Escherichia coli*. *Protein Engr.* **4** 837–841

Tringler B, Liu W, Corral L, Torkko KC, Enomoto T, Davidson S, Lucia M, Heinz D, Papkoff J and Shroyer K 2006 B7-H4 overexpression in ovarian tumors. *Gynecol. Oncol.* **100** 44–52

Van Gool SW, Vandenberghe P, de Boer M and Ceuppens JL 1996 CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model. *Immunol. Rev.* **153** 47–83

Wiendl H, Mitsdoerffer M, Schneider D, Melms A, Lochmuller H, Hohlfeld H and Weller M 2003 Muscle fibres and cultured muscle cells express the B7.1/2-related inducible co-stimulatory molecule, ICOSL: implications for the pathogenesis of inflammatory myopathies. *Brain* **126** 1026–1035

Yi KH and Chen L 2009 Fine tuning the immune response through B7-H3 and B7-H4. *Immunol. Rev.* **229** 145–151

Zheng Y, Katsaros D, Shan SJ, Longrais IR, Porpiglia M, ScorilasA, Kim NW, Wolfert RL, Simon I, Li L, Feng Z and Diamandis EP 2007 A multiparametric panel for ovarian cancer diagnosis, prognosis, and response to chemotherapy. *Clin. Cancer Res*. **13** 6984–6992

Zhu WH, Jiang CL and Zhang C 2008 Expression, purification and activity of recombinant human B7-H4IgV fusion protein. *Chinese J. Biol.* **21** 489–492

Zou W and Chen L 2008 Inhibitory B7-family molecules in the tumour microenvironment. *Nat. Rev. Immunol*. **8** 467–477

Corresponding editor: Rajiv K Saxena