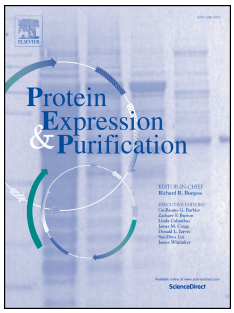
Accepted Manuscript



Expression of 5T4 extracellular domain fusion protein and preparation of anti-5T4 monoclonal antibody with high affinity and internalization efficiency

Ruixue Wang, Qinhuai Lai, Ying Lu, Yan Zhou, Liangze Tang, Yiran Tao, Yuqin Yao, Lin Yu, Yu Liu, Yuxi Wang, Ruirui Zhang, Xiaohua Jiang, Lantu Gou, Jinliang Yang

PII: S1046-5928(18)30212-2

DOI: 10.1016/j.pep.2018.07.003

Reference: YPREP 5296

To appear in: *Protein Expression and Purification*

Received Date: 16 April 2018

Revised Date: 26 June 2018

Accepted Date: 4 July 2018

Please cite this article as: R. Wang, Q. Lai, Y. Lu, Y. Zhou, L. Tang, Y. Tao, Y. Yao, L. Yu, Y. Liu, Y. Wang, R. Zhang, X. Jiang, L. Gou, J. Yang, Expression of 5T4 extracellular domain fusion protein and preparation of anti-5T4 monoclonal antibody with high affinity and internalization efficiency, *Protein Expression and Purification* (2018), doi: 10.1016/j.pep.2018.07.003.

Expression of 5T4 extracellular domain fusion protein and preparation of anti-5T4 monoclonal antibody with high affinity and internalization efficiency

Ruixue Wang[1], Qinhuai Lai[1], Ying Lu[1], Yan Zhou[2], Liangze Tang[1], Yiran Tao[1], Yuqin Yao[1,3,4], Lin Yu[1], Yu Liu[1], Yuxi Wang[1], Ruirui Zhang[1], Xiaohua Jiang[1], Lantu Gou[1], Jinliang Yang[1,4]

[1]Department of Biotherapy, Cancer Center/Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University, Chengdu, China;

[2]The Gastroenterology Tumor and Microenvironment Laboratory, Department of Gastroenterology, the First Affiliated Hospital of Chengdu Medical College, Chengdu Medical College, Chengdu, China

[3]Research Center for Public Health and Preventive Medicine, West China School of Public Health and Healthy Food Evaluation Research Center, NO. 4 West China Teaching Hospital, Sichuan University, Chengdu, China;

[4]Guangdong Zhongsheng Pharmaceutical Co., Ltd., China.

Corresponding author: Jinliang Yang, Department of Biotherapy, Cancer Center/Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan, 610041, 3-17 People Road, China, Tel: + 86 28 85502796; fax: +86 28 85502796; e-mail: jlyang01@163.com

Abbreviations: ADC, Antibody-Drug Conjugate; IHC, Immunohistochemistry; FCM, Flow Cytometry; CHO, Chinese Hamster Ovary; ECD, Extracellular Domain; ELISA, Enzyme-linked immunosorbent assay; LRR, leucine-rich repeats; EMT, Epithelial-to-Mesenchymal Transition; PEI, Polyethyleneimine; BSA, Bovine Serum Albumin; TBS, Tris Buffered Saline; SPR, Surface Plasmon Resonance; IHC, Immunohistochemistry; mAbs, monoclonal antibodies

Ethics approval and consent to participate

Animal experiments have been approved by Institutional Animal Care and Treatment Committee of Sichuan University, China.

Competing interests

The authors declare that they have no competing interests.

Grant sponsors: National Major Scientific and Technological Special Project; **Grant numbers:** 2007ZX09302-010-005-003; **Grant sponsors:** National Natural Science Foundation of China; **Grant numbers:** 81372822, 81501368, and 81672722; **Grant sponsors:** Sichuan Science and Technology Program; **Grant numbers:** 2016JY0090

Keywords: 5T4, Mammalian expression, Hybridoma, Monoclonal antibody

Abstract

5T4, a membrane protein, is overexpressed in many tumor tissues but rarely expressed in normal tissues. Here, CHO-5T4^{+} cells were generated and served as the antigen to immunize mice. Hybridoma techniques were employed to produce monoclonal antibodies (mAbs). The recombinant protein of human IgG Fc-fused extracellular domain of 5T4 (5T4 ECD-Fc) was obtained from transient expression in HEK293F cells. The fusion protein 5T4 ECD-Fc and CHO-5T4^{+} cells were respectively utilized to screen anti-5T4 antibodies that could bind to the native antigen. In preliminary screening, three hundred and fifty mAbs were obtained. Via surface plasmon resonance and flow cytometry screening, seven anti-5T4 mAbs stood out. Among them, H6 showed a high affinity (K_D=1.6×10^{-11} M) and internalization percentage (36% for 1 h and 80% for 4 h). The molecular weight and isoelectric point of H6 were determined by LC-MS and iCIEF. Moreover, the specific reactivity of H6 was demonstrated by western blotting, flow cytometry, and immunohistochemistry, respectively. In conclusion, we produced human recombinant protein of 5T4 extracellular domain and developed high-affinity

internalizing monoclonal antibodies which may be applied in the 5T4-targeting ADC therapy and basic research.

1. Introduction

5T4, also known as Trophoblast glycoprotein, was discovered in the identification of the surface molecules shared between human trophoblast and cancer cells [1]. 5T4 is an N-glycosylated protein with an apparent molecular weight of 72 kD [2-4]. It has been shown that 5T4 is overexpressed in many tumor tissues such as gastric cancer, ovarian cancer, and renal cancer, but with a restricted pattern of expression in normal tissues [5-6]. Furthermore, 5T4 may serve as a marker of cancer stem cells in NSCLC, head and neck tumor, etc. [7-8]. Moreover, 5T4 overexpression is closely correlated to poor clinical outcome in gastric cancer, colorectal carcinoma, and head and neck tumor [9-11].

5T4 protein, whose extracellular domain contains seven leucine-rich repeat regions (LRRs) flanked by cysteine-rich region, which is considered to participate in epithelial-to-mesenchymal transition (EMT) process through protein-protein interaction [12-14]. The cytoplasmic region of 5T4 contains a PDZ domain-binding motif [15], which might promote cancer metastasis; 5T4 interacts with the chemokine CXCL12 signaling and promotes the spread of 5T4-positive tumors [16-17]. Besides, 5T4 is also involved in modulation of β-catenin-independent Wnt signaling [18]. The prevalence of 5T4 and its role in carcinogenesis prompted the development of 5T4-targeting therapy such as antibody-superantigen ABR-217620 (Anyara™), vaccine MVA-5T4 (TroVax™) anti-5T4 ADCs, and the CAR-T [6, 7, 19-21]. These reports suggested 5T4 may act as a potential target for targeted therapy.

