



Original Article

Production of monoclonal antibodies against carcinoembryonic antigen-related cell adhesion molecule 6 and detection of their binding affinities

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ABSTRACT

Objectives: Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is a glycoposphatidylinositol-anchored member of the immunoglobulin superfamily, often overexpressed in various malignancies. Targeting CEACAM6 by suppressing its expression can potentially reverse these effects, making it a promising therapeutic target. In this study, we generated five monoclonal antibodies (CEAS1, CEAS2, CEAS3, CEAS4, and CEAS5; CEAS1-S5) against the recombinant CEACAM6 protein. **Materials and Methods:** Through enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) assay, we demonstrated that each antibody specifically binds to CEACAM6 without interfering with the binding of others. SPR analysis further revealed the rate of association (K_a), dissociation (K_d), and equilibrium dissociation constants (K_D) for each antibody. **Results:** The K_D values ranged from 5.089×10^{-11} to 1.213×10^{-13} M, with CEAS5 exhibiting the highest binding affinity. In addition, CEAS5, unlike CEAS1-S4, could bind to both CEACAM5 and CEACAM6, indicating its bivalent nature. **Conclusion:** These findings highlight the strong antigen-binding capabilities of CEAS1-S5, warranting further investigation.

KEYWORDS: *Carcinoembryonic antigen-related cell adhesion molecule, Enzyme-linked immunosorbent assay, Monoclonal antibodies, Surface plasmon resonance*

Submission : 20-Dec-2024
Revision : 20-Jan-2025
Acceptance : 31-Mar-2025
Web Publication : 17-Jun-2025

INTRODUCTION

Malignant tumors are a leading cause of death in many countries. The treatment approach for cancer patients – whether surgery, radiation therapy, or chemotherapy – depends on the type and stage of the tumor [1]. The use of antibodies in cancer diagnosis and treatment is of immense value. Targeted therapies, primarily developed from monoclonal antibodies, are employed either as standalone treatments or in combination with other therapies [2]. Consequently, identifying new tumor markers and developing targeted therapeutic antibodies remain crucial objectives for medical researchers.

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is a member of the immunoglobulin superfamily, linked to the cell membrane through a glycoposphatidylinositol (GPI) anchor [3]. Human CEACAM6 is a GPI-anchored membrane protein with a molecular weight of approximately 90 kDa [4]. Its N-terminus consists of 34 amino acid residues, followed by

a signal sequence and an extracellular domain (286 amino acid residues). The C-terminus contains a hydrophobic pro-peptide composed of 24 amino acid residues, which, after cleavage, allows the protein to attach to the cell membrane through the GPI anchor. The amino acid residues 35–142 of CEACAM6 form V-type Ig-like domains (IgV), while residues 145–232 and 237–314 constitute the first and second C-type immunoglobulin-like domains (C2 type 1 and C2 type 2), respectively. Although GPI-anchored proteins lack transmembrane and intracellular domains, they can influence intracellular signaling through “lipid rafts” on the cell membrane, thereby affecting the biological behavior of cells.

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Supplementary material available online

Access this article online

Quick Response Code:



Website: www.tcmjmed.com

DOI: 10.4103/tcmj.tcmj_331_24

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How to cite this article: Wang YH, Chen YH. Production of monoclonal antibodies against carcinoembryonic antigen-related cell adhesion molecule 6 and detection of their binding affinities. *Tzu Chi Med J* 2025;37(4):395-402.

CEACAM6 is frequently overexpressed in various malignant tumors, and its upregulation is linked to the epithelial-mesenchymal transition (EMT), which enhances invasiveness, chemoresistance, anoikis resistance, and metastatic potential. These effects have been observed in several cancers, including colorectal cancer [5-7], breast cancer [6,8,9], non-small cell lung cancer (NSCLC) [10], intrahepatic cholangiocarcinoma [11], pancreatic adenocarcinoma [12,13], head and neck cancer [14], and gastric adenocarcinoma [15]. Targeting CEACAM6 to suppress its expression could counteract these processes, positioning it as a promising therapeutic target. Given these findings, CEACAM6 shows significant potential for cancer detection and treatment.

In addition to its role in cancer, CEACAM6 is involved in modulating immune responses. It can influence immune cell functions, such as neutrophil adhesion and migration [16]. CEACAM6 is also expressed in intestinal epithelial cells, where it plays a role in modulating immune activity and preventing pathogen infections [17]. In certain cancers, however, CEACAM6 expression may help tumor cells evade immune surveillance by disrupting normal immune functions [18].

Beyond CEACAM6, CEACAM5 has also been identified as a key marker associated with malignancies, making it a promising target for therapeutic development using monoclonal antibodies [7,19,20]. However, there have been relatively few bispecific monoclonal antibodies developed to target both CEACAM5 and CEACAM6 simultaneously. In this study, we aim to design and produce novel monoclonal antibodies that specifically target CEACAM6. Furthermore, we seek to develop a new bispecific monoclonal antibody capable of recognizing both CEACAM5 and CEACAM6, potentially expanding its therapeutic applications.

