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COMMUNICATION

Development of a highly thermostable immunoassay based on a nanobody-alkaline phosphatase fusion protein for carcinoembryonic antigen detection

Jingtao Lin[1] · Jianli Yu[2,3] · Huan Wang[4] · Yanru Xu[1] · Fei Li[4] · Xiaoheng Chen[4] · Yunlong Liang[2] · Jinsong Tang[1] · Lili Wu[1] · Zhengwei Zhou[1] · Cailing Chen[1] · Minjuan Liu[1] · Xuan Chun[1] · Rui Nian[2] · Haipeng Song[4]

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Abstract

Carcinoembryonic antigen–related cell adhesion molecule 5 (CEACAM-5) assays are employed in routine clinical settings to diagnose tumor. We selected two nanobodies with high-affinity to CEACAM-5, termed Nb11C12 and Nb2D5, using phage-display technology. The Nb2D5 fused with calf intestinal alkaline phosphatase (CAP), human placental alkaline phosphatase (HAP), or *Pyrococcus abyssi* alkaline phosphatase (PAP) were expressed in human embryonic kidney (HEK293) cells. The enzymatic activity of Nb2D5-HAP fusion protein was the best and remained stable at 60 °C for 7 days. The affinity of Nb2D5-HAP fusion protein to CEACAM-5 reached 42 pM. A chemiluminescent enzyme immunoassay (CLEIA) based on Nb2D5-HAP fusion protein was established for quantitative CEACAM-5 assay in clinical settings. The CLEIA exhibited a wide linear range of 0.31–640 ng/mL toward CEACAM-5, with a limit of detection (LOD) of 0.85 ng/mL. No cross-reactivity occurred with CEACAM-1, CEACAM-3, CEACAM-6, or CEACAM-8, and no interference was observed with rheumatoid factors. The CLEIA based on Nb2D5-HAP fusion protein was stable for 8 weeks at 37 °C and 50% relative humidity. The CLEIA developed from Nb2D5-HAP fusion protein had much better stability and linearity with similar reproducibility compared with the enzyme-linked immunosorbent assay developed from conventional monoclonal antibodies, which have been widely used in clinics over the past several decades.

Keywords Nanobody · Alkaline phosphatase · Thermostability · Chemiluminescent enzyme immunoassay · Carcinoembryonic antigen

Jingtao Lin and Jianli Yu contributed equally to this work.

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✉ Rui Nian
nianrui@qibebt.ac.cn

✉ Haipeng Song
patrick.song@nanobodi.com

[1] Dalang Hospital of Dongguan, No. 85 Jinlangzhong Road, Dalang, Dongguan 523770, Guangdong, China

[2] CAS Key Laboratory of Biobased Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, No. 189 Songling Road, Qingdao 266101, Shandong, China

[3] University of Chinese Academy of Sciences, No.19 (A) Yuquan Road, Beijing 100049, China

[4] Shenzhen Innova Nanobodi Co., Ltd., No. 7018 Caitian Road, Shenzhen 518000, Guangdong, China

Introduction

Carcinoembryonic antigen–related cell adhesion molecule 5 (CEACAM-5), also known as CEA, is one of the most extensively used clinical biomarkers for cancer, and tests for blood levels of CEACAM-5 have been widely used to evaluate treatment effectiveness, monitor recurrence after therapy, and determine cancer [1]. Antibody-based analytical methods, such as enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescent immunoassay, have been established using various labeling methods to analyze CEACAM-5 in the serum samples. Antibodies for antigen detection are often chemically labeled with enzymes, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). However, the labeling step can affect activities of antibody and enzyme. Furthermore, a subsequent purification step is necessary to remove unlabeled antibody and free enzyme.

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The stability of immunoassay using these antibodies is low, so a temperature-controlled supply chain or "cold chain" is required for transportation and storage. The variable region of single-domain antibodies, termed nanobody (Nb), is the smallest antibody format that harbors the full antigen-binding capacity. Nanobody can be engineered faster and produced cheaper than conventional monoclonal antibody [2]. The fusion of nanobody to alkaline phosphatase instead of labeling chemistry obviates complicated labeling and purification steps, and thus protects the activities of nanobody and enzyme. Nb-AP fusion proteins have previously been used in immunoassays to detect environmental chemicals and human disease–related antigens [3–7]. Although Nb-AP fusion proteins significantly enhance the sensitivity of detection, the stability of the fusion protein depends largely on the property of fused AP [8]. Commercial calf intestinal alkaline phosphatase (CAP) is routinely used for chemical conjugation of antibody, and human placental alkaline phosphatase (HAP) is the heat-stable enzyme of the alkaline phosphatase isoenzymes in man [9]. Alkaline phosphatase from hyperthermophilic euryarchaeon *Pyrococcus abyssi* (PAP) was found to be the most thermostable AP with half-lives at 100 °C for 18 h [10]. However, the stability and activity of these Nb-AP fusion proteins are not clear.