Antibody-drug conjugate (ADC), an emerging strategy for cancer immunotherapy, selectively delivers chemotherapeutic payloads into tumor cells and eliminates them. A successful ADC depends on an antibody with high affinity, internalization rate, and specificity. Highly specific mAbs in ADCs are favorable to deliver extremely cytotoxic drugs to the tumor cells through antibody-mediated internalization. Significantly, the high affinity of the antibody might favor the internalization efficiency and promote therapeutic efficacy if changes do not alter property of the antibody after conjugating with a payload [22]. Recently, much attention has been paid to 5T4-targeting therapy for cancers. Some

studies have demonstrated the potent antitumor efficacy of anti-5T4 antibody-drug conjugates such as A1-mcMMAF and 5T4-PBD in non-small cell lung cancer, head and neck tumor, etc. [7, 19, 23-24].

In our study, stable CHO-5T4$^+$ cells were generated and utilized as the antigen to immunize mice, human recombinant 5T4 fused to Fc region of human IgG was expressed in HEK-293F cells. 5T4 ECD-Fc with a high degree of glycosylation and CHO-5T4$^+$ cells were utilized to screen antibodies with high affinity and internalization rate via ELISA, FCM, and SPR. Seven anti-5T4 monoclonal antibodies were selected. The high affinity and internalization percentage of antibodies were determined by SPR and FCM. The molecular weight and isoelectric point of mAb H6 were determined by LC-MS and iCIEF analysis. Furthermore, high specificity of H6 for 5T4 was confirmed.

2. Materials and methods

2.1 Cell culture and stable cell lines generation

CHO cell, human breast cancer cell MDA-MB-231, colorectal cancer cell LoVo, and ovarian cancer cell SKOV3 were cultured in DMEM medium supplemented with 10% FBS and were incubated at 37 ℃, 5% CO_2 in a humidified incubator. Mouse myeloma cell SP2/0 and human lymphoma cells (Raji) were cultured in RPMI-1640 medium supplemented with 10% FBS at 37 ℃ in a humidified incubator with 5% CO_2. FreeStyle^{TM} 293F Cell and FreeStyle^{TM} 293 expression medium were purchased from Thermo Fisher Scientific. CHO cells were infected with lentivirus particles containing the human 5T4 encoding cDNA sequence (GeneChem, China) to generate stable 5T4-expressing cells. After selection with 4 μg/mL of puromycin, CHO-5T4$^+$ cells were identified by flow cytometry (FCM).

2.2 Expression and purification of 5T4 ECD-Fc

The extracellular domain of human 5T4 (residues 32-355) was fused into the C-terminal human IgG Fc tag to construct 5T4-ECD-Fc (Genscript, China). FreeStyle^{TM} 293F cells were seeded at the density of 8.5×10^5 cells/ml in FreeStyle^{TM} 293 expression medium. After 24 h, DNA and polyethyleneimine (PEI) were dissolved at 3.25:1 PEI-DNA mass ratio in the medium (1 μg DNA per milliliter), incubated for 10 min at

room temperature and then added into cells drop by drop. Cells were incubated at 37 ℃, 125 rpm, 75% humidity, and 5% CO_2 levels.

The culture supernatants were collected, filtered, and then purified by HiTrap™ rProtein A FF on ÄKTA™ purifier according to the manufacturer's instructions. Briefly, the sample was loaded into a pre-equilibrated HiTrap™ rProtein A FF column with buffer A (20 mM Sodium Phosphate, 150 mM NaCl, pH7.0). After being washed with buffer A, bound proteins were eluted with buffer B (0.1 M sodium citrate, pH 3.0), followed by ultrafiltration with PBS (pH 7.8). The purified proteins were determined by 10% SDS-PAGE gels.

2.3 Production of monoclonal antibodies

Six-week-old female BALB/c mice were immunized by intramuscular injection with 1×10^7 or 2×10^7 CHO-5T4$^+$ cells mixed with fast antibody adjuvant (Beijing Biodragon Immunotechnologies, China) for a total of 3 times at three-week interval. Two weeks later, 5×10^6 CHO-5T4$^+$ cells were used to boost the immunity. The serum was collected from the tail vein of vaccinated mice to detect the antibody titer using indirect ELISA. Four days after the last injection, the splenocytes were harvested and fused with the prepared myeloma cells SP2/0. Cells were cultured for 10 to 14 days. Next, the antibodies were screened out by ELISA, SPR, and FCM, and then the cells were sub-cloned at least two times by limiting dilution method.

For large-scale production of the monoclonal antibody, hybridoma cells were injected intraperitoneally into female BALB/c mice that were previously primed with incomplete Freund's Adjuvant (approximately $1\text{-}2\times10^6$ cells per mouse). About ten days later, ascitic fluids were harvested and centrifuged, and the supernatants were collected for purification by HiTrap™ Protein G HP (GE Healthcare, USA) [25].

2.4 Enzyme-linked immunosorbent assay (ELISA)

Antibody screening and the antibody titer were examined using an indirect ELISA. Microplates (Corning, USA) were coated with 5T4 ECD-Fc (1 μg/mL, 50 μL/well) in 20 mM PBS (pH 7.8) overnight at 4 ℃ and blocked with 2% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 (PBST) for 2 h at 37 ℃. Diluted serum or

hybridoma supernatant was added to the plates and incubated for 1 h at 37 ℃, followed by 100 μL of goat anti-mouse horseradish peroxidase-conjugated IgG (ThermoFisher Scientific, USA). After a final wash, 100 μL of 3,3',5,5'-tetramethylbenzidine substrate was added and the absorbance was determined at 450 nm.

2.5 Flow cytometry (FCM)

Cells were collected and washed with trypsin and PBS, respectively. 1×10^6 cells were incubated with control antibody (Bioss, China), anti-5T4 mAb (Abcam, UK) or antibody H6 (prepared in our laboratory) at 4 ℃ for 30 min, followed by FITC-conjugated anti-rabbit or anti-mouse antibody (ZXBIO, China) for 30 min at 4 ℃. The cells were suspended in PBS for analysis by FCM (Novocyte, China). For the internalization percentage of antibody, CHO-$5T4^+$ cells were incubated with antibody at 4 ℃ for 30 min, washed by PBS, and then transferred to 37 ℃ or stay at 4 ℃ for 1 h or 4 h, respectively. After centrifugation, they were incubated with FITC-conjugated anti-mouse antibody (ZXBIO, China) at 4 ℃ for 30 min. The formula was utilized to calculate the internalization percentage of the antibody: internalization percentage (%) = [(fluorescence intensity of control group - fluorescence intensity of experimental group)/fluorescence intensity of control group] × 100% [26].