MATERIALS AND METHODS

Production of recombinant His-tagged carcinoembryonic antigen-related cell adhesion molecule 6 and carcinoembryonic antigen-related cell adhesion molecule 5 protein

To produce a mature form of recombinant CEACAM6 and CEACAM5 protein, PCR fragments for the extracellular domain of CEACAM6 cDNA (corresponding to amino acid residues 35–320) and CEACAM5 cDNA (corresponding to amino acid residues 35–685) were prepared by PCR from full-length cDNA clones of CEACAM6 (NM_002483.7) and CEACAM5 (NM_001291484.3) (OriGene Corp., Rockville, MD) using following primers containing restriction-recognition sites for NdeI and XhoI, CEACAM6 NdeI F: 5'CATATGAAGCTCACTATTGAATCCACGCC3' and CEACAM6 XhoI R: 5'CTCGAGTTATCCAGAGACTGTGATCATCGTGAC3' for CEACAM6; CEACAM5 NdeI F: 5'CATATGAAGCTCACTATTGAATCCACGCC3' and CEACAM5 XhoI R: 5'CTCGAGGAGAAGTTCCAGATGCAGAGACTG3' for CEACAM5, respectively, and inserted into pGEM®-T-easy plasmid (Promega, Madison, WI, USA). The cDNA fragments were excised from the pGEM®-T-easy plasmid using NdeI and

XhoI restriction enzymes. The resulting DNA fragments were then purified and subsequently ligated into the corresponding sites of the pET-15b expression vector (Novagen, Madison, WI, USA). The expression constructs described above were verified by DNA sequencing and transformed into *Escherichia coli* for protein expression. After verification by DNA sequencing, this expression construct was transformed into *E. coli* (Rosetta-gami2(DE3) pLysS). His-tagged fusion CEACAM6 and CEACAM5 proteins were expressed in *E. coli* with 1 mM isopropyl-β-D-thiogalactopyranoside for 16 h at 30°C. Following the induction, the bacterial cells were subjected to lysis by sonication in equilibration buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7). To purify the His-CEACAM6 protein, the soluble fraction was loaded onto a TALON® Metal Affinity Resin (Clontech, Palo Alto, CA) and was eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7, 500 mM imidazole). The resulting purified His-CEACAM6 protein in the eluate was dialyzed against PBS and used for mouse immunization. To produce recombinant CEACAM6 protein used in ELISA and surface plasmon resonance (SPR) assay, the cDNA fragment was excised from the pGEM®-T-easy plasmid using the restriction enzymes NdeI and XhoI. The resulting DNA fragments were then purified and subsequently ligated into the NcoI and XhoI sites of the pET-32b expression vector (Novagen, Madison, WI, USA). After transformation into *E. coli* (Rosetta-gami2(DE3)pLysS), expression of the recombinant fusion proteins, N-terminally tagged with Thioredoxin (Trx) and C-terminally tagged with His-tag, for CEACAM6, was induced with 0.6 mM isopropyl-β-D-thiogalactopyranoside for 16 h at 30°C. Following the induction, the bacterial cells were subjected to lysis, purification, and dialyzed as described above. Recombinant Trx was also induced and purified as a control.

Production of recombinant carcinoembryonic antigen-related cell adhesion molecule 6 containing different structural domains

To produce recombinant CEACAM6 with structural domain V-type Ig-like domain (V) spanning amino acid residues 35-142, primers CEACAM6 NdeI F: 5'CATATGAAGCTCACTATTGAATCCACGCC3' and CEACAM6 XhoI V R: 5'CTCGAGTTACGGGTATACATGGAAGTGTCC3' were used in PCR and the cDNA containing CEACAM6 V domain was inserted into the pET-15b as described above. To produce recombinant CEACAM6 with structural domain C-type Ig-like domains type 1 (C2 type 1 spanning amino acid residues 145-232) and type 2 (C2 type 2 spanning amino acid residues 237-314), fused to His-tag and T7 gene 10 protein, primers CEACAM6 NdeI C1 F: 5'CATATGGAGCTGCCAAGCCCTCC3' and CEACAM6 XhoI C1 R: 5'CTCGAGTTAATTCAGGGTGACTGGGT CAC3' were used in PCR and the cDNA containing CEACAM6 V domain, and primers CEACAM6 NdeI C2 F: 5'CATATGGTCTCTATGGCCC3' and CEACAM6 XhoI R: 5'CTCGAGTTATCCAGAGACTGTGATCATCGTGAC3', was inserted into the pScreen1b (Novagen, Madison, WI, USA), respectively, as described above.

Production of monoclonal antibodies

We sent the recombinant protein His-CEACAM6 to a company for customized monoclonal antibodies production (Yao-Hong Biotechnology Inc., Taipei, Taiwan). In brief, mice were immunized by injecting recombinant protein His-CEACAM6 for several weeks. Afterward, the antibody titers in the mice serum were measured using an enzyme-linked immunosorbent assay (ELISA) with detection by a SpectraMax microplate spectrophotometer. Spleen cells from high-titer mice were extracted and fused with myeloma cells in a PEG-containing solution. Fusion cells secreting anti-His-CEACAM6 antibodies were selected using a HAT culture medium, resulting in five fusion cell lines. The monoclonal antibodies produced and purified with Protein G were CEAS1, CEAS2, CEAS3, CEAS4, and CEAS5 (CEAS1-S5). The use of small vertebrate animals was approved by the Koo Foundation Sun Yat-Sen Cancer Center Institutional Animal Care and Use Committee (ID number: 20100908-2)

Western blotting for purified recombinant protein

To detect purified CEACAM6, a Western blot was performed using a biotin-labeled anti-CEACAM6 polyclonal antibody (LS-C442291, LS bio, Shirley, MA). Following electrophoretic separation on an SDS-PAGE gel, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with the biotin-labeled anti-CEACAM6 antibody at the appropriate dilution, followed by detection using streptavidin-conjugated alkaline phosphatase and a chromogenic substrate.