In this study, six nanobodies with high affinity and specificity against CEACAM-5 were generated using phage-display technology. Two of them, termed Nb11C12 and Nb2D5, were chosen to recognize different epitopes of CEACAM-5. The Nb-AP fusion protein was constructed using Nb2D5 and expressed in human embryonic kidney (HEK 293) cells. After purification and characterization, the Nb-AP fusion protein was used to develop a chemiluminescent immunoassay (CLEIA) to detect CEACAM-5 in serum samples. Thermal stability was also analyzed and compared between the Nb-AP fusion protein and the labeled antibody from a commercially available ELISA kit.

Materials and methods

Reagents and chemicals

Recombinant human CEACAM-1, CEACAM-3, CEACAM-5, CEACAM-6, and CEACAM-8 were purchased from Sino Biological Inc. (Beijing, China). T_4 DNA ligase, Pst I, EcoR I, Hind III, Xba I, and BstE II endonucleases and Taq DNA polymerases were all obtained from New England Biolabs Inc. (Beverly, MA, USA). Freund's adjuvant, isopropyl-β-D-1-thiogalactopyranoside (IPTG), and BM chemiluminescence ELISA substrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lymphocyte separation medium was obtained from Jingyang Inc. (Tianjin, China). VCSM13 helper phage and HRP-labeled mouse anti-M13 monoclonal antibody were acquired from GE (Pittsburgh, PA USA). RNA purification and reverse transcription kits were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). TG1 and BL21 (DE3) *E.coli* competent cells were obtained from TransGen Biotech Inc. (Beijing, China). Human embryonic kidney (HEK 293) cell line was purchased from ATCC (reference ATCC-CRL-11268). Plasmid purification kits were obtained from Tiangen Biotech Inc. (Beijing, China), and the Ni-NTA resin was obtained from GeneScript Inc. (Nanjing, Jiangsu, China). AP dilution reagent was obtained from Biodragon Immunotechnologies Inc. (Beijing, China). The kit for detecting AP activity was obtained from MultiSciences Biotech Inc. (Hangzhou, Jiangsu, China). The chemiluminescent microparticle immunoassay (CMIA) kit and Architech i2000SR were obtained from Abbot Laboratories Inc. (Chicago, IL, USA). ELISA kits for CEA detection were purchased from Autobio Inc. (Zhengzhou, Henan, China). Dalang Hospital of Dongguan provided all human sera.

Generation of nanobodies to CEACAM-5

Nanobodies specific to CEACAM-5 were screened out from an immunized alpaca with phage-display technology. Affinities of nanobodies to CEACAM-5 expressed from *E.coli* were assayed through surface plasmon resonance (SPR) on a Biacore T-100 (GE, Pittsburgh, PA, USA). All protocols are detailed in Electronic Supplementary Material (ESM).

Construction and characterization of Nb-AP fusion proteins

DNA of HAP, CAP, and PAP were synthesized by Genwiz Inc. (Suzhou, Jiangsu, China). The recombinant plasmids were constructed by fusing the N-terminus of Nb to the C-terminus of AP in plasmid pCDNA3.1, and transfected into HEK 293 cells for transient expression. After purification via DEAE-cellulose anion exchange chromatography from the harvested medium, the purities and affinities of Nb-AP fusion proteins were evaluated through sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) and SPR, respectively. The enzymatic activity of the Nb-AP fusion proteins was measured using colorimetric analysis. Briefly, serially diluted Nb-AP fusion protein (50 μL) and 100 μL of substrate solution (0.1 M glycine, 50 mM $MgCl_2$, 50 mM $ZnCl_2$, and 3.8 μM p-nitrophenyl phosphate, pH 10.4) were added in a 96-well microplate. After incubation at 37 °C for 30 min, the reaction was stopped with 50 μL of NaOH (4 M). The absorbance at 405 nm was measured on a microplate reader (Spark 10 M, Teacan, Austria).