2.6 Surface plasmon resonance (SPR)

SPR was performed to measure binding kinetics using Biacore T200 or X100 system (GE Healthcare, USA). 5T4 ECD-Fc was immobilized onto CM5 chip by amine-coupling in 10 mM sodium acetate buffer at pH 4.5. Different concentrations of the anti-5T4 antibodies (0.25 nM, 0.5 nM, 1 nM, 2 nM, 4 nM, 8 nM, 16 nM, 32 nM) were put through the control and detection channel at 20 μL/min. The regeneration was performed using glycine-HCl solution (pH 2.5).

2.7 Western blotting

Protein extraction was conducted using RIPA buffer (Sigma, USA) with protease inhibitors and followed by incubation with loading buffer. The proteins were separated on a 10% SDS-PAGE under non-reducing and reducing conditions and were transferred

onto a polyvinylidene difluoride (PVDF) membrane. After blocked with 5% (w/v) non-fat milk in TBST at 4 ℃ overnight, the membranes were incubated with anti-5T4 mAbs (Abcam, UK) or H6 antibody (prepared in our laboratory), followed by incubation with HRP-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, USA) or goat anti-mouse IgG (ZXBIO, China), respectively. Finally, ECL solution was added to reveal the antibody reaction.

2.8 Immunohistochemistry (IHC)

The formalin-fixed, paraffin-embedded cancer tissue slides derived from lung cancer and colorectal cancer patients were purchased from Alenabio, China. The slides were deparaffinized, rehydrated, infiltrated with target retrieval solution, blocked with goat serum, and incubated with anti-5T4 antibody H6 developed in the study or control IgG (Bioss, China) at 4 ℃ overnight. After washes, the slides were infiltrated with HRP-conjugated anti-mouse and rabbit antibody (Dako REALTM EnVisionTM Detection System, USA) for 60 min at room temperature and visualized with diaminobenzidine tetrahydrochloride (Dako REALTM EnVisionTM Detection System, USA).

2.9 iCIEF analysis

The profiling of charge heterogeneity was performed on an iCE3 system (Protein Simple). The instrument parameters were set as follows: pre-focusing time 1 min at 1500 V, focusing time 6 min at 3000 V. The CIEF ampholyte solution consisted of 4% of Carrier Ampholytes with pH 3-10, pI marker 5.12, pI marker 7.65, 0.35% of methylcellulose and 4 M urea in water. Before analysis, intact protein samples were mixed with ampholyte solution at a final concentration of 0.25 mg/mL and centrifuged at 10,000 rpm for 5 min. The electropherograms were calibrated with the pI markers and converted to Empower software (Version: 4.1.1) for data analysis.

3. Results

3.1 Expression, purification, and characterization of human recombinant 5T4 extracellular domain

The pFuse hIgG1-Fc2/5T4ECD expression vector was constructed. Next, the supernatant of transfected HEK293F cells was collected, concentrated and purified by protein A affinity chromatography (Fig. 1A). The purified 5T4 ECD-Fc showed that the protein under non-reducing condition was larger than their counterparts under reducing condition. This may be attributed to polymerization under non-reducing condition. (Fig. 1B). At last, 60 mg of pure 5T4 ECD-Fc were acquired at a final concentration of 6 mg/mL with 10 mL with an 83% total yield (Table 1). Besides, Western blot assay showed that purified 5T4 ECD-Fc harbored a larger molecular weight than 65 kD as predicted. This may be attributed to glycosylation of the protein. (Fig. 1C), Moreover, the pI was around 7.2 to 7.4 as identified by iCIEF (Fig. 1D).

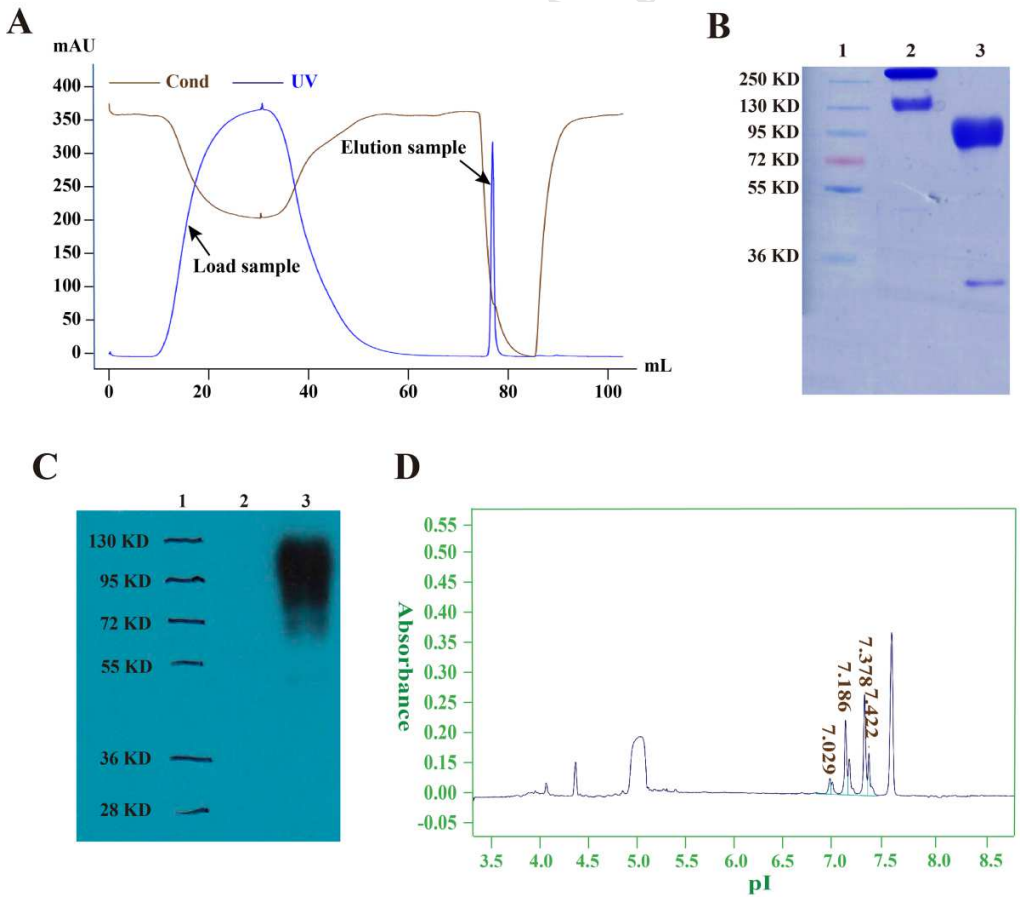


Fig. 1 Purification and identification of human recombinant 5T4 extracellular domain. (A) 5T4 ECD-Fc Purification by HiTrap™ rProtein A FF column. (B) 5T4 ECD-Fc detected by SDS-PAGE. Line 1, prestained protein ladder; line 2, non-reducing 5T4 ECD-Fc, line 3, reducing 5T4 ECD-Fc. (C) Western blotting analysis of the purified 5T4 ECD-Fc with anti-5T4 mAbs as the primary antibody. Line 1, protein ladder; line 2, the concentrated supernatant of non-transfected HEK 293F cells under reducing condition; line 3, the purified 5T4 ECD-Fc under reducing condition. (D) The pI of 5T4 ECD-Fc analyzed by capillary isoelectric focusing electrophoresis.