Enzyme-linked immunosorbent assay for evaluation of monoclonal antibodies binding to recombinant carcinoembryonic antigen-related cell adhesion molecule 6

To assess the binding of the primary antibodies CEAS1-S5 to recombinant CEACAM6, an ELISA assay was performed. Each well of an ELISA plate was coated with 50 μ L of recombinant CEACAM6 protein in PBS-azide (0.02%) and incubated overnight at 4°C (some wells were coated with recombinant Trx as negative control). The assays were conducted at room temperature. After three washes with 150 μ L washing buffer (PBS containing 0.2% Tween-20), each well was blocked with 150 μ L blocking buffer (PBS containing 2% BSA and 0.05% Tween-20) for 30 min. Following three additional washes, 50 μ L of CEAS1-S5 was added to each well in varying concentrations. Wells were incubated for 20 min and then washed three times. Subsequently, each well was incubated with 50 μ L of goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (ThermoFisher Scientific) diluted 1:500 in blocking buffer for 45 min. Finally, each well was incubated with 100 μ L alkaline phosphatase substrate for 60 min, and the absorbance was measured at 405 nm using a microplate reader. All experiments were in triple-repeated. Data were presented as average \pm SD, using Microsoft Excel software.

Covalent immobilization of thioredoxin-tagged carcinoembryonic antigen-related cell adhesion molecule 6 onto a sensor chip

Immobilization onto a sensor chip CM5 (GE Healthcare) was performed using a Biacore T100 (GE Healthcare) via amine coupling using an Amine coupling kit (GE Healthcare) according to the manufacturer's instructions. The Biacore T100 software was used to create an immobilization method, specifying the chip type and target immobilization level. The thioredoxin-tagged CEACAM6 was immobilized in Flow Cell 2, while Trx was Flow Cell 1 to serve as a blank.

The kinetic and affinity evaluation by surface plasmon resonance

Experiment design for kinetic and affinity evaluation of CEAS1, CEAS2, CEAS3, CEAS4, or CEAS5 binding to Trx-tagged CEACAM6 was conducted using SPR on a Biacore T100 instrument (GE Healthcare) using Biacore T100 control software version 2.0.1 and multi-cycle analysis mode. The resulting sensograms were analyzed by Biacore T100 evaluation software version 2.0.1. Briefly, CEAS1, CEAS2, CEAS3, CEAS4, or CEAS5 as analytes were injected over the chip surface at concentrations of 1000 nM, 500 nM, 250 nM, 125 nM, 62.5 nM, and 0 M at a flow rate of 30 mL/min in a running buffer containing 1X HBS-P (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20) (GE Healthcare). The binding responses were recorded in real-time to generate sensorgrams, allowing the determination of association (K_a) and dissociation (K_d) rate constants. These values were used to calculate the equilibrium dissociation constant (K_D), providing a measure of the affinity of each CEAS for the immobilized CEACAM6. Flow Cell 1, containing only thioredoxin, was used as a blank to account for non-specific binding.

RESULTS

Preparation of carcinoembryonic antigen-related cell adhesion molecule 6 antigen and the production of monoclonal antibodies

Human CEACAM6 is a 344 amino-acids (a.a.) polypeptide (1–344 a.a.), which will undergo proteolytic cleavage at both N- and C-terminal (35–320 a.a., ~34 kDa). Then the proteolytic cleavage product (35–320 a.a.) will be modified with a GPI anchor on the C-terminal, and consequently become a mature form of CEACAM6 [Figure 1a]. To increase the solubility of the CEACAM6 recombinant protein, Thioredoxin (Trx, ~14 kDa) and His-tag were fused to the N- and C-terminals, respectively [Figure 1a]. To produce the Trx-CEACAM6-His recombinant protein as antigens, the extracellular region (extracellular region) of Trx-CEACAM6-His recombinant protein (including amino acid residues 35 to 320) was purified using the *E. coli* expression system and nickel ion affinity column chromatography. After SDS-PAGE analysis, a ~48 kDa protein (indicated to be Trx-CEACAM6-His, 14 + 34 = 48 kDa) was purified and observed [Figure 1b]. Subsequently, this ~48 kDa was confirmed as Trx-CEACAM6-His recombinant protein by Western blotting analysis [Figure 1c]. It was used as an antigen to immunize mice to produce anti-CEACAM antibodies. Then, the antibody titer in the serum was measured using