Thermostability of Nb-AP fusion protein

The Nb-AP fusion protein was stored at 37 °C or 60 °C for 1, 3, 7, and 14 days, followed by cooling to ambient temperature. The affinity of Nb-AP fusion protein to CEACAM-5 was subsequently evaluated by SPR. Meanwhile, the enzymatic activity was measured by the colorimetric analysis as above.

Chemiluminescent enzyme immunoassay for CEACAM-5 based on Nb-AP fusion protein

For this immunoassay, a black opaque 96-well microplate was incubated with 100 μL of 10 μg/mL Nb11C12 with Fc tag per well at 4 °C overnight. The plate was blocked with 2% (w/v) bovine serum albumin (BSA) in Tris-buffered saline at 37 °C for 1 h. After washing with Tris-buffered saline containing 0.05% Tween-20 (TBST), 2-fold serial dilutions of CEACAM-5 (50 μL/well) in TBST were added, followed by the addition of 50 μL of Nb-AP fusion protein (2 μg/mL) in TBST. After incubation at ambient temperature for 1 h, the plate was washed and incubated with 150 μL of BM chemiluminescence ELISA substrate (Roche, Basel, Switzerland) at ambient temperature for 10 min. Disodium 3-(4-methoxyspiro {1, 2-dioxetane-3, 2′-(5′-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CSPD) of the BM chemiluminescence ELISA substrate was dephosphorylated by alkaline phosphatase. The resulting instable dioxetane anion decomposed and emitted light at 477 nm which was measured on a microplate reader (Spark 10M, Teacan, Austria). All concentrations performed in triplicate and calibration curve was established by plotting the value of relative light units (RLU) against the CEACAM-5 concentration.

Cross-reactivity

The specificity was evaluated by determining the selectivity coefficient ($K_{A,\,I}$) [12] of the CLEIA with a group of structure analogues in a range of 0–5000 ng/mL. The $K_{A,\,I}$ was calculated with the equation as follows: $K_{A,\,I}\ (\%) = [k_{(\text{interfering agent})}/k_{(\text{CEACAM-5})}] \times 100$.

Validation study

For recovery study, CEACAM-5 was spiked into the blank human serum collected by Dongguan Dalang Hospital of China at four levels: 8 ng/mL, 16 ng/mL, 32 ng/mL, and 64 ng/mL. The resulting spiked samples were directly analyzed by the CLEIA based on Nb-AP fusion protein. In addition, the CLEIA was also applied to analyze thirty-two human serum samples collected by Dongguan Dalang Hospital of China. Their CEACAM-5 concentrations were determined by the CMIA kit approved by National Medical Products Administrate (NMPA) of China.

Long-term thermostability

Nb-AP fusion protein was diluted to working concentration (2 μg/mL) and aliquoted into 20 glass bottles. After sealing, the bottles were stored at 37 °C and 50% relative humidity. Quality control serum (QCs) containing 15 ng/mL CEACAM-5 was prepared freshly and calibrated by a commercially available CMIA kit before every use. QCs were detected by CLEIA using the stored Nb-AP fusion protein every week in five replicates. Detecting antibody from a commercially available ELISA kit approved by NMPA of China was stored at the same condition and utilized for comparison.

Results and discussion

Generation of anti-CEACAM-5 nanobodies

The library size was 1.5×10^7 colony-forming units (CFU), and seventeen of eighteen colonies which were picked randomly had the expected DNA inserts, demonstrating that the library was constructed successfully (see ESM Fig. S1). After the third round of panning, six nanobodies were identified. The affinities of nanobodies to CEACAM-5 were in the nanomolar range, which afforded sufficient sensitivity for biological applications (see ESM Table S1). Two nanobodies, termed Nb11C12 and Nb2D5, bound to different epitopes of CEACAM-5, and were chosen to construct an immunoassay.

Characterization and thermostability analysis of Nb-AP fusion proteins

High-purity Nb2D5-AP fusion proteins were obtained via DEAE-cellulose anion exchange chromatography (see ESM Fig. S2). AP is one of the most ubiquitous enzyme families that process phosphate groups, one magnesium ion and two zinc ions binding sites. All APs share the same general folding motif of the catalytic core [11]. Nb2D5-CAP fusion protein and Nb2D5-PAP fusion protein had very low enzymatic activity, but the enzymatic activity of Nb2D5-HAP fusion protein was unaffected (Fig. 1a), indicating that the fusion of a nanobody at the N-terminus may disrupt the motif of the catalytic cores of the CAP and PAP, but not of the HAP. PAP is a highly thermostable alkaline phosphatase from the hyperthermophilic euryarchaeon, *Pyrococcus abyssi*, with an optimum reaction temperature of 70 °C. The enzymatic activity of Nb2D5-PAP fusion protein was tested again at 70 °C, but the higher temperature caused no detectable increase. The affinity of Nb2D5-HAP fusion protein to CEACAM-5 reached to 42 pM and was higher than that of Nb2D5. Alkaline phosphatase is a homodimeric protein enzyme, and dimerization of monomer subunits turned the fusion protein into an antibody-like structure with two antigen-binding