Table 1. Purification of Fc-tagged 5T4 ECD expressed in HEK293F cells.

Sample	Volume (mL)	Protein concentration (mg/mL)	Total amount (mg)	Purity (%)	Yield (%)
Culture medium	1000	0.6	600	12[a]	/
Elution 5T4 ECD-Fc	26	2.5	65	95	90[b]
Final product of 5T4 ECD-Fc after concentration and dialysis	10	6	60	95	83[c]

The final storage buffer of 5T4 ECD-Fc: PBS, pH 7.8.
[a]The percentage of 5T4 ECD-Fc in total protein is about 12%.
[b]The total amount of 5T4 ECD-Fc in culture medium is 600×12%=72 mg, and the yield of elution 5T4 ECD-Fc is 65/(600×12%)=90%.
[c]The yield of 5T4 ECD-Fc in the final product is 60/(600×12%)=83%.

3.2 Preparation of monoclonal antibodies against human 5T4

To prepare monoclonal antibodies against human 5T4, CHO-5T4$^+$ cells were identified with a high level of 5T4 expression on the cell surface by FCM (Fig. 2). Next, BALB/c mice were immunized with CHO-5T4$^+$ cells and antibodies were obtained by hybridoma technology. Firstly, ELISA was used to select antibodies that could bind to 5T4 ECD-Fc, and FCM was performed to screen antibodies with high binding ability to CHO-5T4$^+$. And then, SPR was utilized to screen antibodies with high affinity. Finally, FCM was used to screen antibodies with robust internalization. The hybridoma cells were sub-cloned at least two times by limiting dilution method. Thus, seven monoclonal antibodies stood out with high affinity and internalization percentage. Then, antibodies with high purity were prepared from the ascitic fluids using protein G affinity chromatography for accurate analysis of affinity and internalization percentage (Fig. 3A).

Two bands of about 50 and 25 kD were observed by SDS-PAGE analysis, which corresponded to the heavy chain and light chain of the antibodies. Besides, the purity of the antibodies was more than 95% as determined by SDS-PAGE (Fig. 3B).

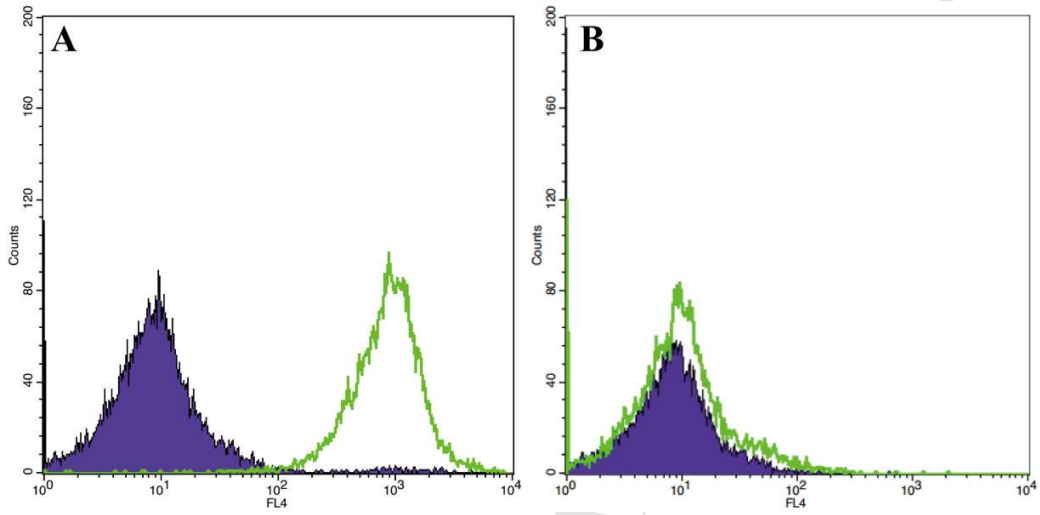


Fig. 2 Identification of CHO-5T4^{+} cell lines by FCM. The purple represented cells incubated with control IgG as the primary antibody for 30 minutes on the ice. The green represented cells incubated with anti-5T4 mAbs for 30 minutes on the ice. (A) CHO cells infected with lentivirus containing human 5T4 gene. (B) CHO cells infected with lentivirus containing the empty vector.

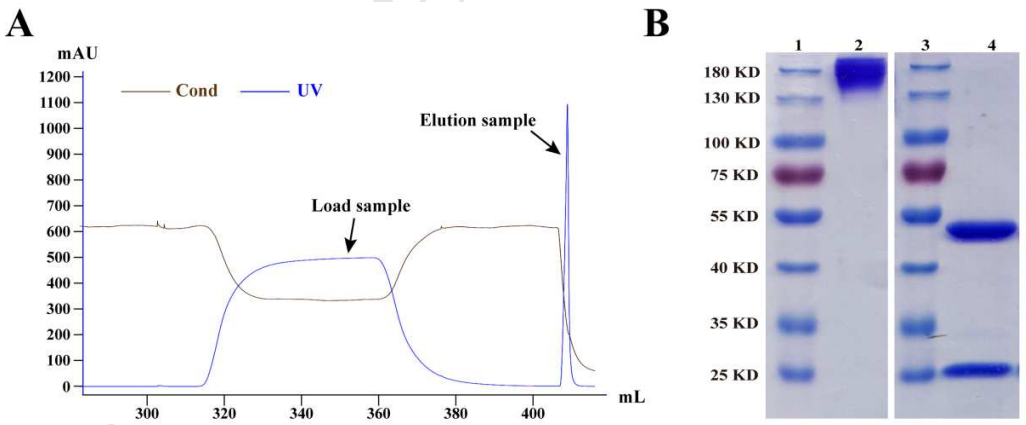


Fig. 3 Production and purification of the anti-5T4 antibody. (A) Purification of the anti-5T4 antibody by protein G affinity chromatography. (B) SDS-PAGE analysis of anti-5T4 antibody. The purity of anti-5T4 antibody was above 95%. Line 1 and 3, protein ladder; line 2, antibody under non-reducing condition; line 4, antibody under reducing condition.