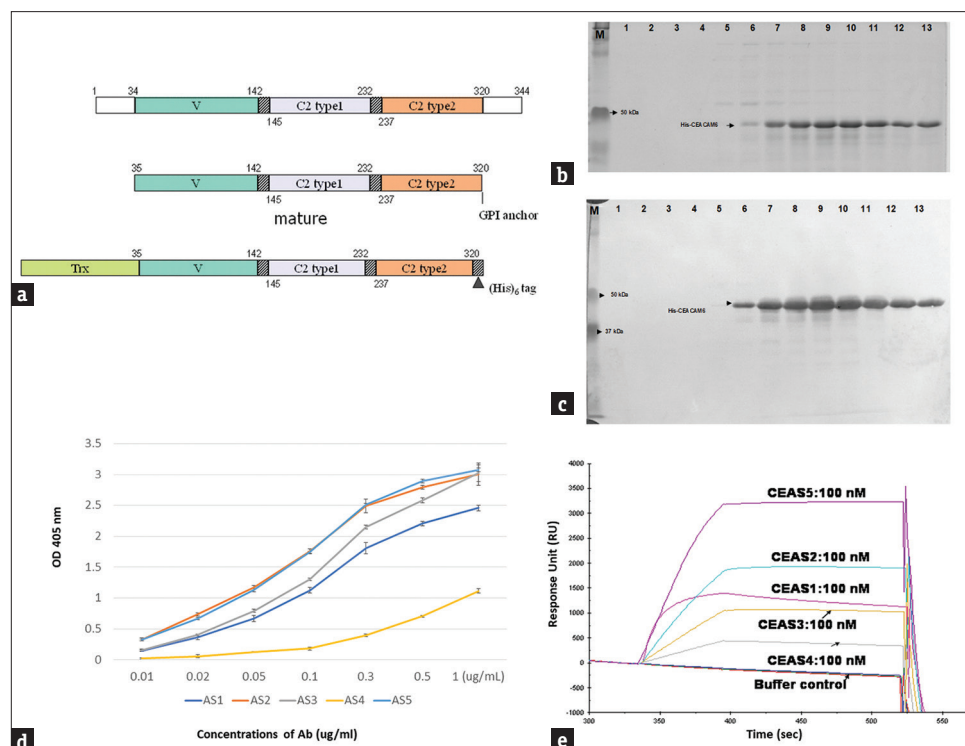


Figure 1: Characterizations of recombinant human thioredoxin-carcinoembryonic antigen-related cell adhesion molecule 6 (Trx-CEACAM6-His) and evaluation of the binding activities of CEAS1-S5 to Trx-CEACAM6-His. (a) Schematic illustration of human CEACAM6 (full-length human CEACAM6, 1–344 a.a., top panel) and mature form of human CEACAM6 (35–320 a.a., middle panel). Mature form of human CEACAM6, including V-type Ig-like domain, C-type Ig-like domains type 1 and type 2 are fused to His-tag and Trx as indicated (thioredoxin-fused human CEACAM 6 (Trx-CEACAM6, bottom panel). (b) This recombinant protein was purified from TALON® Metal Affinity Resin, resolved by SDS-PAGE and subjected to Coomassie brilliant blue staining. (c) Immunoblotting assays were conducted using mouse monoclonal antibodies recognizing His-tag. Immunoblotting were developed by alkaline phosphatase-conjugated secondary antibodies against mouse IgG. M: Molecular weight marker; Lane 1 ~ 13, eluted fractions from TALON® Metal Affinity Resin. (d and e) For enzyme-linked immunosorbent assay (ELISA) experiment, ELISA plate was coated with Trx-fused human CEACAM 6 (Trx-CEACAM6) in PBS-azide (0.02%) overnight at 4°C. CEAS1-S5 were added into each well at different concentrations as indicated. Each well was incubated with diluted anti-mouse antibodies with alkaline phosphatase conjugate and detected with alkaline phosphatase substrate. Absorbance of each well was measured at 405 nm in a microplate reader. (f) Surface plasmon resonance (SPR) analysis, His-CEACAM6 was captured by Ni²⁺-NTA sensor chip. The overlay plot shows sensorgrams of CEAS1-S5 binding to CEACAM 6 versus time (second). Binding response (changes in mass concentration detected as differences in refractive index by SPR) is shown in resonance units (RU). Reference-corrected sensorgrams for 100 nM of CEAS1-S5 antibodies were monitored on a His-CEACAM6 surface. Buffer controls were also injected

an immunoenzyme method (Enzyme-Linked Immunosorbent Assay, ELISA). The spleen cells of high-titer mice were removed and fused with myeloma cells in a PEG solution. The fusion tumor cell lines that secreted anti-CEACAM6 antibodies were screened in a culture medium containing HAT. A total of five fusion tumor cell lines were obtained. The monoclonal antibodies produced by each hybridoma cell line and purified with Protein G were named CEAS1, CEAS2, CEAS3, CEAS4, and CEAS5 (CEAS1-S5).

Next, we conducted the ELISA experiments to evaluate the antigen-binding abilities of each monoclonal antibody (CEAS1-S5). The His-CEACAM6 recombinant protein was used as an antigen for coating on the ELISA plates. At low concentration (0.01 µg/mL) of antibodies, the intensities of OD₄₅₀ were 0.148 ± 0.015 (CEAS1), 0.323 ± 0.015 (CEAS2), 0.157 ± 0.015 (CEAS3), 0.023 ± 0.012 (CEAS4), and 0.33 ± 0.026 (CEAS5). At high concentration (0.01 µg/mL), CEAS2 (3.003 ± 0.0182), CEAS3 (3.023 ± 0.0136), and CEAS5 (3.077 ± 0.030) had higher OD intensities. The results suggested that the antigen-binding activities of CEAS2, AS3, and AS5 are better than CEAS1 and CEAS4 [Figure 1d]. In addition,

the SPR biosensor was used to evaluate the interaction between antigens (recombinant CEACAM6) and antibodies (CEAS1-S5). As shown in Figure 1e, no resonance unit (RU) was detected after the buffer was injected (Buffer control). However, there were quite obvious reaction signals (500 ~ 2500 RU) after CEAS1-S5 were injected. The results showed that all monoclonal antibodies (CEAS1-S5) were able to bind His-CEACAM6, and CEAS5 had the highest binding affinity [Figure 1e].