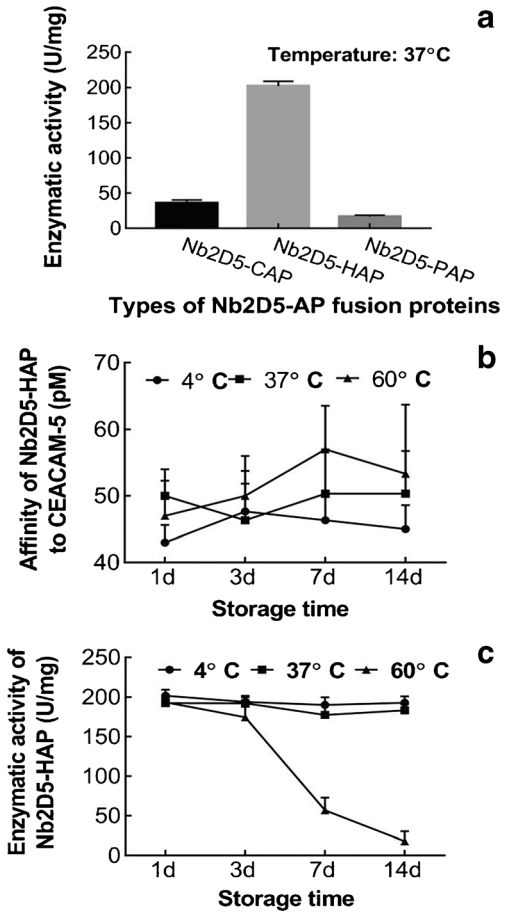

Fig. 1 Characterization of Nb2D5-AP fusion proteins. Colorimetric assay was performed to measure the enzymatic activity of three Nb2D5-AP fusion proteins (**a**). The result showed that Nb2D5-HAP was the best and its thermostability about enzymatic activity (**b**) and affinity (**c**) were further analyzed. The data are average values of triplicate samples

motifs at the N-terminus. This can increase the avidity of the fusion protein. The affinity of Nb2D5-HAP fusion protein remained stable at 37 °C and 60 °C for over 2 weeks (Fig. 1b). But the enzymatic activity decreased significantly at 60 °C on the 14th day indicating that alkaline phosphatase of Nb2D5-HAP fusion protein is sensitive to high temperature.

Chemiluminescent enzyme immunoassay for CEACAM5 based on Nb-AP fusion protein

Nb11C12 with Fc tag (see ESM Fig. S2) was coated on the plate at 10 μg/mL to capture CEACAM-5 and Nb2D5-HAP fusion protein was diluted to 2 μg/mL to detect CEACAM-5 (see ESM Fig. S4). This CLEIA had no hook effect at CEACAM-5 concentrations up to 81,920 ng/mL, and the linear range of the CLEIA was 0.31–640 ng/mL with a good correlation coefficient of 0.994 (see ESM Fig. S5). Herein, the limit of detection (LOD) refers to computed CEACAM-5 concentration corresponding to signal response of the blank human serum plus three times of its standard deviation. The LOD was 0.85 ng/mL in human serum ($n = 20$) and approximately 3 times lower than that of commercially available immunoassay kits for CEACAM-5 (2.78 ng/mL and 3.15 ng/mL), which indicated that the CLEIA had higher sensitivity to CEACAM-5. The high sensitivity can be vital in real sample detection since it allows high dilution of immunoreagents and cost saving [12]. No cross-reactivity with CEACAM-1, CEACAM-3, CEACAM-6, or CEACAM-8 and no interference from rheumatoid factors were found (see ESM Table S2), demonstrating that the CLEIA was highly CEACAM5-specific.