3.3 Affinity and internalization percentage of monoclonal antibodies

The accurate affinity and internalization percentage were detected using SPR and FCM, respectively. The affinity and internalization percentage of antibodies were listed in Table 2. Among the obtained antibodies, H6 showed high affinity and internalization percentage (Table 2). The K_a, K_d, and K_D of H6 against 5T4 were 8.21×10^{5} 1/MS, 1.31×10^{-5} 1/S, and 1.6×10^{-11} M separately calculated by Biacore-evaluation Software (Fig. 4A). FCM was performed to further characterize the affinity of H6 to CHO-5T4^{+} cells. As shown in Fig. 4B, monoclonal antibody H6 bound to CHO-5T4^{+} cells in a concentration-dependent manner. The antibody concentration of H6 required for 50% binding activity was 3.6 μg/mL (Fig. 4B). The internalization percentage was detected about 36% for 1 h and 80% for 4 h in CHO-5T4^{+} cells, respectively (Fig. 4C).

Table 2. Affinity and internalization percentage of candidate anti-5T4 antibodies.

Antibody name	K_a (1/MS)	K_d (1/S)	K_D (M)	Internalization percentage (%) for 4 h
A2	6.15×10^{4}	1.60×10^{-4}	2.60×10^{-9}	50
R3	6.03×10^{4}	4.73×10^{-5}	7.84×10^{-10}	66
G4	1.97×10^{5}	1.28×10^{-4}	6.50×10^{-10}	58
G5	1.54×10^{5}	2.41×10^{-6}	1.56×10^{-11}	66
P3	1.61×10^{5}	2.27×10^{-6}	1.41×10^{-11}	45
W5	1.24×10^{4}	2.05×10^{-7}	1.65×10^{-11}	65
H6	8.21×10^{5}	1.31×10^{-5}	1.6×10^{-11}	80

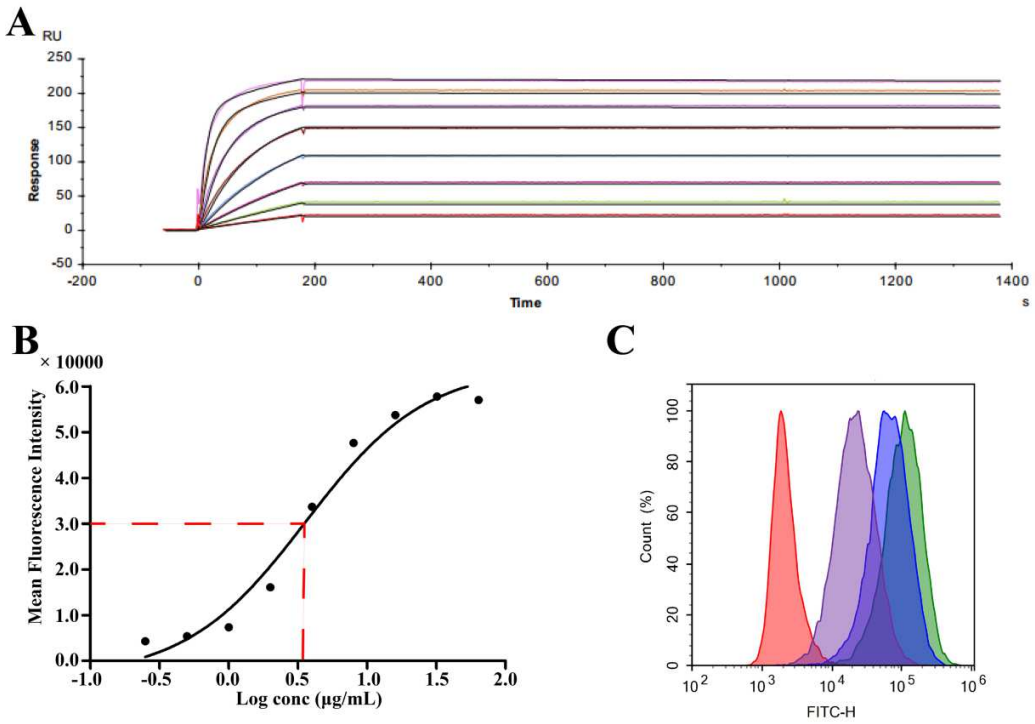


Fig. 4 Affinity and internalization analysis of mAb H6. (A) Biacore analysis of the binding kinetics of H6 to 5T4 ECD-Fc. H6 was assayed at different concentrations. The lines from top to the bottom represented H6 at the concentration of 64 nM, 32 nM, 16 nM, 8 nM, 4 nM, 2 nM, 1 nM, 0.5 nM, 0.25 nM, respectively. (B) Binding of H6 to CHO-5T4^{+} cells by FCM. The black solid dot from left to the right represented the mean fluorescence intensity of H6 at the concentration of 0.25 μg/mL, 0.5 μg/mL, 1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL, 32 μg/mL, and 64 μg/mL, respectively. The red line represented the antibody concentration of H6 required for 50% binding activity. (C) Internalization of H6 on CHO-5T4^{+} cells by FCM. Red line represented cells incubated with hIgG isotype, green line with H6 on the ice, blue line with H6 shifted to 37 ℃ for 1 h, purple line with H6 shifted to 37 ℃ for 4 h.

3.4 Molecular weight and isoelectric point of mAb H6

LC-MS and iCIEF were respectively performed to determine the molecular weight and isoelectric point of mAb H6. Isoelectric point (pI) of H6 was 6.2 as identified by iCIEF (Fig. 5A). Besides, the molecular weight of the entire antibody H6 was accurately determined 148.6 kD by LC-MS (Fig. 5B). These results reflected the homogeneity of antibody H6.