Dynamic analysis of the binding affinity between monoclonal antibodies (CEAS1-S5) and carcinoembryonic antigen-related cell adhesion molecule 6

To understand the binding affinity between CEAS1-S5 and CEACAM6, the SPR biosensing method was used for dynamic analysis to detect antigen-antibody binding. Regarding the binding activity (association constant, shown as K_a) and the dissociation activity (dissociation constant, shown as K_d) to the antigen, it was found that all antibodies (CEAS1-S5) possess extremely excellent antigen-binding abilities (equilibrium dissociation constant, K_D, decreasing to approximately the picomolar range). In particular, the K_D of CEAS5 is 1.213 × 10⁻¹³M (the lowest among all tested antibodies),

suggesting that monoclonal CEAS5 has the highest binding affinity to CEACAM6 [Supplementary Table 1].

Identification of the binding regions for monoclonal antibodies CEAS1-S5

To further identify the antigen recognition regions of monoclonal antibodies CEAS1-S5, three expression vectors that encoded different regions of recombinant protein CEACAM6 were constructed. The three regions are full length (FL) of His-CEACAM6 (contained V, C2 type I, and C2-type II); (1) amino acid residues 35–142 (V-type Ig-like domains); (2) amino acid residues 145 to 232 (C-type immunoglobulin-like region type I, C2 type I); and (3) amino acid residues 237 to 314 (C2 type II) [Figure 2a]. Then, these expression vectors were expressed and purified in *E. coli*, and stained with Coomassie blue [Figure 2b]. Western blotting experiments showed that monoclonal antibodies (CEAS1-S5) could recognize the full-length of His-CEACAM6 recombinant protein [\sim 34 kDa, Figure 2c-g]. In addition, ELISA and SPR biosensing experiments were performed to record the binding affinities between these five antibodies and three different regions of the antigen (His-CEACAM6). The ELISA results showed that CEAS1 only binds to the C2 type I region [Figure 3a]. In contrast, monoclonal antibodies, CEAS2, CEAS3, and CEAS5 (0.05 and 0.5 μ g/mL) have binding abilities to these three regions (C2 type I, C2 type II, and V-type) [Figure 3b-e].

CEAS4 can bond to these three regions, but only in higher concentrations (0.5 and 5 μ g/mL) [Figure 3d]. Similar results were also observed in the SPR experiments, indicating that CEAS1 only binds to the C2 type I region and CEAS5 has the highest binding activity to CEACAM6, no matter which regions (C2 type I, C2 type II, and V-type) [Figure 3f-h].

CEAS1-S5 monoclonal antibodies bound to carcinoembryonic antigen-related cell adhesion molecule 6 at different binding positions

Next, we used the SPR biosensing method to detect whether CEAS1-S5 monoclonal antibodies compete with each other for the same antigen binding positions or not. As shown in Figure 4, a signal (\sim 1300 RU) was observed at around 450 s, indicating that CEAS1 bound to CEACAM6 and formed a CEAS1-CEACAM6 complex. When CEAS1 was injected again, because the binding site on the CEACAM6 is occupied by CEAS1, the RU value did not change a lot (please see the signals at the position of around 550–700 s, bottom line). Instead, another antibody (CEAS2) was injected again, and an increasing RU volume was observed, indicating that the CEAS1 and CEAS2 bound to different epitopes; it may form a CEAS1-CEAS2-CEACAM6 complex (please see the signals and the represented art work at the position of around 550–700 s, upper line). Repeating this method for testing, it was found that the antigen-binding regions of the CEAS1-S5 monoclonal antibodies did not overlap with each other. Taken