Validation study

In order to evaluate the reliability of the CLEIA for CEACAM-5, the immunoassay based on Nb2D5-HAP fusion protein was performed to detect CEACAM-5 in the spiked human serum. Before spiking, the human serum was confirmed to be free of CEACAM-5 by a commercially available CMIA kit. The average recoveries of the CLEIA ranged from 91 to 109% and the coefficients of variation ranged from 0.024 to 0.1 (see ESM Table S3). In addition, thirty-two clinical serum samples detected by the CLEIA have shown a high degree of consistency with the existing mainstream detection methods approved by NMPA in Abbott Architect i2000SR (Fig. 2). These data indicates that the CLEIA was precise, repeatable, and suitable for in vitro detection of CEACAM-5 in clinical patient samples for tumor screening.

Long-term thermostability

The CLEIA based on Nb2D5-HAP fusion protein was stable for 8 weeks at 37 °C and 50% relative humidity (Table 1). As control subject, the commercially available ELISA kit could not detect CEACAM-5 of the QCs after 2 weeks at 37 °C. Besides the intrinsic stability of nanobody [13], dimerization

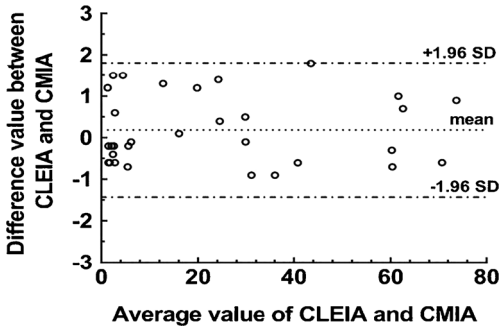

Fig. 2 Values of CEACAM-5 in thirty-two clinic human serum samples detected by the chemiluminescent enzyme immunoassay (CLEIA) based on Nb2D5-HAP fusion protein and commercially available chemiluminescent microparticle immunoassay (CMIA) were analyzed by Bland-Altman Method

Table 1 Determination of CEACAM-5 in quality control serum (QCs) by CLEIA and ELISA using the stored Nb2D5-HAP fusion protein and conventional-labeled antibody respectively

Storage time at 37 °C (weeks)	CEACAM-5 concentration in QCs[a] (ng/mL)	Measured by CLEIA (mean ± SD) (ng/mL) ($n = 5$)	Measured by ELISA (mean ± SD) (ng/mL)
0	15	15.1 ± 0.5	15.0 ± 0.2
1		14.8 ± 0.7	15.0 ± 1.0
2		14.8 ± 0.3	18.0 ± 0. 9 *
3		14.9 ± 0.8	ND[b]
4		15.0 ± 0.8	ND[b]
5		15.3 ± 0.4	ND[b]
6		15.3 ± 0.5	ND[b]
7		15.5 ± 0.9	ND[b]
8		15.3 ± 0.9	ND[b]
9		18.9 ± 1.3 *	ND[b]

[a] QCs was calibrated by a commercially available chemiluminescent microparticle immunoassay (CMIA) kit approved by National Medical Products Administration (NMPA) of China before every use

[b] *ND* not detectable

*$p < 0.1$, indicate significant difference

of AP provides excellent resistance to digestion of proteases and thus contributes to the long-term storage [14]. Overall, the CLEIA for CEACAM-5, which we developed from Nb2D5-HAP fusion protein, had better stability and reproducibility than that of the control immunoassay developed from conventional-labeled antibodies which has been extensively used in clinics for the past several decades.

Conclusion

We developed a sensitive and thermostable CLEIA for CEACAM-5. Although Nb is more thermally stable than conventional monoclonal antibodies, the stability of an immunoassay developed from Nb-AP fusion protein is highly dependent on the enzymatic stability of AP. Of the three Nb2D5-AP fusion proteins that we tested, Nb2D5-HAP fusion protein was the best. The Nb2D5-HAP fusion protein was stable at 37 °C for at least 8 weeks and reliable to be used in quantitative detection of CEACAM-5. This result paves the way for novel, thermally stable, highly valuable applications in diagnostic detection of CEACAM-5. After further development, this methodology may also be used to automatically detect CEACAM-5 in patient serum samples with high sensitivity, specificity, accuracy, and reproducibility. Furthermore, this method is applicable in immunoassays of other antigens with clinical significance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Research involving human participants All procedures involving humans were conducted in compliance with the relevant Chinese laws and the Chinese Academy of Sciences regulations. This study received ethical approval from the Ethics Committee of Chinese Academy of Sciences and the local Ethical Board of Dongguan Dalang Hospital. Signed informed consent of all individual participant samples was obtained.

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