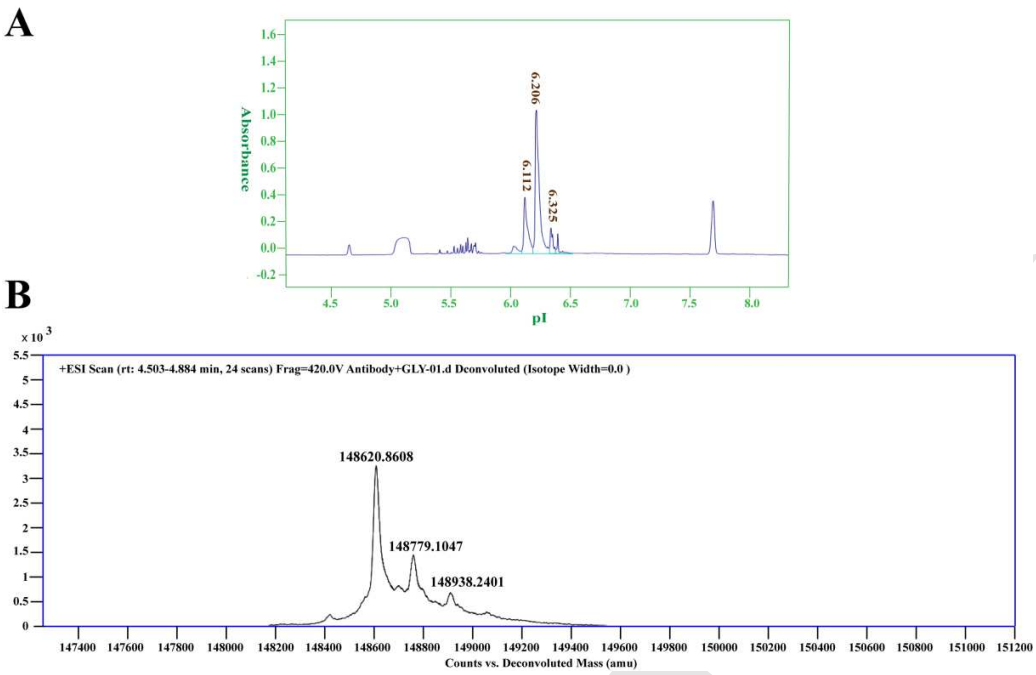


Fig. 5 The pI and molecular weight of mAb H6. (A) The pI of mAb H6 was analyzed by capillary isoelectric focusing electrophoresis. Experimental conditions are described in the materials and methods. (B) LC-MS analysis of antibody H6. The glycosylated form of intact antibody H6 was analyzed by an Agilent 1290 Infinity LC coupled with an Agilent 6550 iFunnel Q-TOF LC/MS System.

3.5 Specificity of mAb H6

To characterize the specific reactivity of H6 against 5T4, western blotting, FCM, and IHC analysis were performed using H6 as the primary antibody. The protein band was detected in the lysates of CHO-5T4$^+$ but not CHO-neo cells by western blotting, suggesting the specific immunoreactivity of H6 for 5T4 (Fig. 6A). Moreover, H6 could only recognize 5T4 under the non-reducing condition but not the reducing condition, suggesting H6 is a conformation-dependent antibody.

Here, the FCM results showed that H6 bound to 5T4-positive cells such as CHO-5T4$^+$, MDA-MB-231, and SKOV3 but did not bind to 5T4-negative cells such as CHO-neo, LoVo, and Raji (Fig. 6B). Formalin fixed paraffin-embedded human lung cancer and colorectal cancer tissue slides were used to test the specificity of H6 by IHC. Positive staining was shown in the membrane and cytoplasm of the tumor cells, but not shown in the adjacent normal tissues (Fig. 6C). Besides, no immunostaining was

observed in IgG control. These results demonstrated mAb H6 was highly specific to 5T4 both in cell lines and tissues.

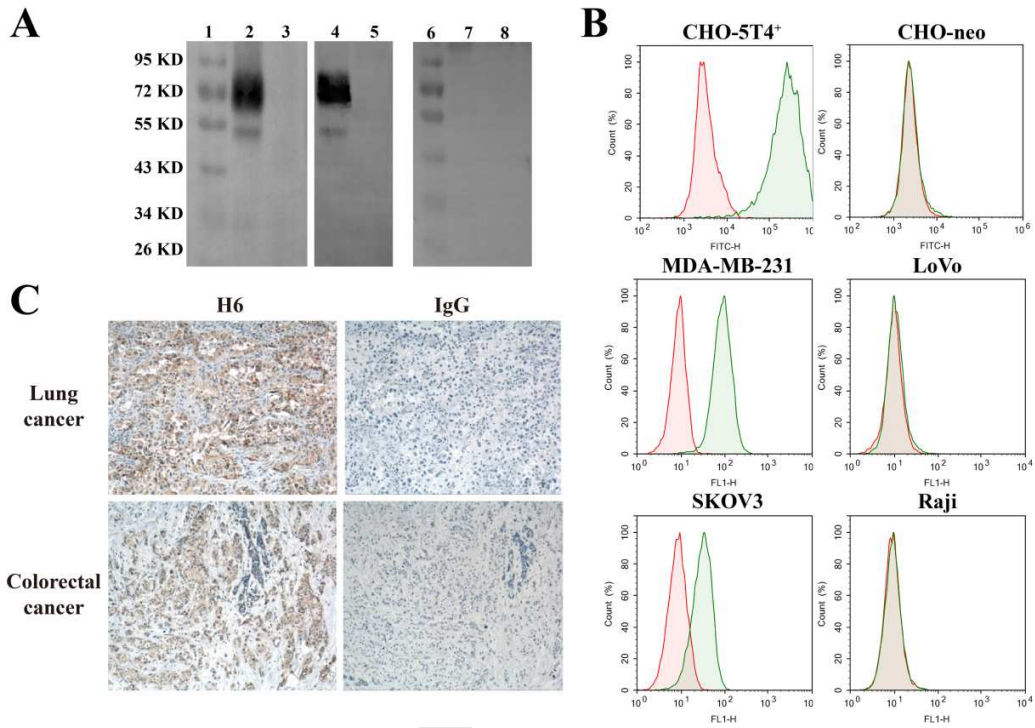


Fig. 6 Specificity of mAb H6 by western blot, FCM, and immunohistochemistry. (A) The specificity of H6 in CHO-5T4^{+} by Western blot. Line 1 and 6 represented protein ladder; 2 and 3 represented cell lysates under non-reducing condition incubated with H6 in CHO-5T4^{+} cells and in CHO-neo cells, respectively; 4 and 5 represented cell lysates under non-reducing condition incubated with positive control antibody in CHO-5T4^{+} cells and in CHO-neo cells, respectively; 7 and 8 represented cell lysates under the reducing condition incubated with H6 in CHO-5T4^{+} cells and in CHO-neo cells, respectively. (B) The specificity of H6 to 5T4 on the cell surface by FCM. The red line represented cells incubated with control mouse IgG, the green line with antibody H6. (C) The specificity of H6 by IHC. The slides incubated with antibody H6 (left panel) or isotype IgG (right panel). The formalin-fixed paraffin-embedded lung cancer tissues (upper panel) and colorectal cancer tissues (bottom panel).

4. Discussion

5T4, an N-glycosylated transmembrane protein, is overexpressed in a wide spectrum of cancers, including gastric cancer, renal cancer, etc., but rarely expressed in normal tissues [5-6]. The high expression level of 5T4 on the surface of cancer cells, along with

the efficient internalization mediated by 5T4 has promoted the development of 5T4-targeting antibody-drug conjugates [7, 19, 23-24]. In order to prepare the 5T4-targeting antibody used in ADCs, cell lines stably expressing 5T4 were utilized to immunize mice to produce antibodies that could bind to the native conformation of 5T4, and the methods of ELISA, FCM, and SPR were combined to establish an efficient screening system. The recombinant protein 5T4 ECD-Fc, a highly glycosylated form revealed by a broad range of molecular weight in western blotting (Fig. 1C), favored the antibody selection for the native antigen. Besides, 5T4 ECD-Fc was coated at a low concentration of 50 ng/well in ELISA, which might help to screen the antibodies with strong binding ability. Moreover, it is generally regarded that FCM is an efficient method to identify antibodies against native antigens [27-28]. Thus, FCM was utilized in this study to screen antibodies that bound to 5T4 with native conformation. Next, SPR was performed to measure the affinity kinetics by characterizing the associating and dissociating processes. More than 70% of the antibodies showed good kinetics by SPR assay (data not shown). Finally, the internalization percentage of candidates were determined by FCM. Based on the high-throughput and systematic screening platform, we developed several superior anti-5T4 antibodies.