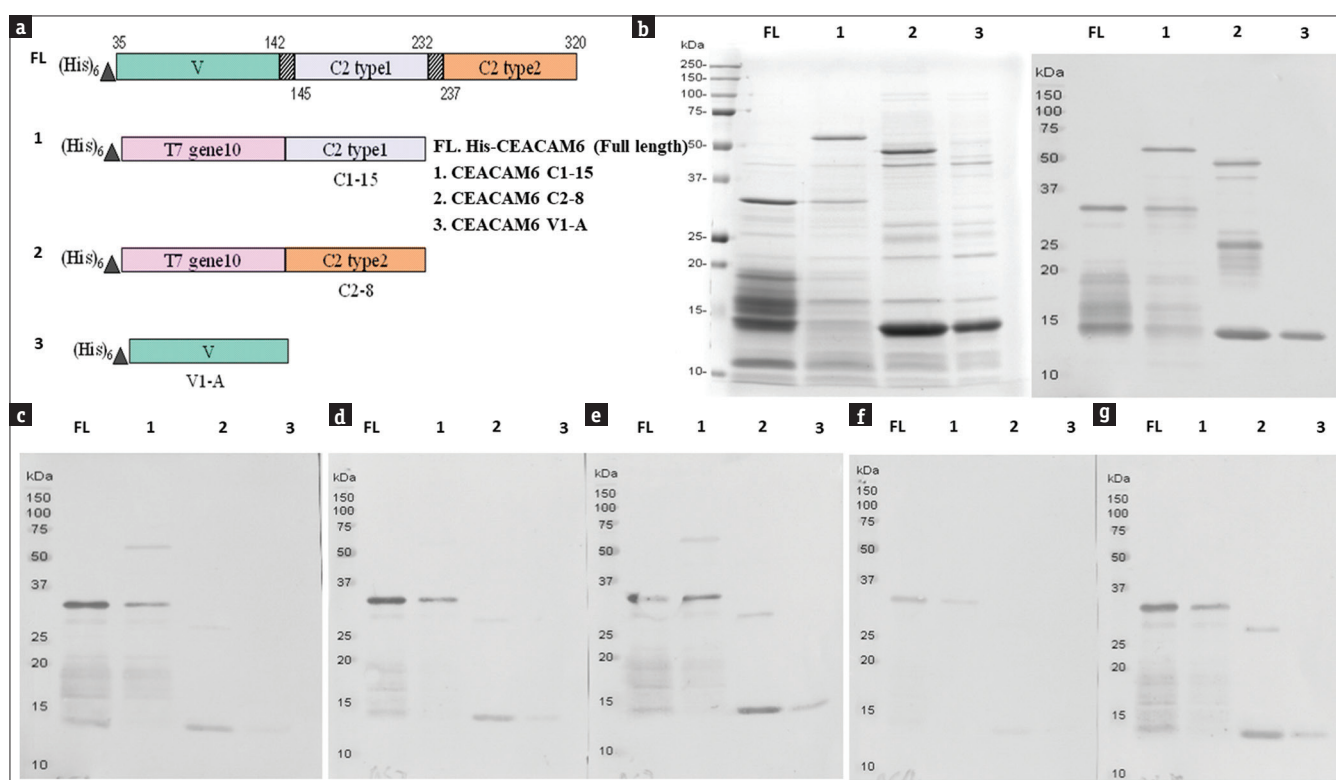


Figure 2: Expression of different domain of human carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) recombinant protein and evaluation of the binding activities of antibodies CEAS1-S5. (a) Four expression plasmids were constructed: full-length human CEACAM6 with a His tag in the N-terminal (His-CEACAM6); (1) T7-gene-10-protein-fused with C-type-Ig-like domains type 1 (CEACAM6 C1-15); (2) T7-gene-10-protein-fused with C-type-Ig-like domains type 2 (CEACAM6 C2-8); and (3) His-tagged V-type Ig-like domains (V1-A) of CEACAM6 (CEACAM6 V1-A). (b) Coomassie blue staining (left panel) or Western blot analysis (right panel) using anti-His antibody was shown. Western blot analysis of monoclonal antibodies (c) CEAS1, (d) CEAS2, (e) CEAS3, (f) CEAS4, and (g) CEAS5 to the different recombinant protein as indicated on the top of each lane

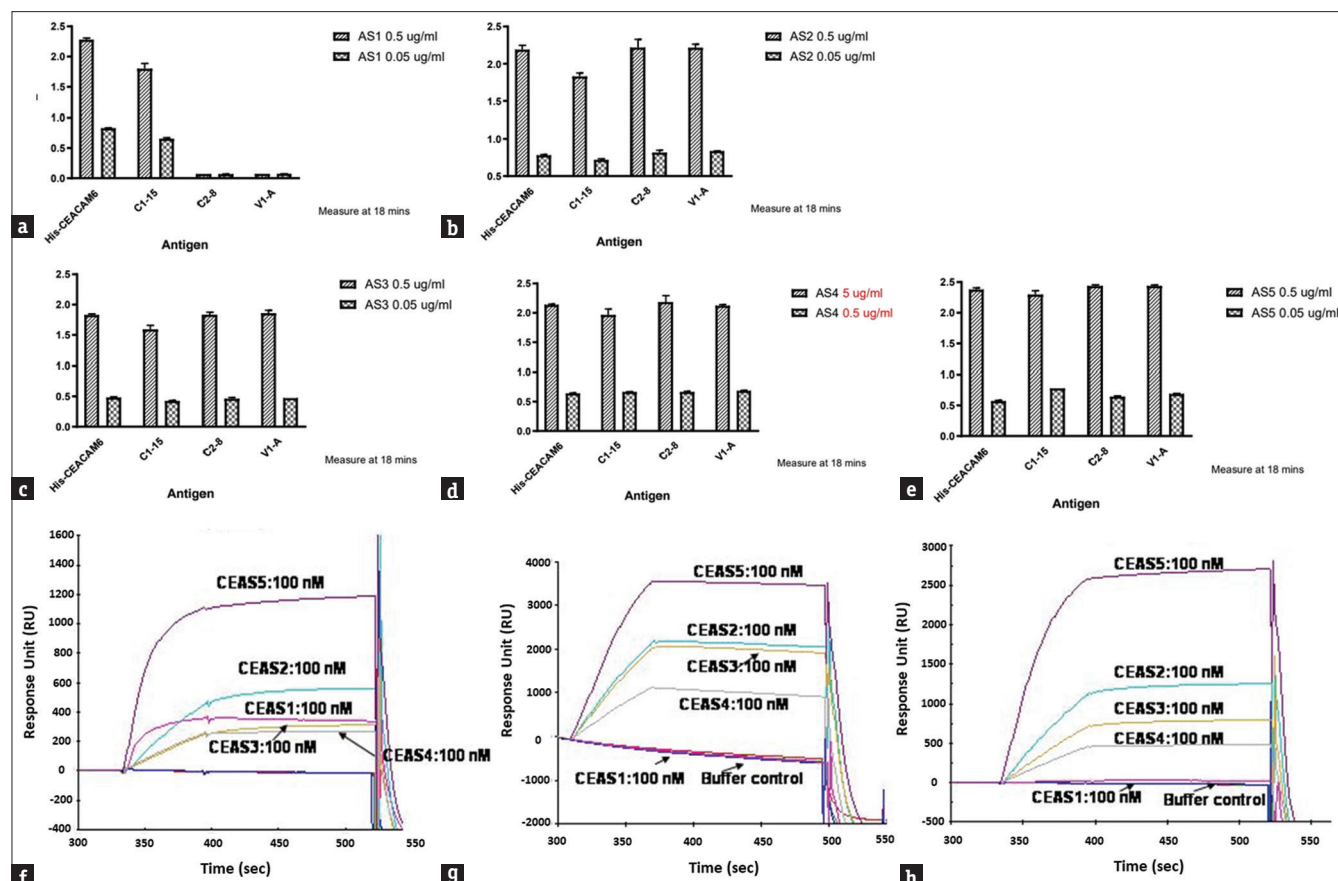


Figure 3: Epitope mapping of CEAS1-S5 to human carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) by enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) analysis. ELISA plates were coated with CEACAM6 (His-CEACAM6), CEACAM6 C1-15, CEACAM6 C2-8, or CEACAM6 V1-A as mentioned in Figure 2. CEAS1 (AS1; a), CEAS2 (AS2; b), CEAS3 (AS3; c), CEAS4 (AS4; d) or CEAS5 (AS5; e) were added to wells at concentrations as described. Each well was incubated with diluted anti-mouse antibodies with alkaline phosphatase conjugate and detected with alkaline phosphatase substrate. Absorbance of each well was measured at 405 nm in a microplate reader. (f-h) CEACAM6 C1-15, CEACAM6 C2-8, and CEACAM6 V1-A were captured by Ni²⁺-NTA sensor chip. The overlay plot shows sensorgrams of CEAS1-S5 binding to CEACAM6 versus time (second). Binding response (changes in mass concentration detected as differences in refractive index by SPR) is shown in resonance units (RU). Reference-corrected sensorgrams for 100 nM of CEAS1-S5 antibodies were monitored on a His-CEACAM6 surface. Buffer controls were also injected

together, we suggested that CEAS1-S5 monoclonal antibodies do not compete with each other for binding to CEACAM6.

Evaluation of the cross-reaction activity of monoclonal antibodies CEAS1-S5 to another high homology protein, carcinoembryonic antigen-related cell adhesion molecule 5

CEACAM5 is a well-known cancer marker, especially for colorectal and non-small cell lung cancer [7,21]. CEACAM6 and CEACAM5 share part of the epitope region [4]; thus, we expressed and purified the extracellular region of CEACAM5. Afterward, the recombinant CEACAM5 was coated on a 96-well plate, and a certain concentration of CEAS1-S5 was added to perform ELISA experiments. As a result, CEAS1-S4 were unable to bind CEACAM5 [Figure 5a-d], only CEAS5 cross-reacted with CEACAM5 [Figure 5e]. This means that CEAS5 can act as a bivalent antibody to recognize both CEACAM5 and CEACAM6.

DISCUSSION

In this study, we developed five monoclonal antibodies (CEAS1-S5) targeting the tumor marker CEACAM6. Each

antibody exhibits a high binding affinity for CEACAM6, with equilibrium dissociation constants (K_D) ranging from 10^{-11} to 10^{-13} [Supplementary Table 1]. Typically, the K_D values for over 60% of antibodies targeting their specific antigens fall between 10^{-7} and 10^{-9} [22], making the affinities of CEAS1-S5 exceptionally high, with CEAS5 showing the greatest binding affinity. High-affinity antibodies form stable complexes with their targets, which enhances the immune response by facilitating more effective neutralization and clearance of pathogens or toxins [23]. Consequently, antibodies with such high affinities benefit vaccine development, detection, and therapeutic applications.

Tumors that are frequently double positive for CEACAM5 and CEACAM6 include colorectal adenocarcinomas, pancreatic ductal adenocarcinoma, gastric carcinoma, NSCLC, and breast cancer [3]. These markers play a significant role in both diagnostic and therapeutic approaches, especially in targeted therapies and monitoring disease progression. Numerous therapeutic strategies have been developed to target CEACAM6, often in combination with other markers rather than as a standalone target. These strategies include

monoclonal antibodies, antibody-drug conjugates, and bispecific antibodies. For instance, an anti-CEACAM6 antibody has been shown to inhibit the growth of xenografted pancreatic ductal adenocarcinoma in a mouse model [24]. CEACAM6 has also been identified as a target for chimeric antigen receptor T (CAR-T) immunotherapy in pancreatic ductal adenocarcinoma [25]. In addition, treatment with an anti-CEACAM6 antibody was reported to inhibit tumor growth in NSCLC xenografted mice [26,27]. Regarding bispecific antibodies, some are designed to target both CEACAM6 and other tumor-associated antigens, such as CEACAM5, enhancing the immune system's ability to recognize and attack CEACAM5 and CEACAM6 double-positive cancer cells.

Additionally, CEACAM6 shares similar domains with CEACAM5 in the N-terminal region. Previous studies have shown that some antibodies targeting the N-terminal region

of CEACAM6 (V-type Ig-like domains, V1-A) also bind to CEACAM5 [28]. Our study demonstrated that CEAS5 can bind to both CEACAM5 and CEACAM6 [Figure 5e], properly because CEAS5 is able to recognize the V1-A domain of CEACAM6 [Figure 2g].

Several anti-CEACAM6 antibodies have been identified in previous studies. For example, the AP11 antibody (which targets CEACAM6) has been reported to recognize paraffin-embedded human tumor tissues [29], and the 2A3 antibody was shown to detect CEACAM6-expressing tumor cells [30]. While we conducted Western blot, ELISA, and SPR experiments to evaluate the binding affinities of the CEAS1-S5 antibodies, a limitation of our study is that we have not yet tested the effectiveness of CEAS1-S5 in cultured human tumor cells or tumor tissue sections. We plan to address this in future work.