A successful ADC usually requires a monoclonal antibody with high affinity and internalization efficiency [29-31]. The high affinity of antibody might also promote the internalization efficiency and ultimately influence the pharmacodynamics of ADCs [32-34]. Therefore, multiple methods were employed to screen antibodies with high affinity including ELISA, FCM, and SPR. The affinity of H6 ($K_D=1.6\times10^{-11}$ M) measured by SPR was higher than that of previously reported anti-5T4 antibody [19]. The antibody concentration of H6 required for 50% binding activity was 3.6 μg/mL (Fig. 4B). These data demonstrated that the high affinity of H6 to 5T4 expressed on the cell surface and 5T4 ECD-Fc recombinant protein.

In addition, the efficient internalization ability plays an in important role in the biodistribution of ADCs [35]. Rapidly internalizing mAbs might promote antibody accumulation in tumors, which may induce the strong anti-tumor activity of ADCs [36]. Thus, in the study, the internalization ability was set as one of the core criterions in the screening procedures. 36% and 80% of H6 can be dramatically internalized once binding

to 5T4 for 1 h and 4 h, respectively (Fig. 4C). Therefore, the mAb H6 with a high internalization rate has the potential to be a carrier of payloads.

Western blotting, FCM, and immunohistochemistry were performed to analyze the reactivity and specificity of H6. Western blotting results exhibited conformation-dependent specificity of H6 to CHO-5T4^{+} lysates rather than CHO-neo lysates. In addition, the specific reactivity of H6 in human lung cancer and colorectal cancer tissues was confirmed by immunohistochemistry. Furthermore, the iCIEF and LC-MS results showed that H6 has a pI of 6.2 and molecular weight of 148.6 kD (Fig. 5A, Fig. 5B), indicating a high homogeneity of H6. However, further studies are needed for precise epitope mapping, for the leucine-rich repeats of the 5T4 molecule are shared by lots of proteins with various function and expression.

In the study, a mammalian expression system was established to produce highly glycosylated human recombinant 5T4 extracellular domain. Next, seven anti-5T4 monoclonal antibodies were obtained by hybridoma technique and the screening methods consisting of ELISA, SPR, and FCM methods. Among the seven anti-5T4 mAbs, H6 showed the high affinity, internalization rate, and specificity. In conclusion, we produced human recombinant 5T4 extracellular protein and prepared high-affinity internalizing monoclonal antibodies which may be applied in the 5T4-targeting ADCs therapy as well as basic research.

Acknowledgments

This study was supported by National Major Scientific and Technological Special Project [grant number 2007ZX09302-010-005-003], National Natural Science Foundation of China [grant numbers 81372822, 81501368, and 81672722], and Sichuan Science and Technology Program [grant numbers 2016JY0090].

References

[1] P.L. Stern, R. Harrop, 5T4 oncofoetal antigen: an attractive target for immune intervention in cancer. Cancer Immunol, Immunother 66 (2017) 415-426.
[2] N. Hole, P.L. Stern, Isolation and characterization of 5T4, a tumour-associated antigen. Int J Cancer 45 (1990) 179-184.
[3] D.M. Shaw, K.A. Woods AMMyers, C. Westwater, V. Rahi-Saund, M.J. Davies, Glycosylation and epitope mapping of the 5T4 glycoprotein oncofoetal antigen. Biochem J 363 (2002) 137-145.

[4] J.M. Boyle, K.H. Grzeschik, P.R. Heath, J.E. Morten, P.L. Stern, Trophoblast glycoprotein recognised by monoclonal antibody 5T4 maps to human chromosome 6q14-q15. Hum Genet 84 (1990) 455.
[5] P. Southall, G. Boxer, K. Bagshawe, N. Hole, M. Bromley, P. Stern, Immunohistological distribution of 5T4 antigen in normal and malignant tissues. Br J Cancer 61 (1990) 89-95.
[6] R.W. Griffiths, D.A. Gilham, V. Ramani, N.W. Clarke, P.L. Stern, R.E. Hawkins, Expression of the 5T4 oncofoetal antigen in renal cell carcinoma: a potential target for T-cell-based immunotherapy. Br J Cancer 93 (2005) 670-677.
[7] S.A. Kerk, K.A. Finkel, A.T. Pearson, K.A. Warner, Z. Zhang, F. Nör, V.P. Wagner, P.A. Vargas, M.S. Wicha, E.M. Hurt, 5T4-targeted therapy ablates cancer stem cells and prevents recurrence of head and neck squamous cell carcinoma. Clin Cancer Res 23 (2017) 2516-2527.
[8] M. Damelin, K.G. Geles, M.T. Follettie, P. Yuan, M. Baxter, J. Golas, J.F. DiJoseph, M. Karnoub, S. Huang, V. Diesl, Delineation of a cellular hierarchy in lung cancer reveals an oncofetal antigen expressed on tumor-initiating cells. Cancer Res 71 (2011) 4236-4246.
[9] T. Starzynska, P. Marsh, P.F. Schofield, S.A. Roberts, K.A. Myers, P.L. Stern, Prognostic significance of 5T4 oncofetal antigen expression in colorectal carcinoma. Br J Cancer 69 (1994) 899-902.
[10]F.V. Castro, O.J. Mcginn, K. Shekhar, M. Georgi, L. Jian, A.J. Rutkowski, E. Eyad, B. Debbie, H. Mark, V. Rasilaben, 5T4 oncofoetal antigen is expressed in high risk of relapse childhood pre-B acute lymphoblastic leukemia and is associated with a more invasive and chemotactic phenotype. Leukemia 26 (2012) 1487-1498.
[11] T. Starzynska, A. Wiechowska-Kozlowska, K. Marlicz, M. Bromley, S.A. Roberts, M. Lawniczak, B. Kolodziej, A. Zyluk, P.L. Stern, 5T4 oncofetal antigen in gastric carcinoma and its clinical significance. European Journal of Gastroenterology & Hepatology 10 (1998) 479.
[12] Y. Zhao, T. Malinauskas, K. Harlos, E.Y. Jones, Structural insights into the inhibition of Wnt signaling by cancer antigen 5T4/Wnt-activated inhibitory factor 1. Structure 22 (2014) 612-620.
[13] B. Kobe, J. Deisenhofer, Proteins with leucine-rich repeats. Current opinion in structural biology 5 (1995) 409-416.
[14] B. Kobe, A.V. Kajava, The leucine-rich repeat as a protein recognition motif. Current opinion in structural biology 11 (2001) 725-732.
[15] A. Awan, M.R. Lucic, D.M. Shaw, F. Sheppard, C. Westwater, S.A. Lyons, P.L. Stern, 5T4 interacts with TIP-2/GIPC, a PDZ protein, with implications for metastasis. Biochemical and biophysical research communications 290 (2002) 1030-1036.
[16] T.D. Southgate, O.J. McGinn, F.V. Castro, A.J. Rutkowski, M. Al-Muftah, G. Marinov, G.J. Smethurst, D. Shaw, C.M. Ward, C.J. Miller, CXCR4 mediated chemotaxis is regulated by 5T4 oncofetal glycoprotein in mouse embryonic cells. PloS one 5 (2010) e9982.
[17] O.J. McGinn, G. Marinov, S. Sawan, P.L. Stern, CXCL12 receptor preference, signal transduction, biological response and the expression of 5T4 oncofoetal glycoprotein. J Cell Sci 125 (2012) 5467-5478.
[18] B. Kagermeierschenk, D. Wehner, G. Ozhankizil, H. Yamamoto, J. Li, K. Kirchner, C. Hoffmann, P. Stern, A. Kikuchi, A. Schambony, Waif1/5T4 inhibits Wnt/β-catenin signaling and activates noncanonical Wnt pathways by modifying LRP6 subcellular localization. Dev Cell 21 (2011) 1129-1143.
[19] P. Sapra, M. Damelin, J. Dijoseph, K. Marquette, K.G. Geles, J. Golas, M. Dougher, B. Narayanan, A. Giannakou, K. Khandke, Long-term tumor regression induced by an antibody-drug conjugate that targets 5T4, an oncofetal antigen expressed on tumor-initiating cells. Mol Cancer Ther 12 (2013) 38-47.
[20] G. Forsberg, N.J. Skartved, M. Wallénohman, H.C. Nyhlén, K. Behm, G. Hedlund, T. Nederman, Naptumomab estafenatox, an engineered antibody-superantigen fusion protein with low toxicity and reduced antigenicity. J Immunother 33 (2010) 492-499.
[21] R.J. Amato, M. Stepankiw, Evaluation of MVA-5T4 as a novel immunotherapeutic vaccine in colorectal, renal and prostate cancer. Future Oncology 8 (2012) 231-237.

[22] J. Harper, S. Mao, P. Strout, A. Kamal, Selecting an Optimal Antibody for Antibody–Drug Conjugate Therapy: Internalization and Intracellular Localization, Humana Press, 2013.
[23] O.J. Mcginn, S. Krishnan, J.P. Bourquin, P. Sapra, C. Dempsey, V. Saha, P.L. Stern, Targeting the 5T4 oncofetal glycoprotein with an antibody drug conjugate (A1mcMMAF) improves survival in patient-derived xenograft models of acute lymphoblastic leukemia. Haematologica 102 (2017) 1075-1084.
[24] G.I. Shapiro, U.N. Vaishampayan, P. LoRusso, J. Barton, S. Hua, S.D. Reich, R. Shazer, C.T. Taylor, D. Xuan, H. Borghaei, First-in-human trial of an anti-5T4 antibody-monomethylauristatin conjugate, PF-06263507, in patients with advanced solid tumors. Investigational new drugs 35 (2017) 315-323.
[25] Y. Shen, Y. Wang, X. Jiang, L. Lu, C. Wang, W. Luo, Y. Zhang, P. Li, Z. Du, T. Dai, Preparation and characterization of a high-affinity monoclonal antibody against human epididymis protein-4. Protein Expression & Purification 141 (2017) 44.
[26] H. Zhang, Y. Wang, Y. Wu, X. Jiang, Y. Tao, Y. Yao, Y. Peng, X. Chen, Y. Fu, L. Yu, Therapeutic potential of an anti-HER2 single chain antibody-DM1 conjugates for the treatment of HER2-positive cancer. Signal Transduction and Targeted Therapy 2 (2017) 17015.
[27] Y. Phung, W. Gao, Y.G. Man, S. Nagata, M. Ho, High-affinity monoclonal antibodies to cell surface tumor antigen glypican-3 generated through a combination of peptide immunization and flow cytometry screening. Mabs 4 (2012) 592-599.
[28] Y. Nakadate, S. Yoshimura, N. Nakatani, M. Azuma, T. Tachibana, Generation of a monoclonal antibody against the extracellular domain of IGF-1R. Monoclon Antib Immunodiagn Immunother 33 (2014) 126-128.
[29] P.J. Carter, P.D. Senter, Antibody-drug conjugates for cancer therapy. The Cancer Journal 14 (2008) 154-169.
[30] E.G. Kim, K.M. Kim, Strategies and Advancement in Antibody-Drug Conjugate Optimization for Targeted Cancer Therapeutics. Biomolecules & Therapeutics 23 (2015) 493-509.
[31] P.J. Carter, P.D. Senter, Antibody-drug conjugates for cancer therapy. The Cancer Journal 14 (2008) 154-169.
[32] S.I. Rudnick, J. Lou, C.C. Shaller, Y. Tang, A.J.P. Kleinszanto, L.M. Weiner, J.D. Marks, G.P. Adams, Influence of Affinity and Antigen Internalization on the Uptake and Penetration of Anti-HER2 Antibodies in Solid Tumors. Cancer Res 71 (2011) 2250.
[33] J. Leelawattanachai, K.W. Kwon, P. Michael, R. Ting, J.Y. Kim, M.M. Jin, Side-by-Side Comparison of Commonly Used Biomolecules That Differ in Size and Affinity on Tumor Uptake and Internalization. Plos One 10 (2015) e0124440.
[34] M.M. Schmidt, G.M. Thurber, K.D. Wittrup, Kinetics of anti-carcinoembryonic antigen antibody internalization: effects of affinity, bivalency, and stability. Cancer Immunology Immunotherapy 57 (2008) 1879.
[35] G.G. Bornstein, Antibody Drug Conjugates: Preclinical Considerations. AAPS Journal 17 (2015) 525-534.
[36] M. Yoshikawa, Y. Mukai, Y. Okada, Y. Tsumori, S. Tsunoda, Y. Tsutsumi, W.C. Aird, Y. Yoshioka, N. Okada, T. Doi, Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody. Blood 121 (2013) 2804-2813.

Highlights:

1. CHO cells stably expressing 5T4 were established and identified
2. 5T4 extracellular domain with high purity was obtained by mammalian expression
3. Anti-5T4 antibodies were prepared with high affinity and internalization rate
4. The molecular weight and isoelectric point of antibody H6 were determined.
5. The specificity of H6 was identified by western blot, FCM, and immunohistochemistry