CONCLUSION

We produced 5 high affinity monoclonal antibodies (CEAS1-S5) against CEACAM6. In special, monoclonal antibody CEAS5 has the highest affinity and can act as a bivalent antibody to recognize both CEACAM5 and CEACAM6.

Data availability statement

Data sharing does not apply to this article as no new data were created or analyzed in this study.

Financial support and sponsorship

The Koo Foundation Sun Yat-Sen Cancer Center (KFSYSCC) supported this study by providing research facilities and instruments.

Conflicts of interest

There are no conflicts of interest.

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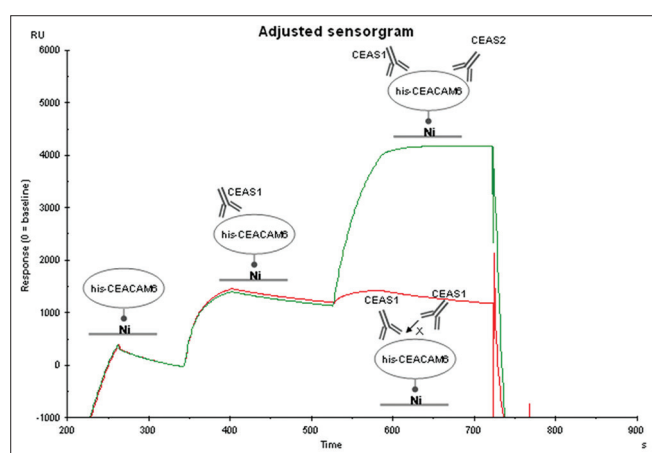


Figure 4: CEAS1 and CEAS2 bind to different epitopes of His-carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6). His-CEACAM6 was captured by Ni²⁺-NTA sensor chip. The overlay plot shows sensorgrams of sequential bindings of monoclonal antibodies as illustrated. First binding: CEAS1 to His-CEACAM6 (red); second binding: CEAS1 (red) or CEAS2 (green) to CEAS1-His-CEACAM6 complex. The binding response (changes in mass concentration detected as differences in refractive index by surface plasmon resonance) is shown in resonance units. Reference-corrected sensorgrams were monitored on a His-CEACAM6 surface

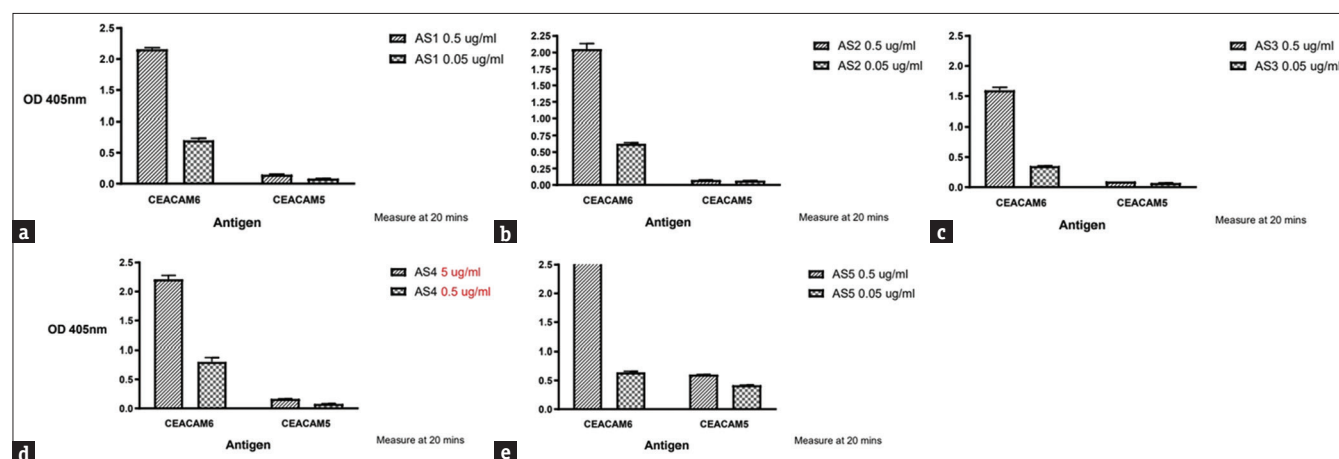


Figure 5: Cross-reactivity of CEAS1, CEAS2, CEAS3, CEAS4 and CEAS5 antibodies to human carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5). Enzyme-linked immunosorbent assay plates were coated with His-CEACAM6 or His-tagged CEACAM5 (containing extracellular domain AA32-685). CEAS1 (a), CEAS2 (b), CEAS3 (c), CEAS4 (d), and CEAS5 (e) antibodies were added to wells at concentrations as described

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SUPPLEMENTARY MATERIAL

Supplementary Table 1: Evaluation of CEAS1, CEAS2, CEAS3, CEAS4, and CEAS5 kinetic and affinity to His-carcinoembryonic antigen-related cell adhesion molecule 6 by surface plasmon resonance assay

Antibody	Rate of association K_a ($M^{-1}s^{-1}$)	Rate of dissociation K_d (s^{-1})	Equilibrium dissociation constant K_D (M)
CEAS1	1.260×10^6	6.411×10^{-5}	5.089×10^{-11}
CEAS2	1.260×10^5	6.919×10^{-8}	5.491×10^{-13}
CEAS3	1.157×10^5	7.277×10^{-7}	6.289×10^{-12}
CEAS4	2.775×10^5	2.700×10^{-6}	9.729×10^{-12}
CEAS5	5.100×10^5	6.186×10^{-8}	1.213×10^{-13}