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Original Article

Epitope Mapping of an Anti-CD44v4 Monoclonal Antibody (C_{44}Mab-108) Using Enzyme-Linked Immunosorbent Assay

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CD44 is a type I transmembrane glycoprotein and possesses various isoforms which are largely classified into CD44 standard (CD44s) and CD44 variant (CD44v) isoforms. Some variant-encoded regions play critical roles in tumor progression. However, the function of CD44 variant 4 (CD44v4)-encoded region has not been fully understood. Using peptide immunization, we developed an anti-CD44v4 monoclonal antibody, C_{44}Mab-108, which is useful for flow cytometry, western blotting, and immunohistochemistry. In this study, we determined the critical epitope of C_{44}Mab-108 by enzyme-linked immunosorbent assay (ELISA). We used the alanine (or glycine)-substituted peptides of the CD44v4-encoded region (amino acids 271–290 of human CD44v3-10) and found that C_{44}Mab-108 did not recognize the alanine-substituted peptides of D280A and W281A. Furthermore, these peptides could not inhibit the recognition of C_{44}Mab-108 in flow cytometry and immunohistochemistry. The results indicate that the critical binding epitope of C_{44}Mab-108 includes Asp280 and Trp281 of CD44v3-10.

Keywords: CD44, CD44 variant 4, monoclonal antibody, epitope, enzyme-linked immunosorbent assay

Introduction

CD44 has various isoforms, which are generated by the alternative splicing of CD44 pre-mRNA.[1] The mRNA of CD44 standard (CD44s) isoform is produced by constant region exons, including the first five (1–5) and the last five (16–20).[2] The mRNAs of CD44 variant (CD44v) isoform are produced by the assembling of variant exons (v1–v10) with the constant region exons of CD44s.[3] CD44s and CD44v receive the posttranslational modifications, including *N*- or *O*-glycosylation.[4] Both CD44s and CD44v can attach to hyaluronic acid, which is important for cellular adhesion, homing, and motility.[5]

CD44v plays important roles in the tumor progression by specific functions of variant exon-encoded regions.[6] The heparin-binding growth factors are recruited to heparan sulphate modified in the v3-encoded region.[7,8] MET, a receptor tyrosine kinase for hepatocyte growth factor, associates with the v6-encoded region.[9,10] These functions are essential for the activation of growth factor signaling and tumor proliferation. However, the roles of CD44 variant 4 (CD44v4)-encoded region have not been investigated. Therefore, specific antibodies against CD44v4 are indispensable for basic research, tumor diagnosis, and therapy.

We have established anti-CD44 monoclonal antibodies (mAbs), which recognize the standard[11,12]—or each variant[13–20]—encoded region. All mAbs can be used for flow cytometry, western blotting, and immunohistochemistry and are expected to contribute not only to basic research but also to phathological diagnosis. We also determined the critical epitopes of C_{44}Mab-5[21] and C_{44}Mab-46.[22,23]

Clinical trials of anti-CD44 mAbs have been conducted.[24] A humanized anti-CD44v6 mAb BIWA4 (bivatuzumab)–mertansine drug conjugate was evaluated, but discontinued due to the severe skin toxicities.[25,26] An anti-pan CD44 mAb, RG7356, exhibited an acceptable safety profile in patients with advanced CD44-expressing solid tumors. However, the study was terminated due to no evidence of a clinical and pharmacodynamic dose–response relationship with RG7356.[27] Therefore, the development of anti-CD44 mAbs with more potent and fewer side effects is required.

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We previously established an anti-CD44v4 mAb, C_{44}Mab-108 (IgG_1, kappa) using the peptide immunization.[19] To clarify further characteristics of C_{44}Mab-108, we performed epitope mapping using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Peptides

The CD44v4 peptide ($_{271}$-AFDHTKQNQDWTQWNPS HSN-$_{290}$) and 20 alanine (or glycine)-substituted peptides (Table 1) were synthesized by utilizing PEPscreen (Sigma-Aldrich Corp., St. Louis, MO, USA). The number of amino acids (aa) is derived from human CD44v3-10 (Accession No.: X66733).

ELISA

The CD44v4 peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a concentration of 10 μg/mL for 30 minutes at 37°C. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc., Kyoto, Japan), wells were blocked with 1% bovine serum albumin (BSA)-containing PBST for 30 minutes at 37°C. The plates were incubated with 10 μg/mL of C_{44}Mab-108, followed by a peroxidase-conjugated anti-mouse immunoglobulins (1:2000 diluted; Agilent Technologies, Inc., Santa Clara, CA, USA). Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

Table 1. Identification of the C_{44}Mab-108 Epitope Using Alanine (or Glycine)-Substituted CD44 Variant 4 Peptides

Peptides	*Sequences*	*C_{44}Mab-108*
WT (271–290)	AFDHTKQNQDWTQWNPSHSN	+++
A271G	GFDHTKQNQDWTQWNPSHSN	+++
F272A	AADHTKQNQDWTQWNPSHSN	+++
D273A	AFAHTKQNQDWTQWNPSHSN	+++
H274A	AFDATKQNQDWTQWNPSHSN	+++
T275A	AFDHAKQNQDWTQWNPSHSN	+++
K276A	AFDHTAQNQDWTQWNPSHSN	+++
Q277A	AFDHTKANQDWTQWNPSHSN	+++
N278A	AFDHTKQAQDWTQWNPSHSN	+++
Q279A	AFDHTKQNADWTQWNPSHSN	+++
D280A	AFDHTKQNQAWTQWNPSHSN	–
W281A	AFDHTKQNQDATQWNPSHSN	–
T282A	AFDHTKQNQDWAQWNPSHSN	+
Q283A	AFDHTKQNQDWTAWNPSHSN	+++
W284A	AFDHTKQNQDWTQANPSHSN	+
N285A	AFDHTKQNQDWTQWAPSHSN	+++
P286A	AFDHTKQNQDWTQWNASHSN	+++
S287A	AFDHTKQNQDWTQWNPAHSN	+++
H288A	AFDHTKQNQDWTQWNPSASN	+++
S289A	AFDHTKQNQDWTQWNPSHAN	+++
N290A	AFDHTKQNQDWTQWNPSHSA	+++

+++, OD655 ≥ 0.6; ++, 0.3 ≤ OD655 < 0.6; +, 0.1 ≤ OD655 < 0.3; –, OD655 < 0.1.
WT, wild type.

Flow cytometry

The N-terminal PA16-tagged CD44v3-10-overexpressed Chinese hamster ovary-K1 (CHO/CD44v3-10)[19] cells were harvested after a brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.) and washed with 0.1% BSA in PBS. C_{44}Mab-108 (10 μg/mL) was incubated with the CD44v4 peptides (10 μg/mL) for 30 minutes at 4°C. The cells were further treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000). Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Immunohistochemical analysis

One formalin-fixed paraffin-embedded (FFPE) tissue of oral squamous cell carcinoma (OSCC) for peptide blocking assay was obtained from Tokyo Medical and Dental University.[28] The tissue slides were autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes for antigen retrieval. After blocking with Super Block T20 (Thermo Fisher Scientific, Inc.), the sections were incubated with C_{44}Mab-108 (10 μg/mL) in the presence or absence of the CD44v4 peptides (10 μg/mL) and then treated with the EnVision+ Kit for mouse (Agilent Technologies, Inc.) for 30 minutes. The color was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.). Counterstaining was performed with hematoxylin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Leica DMD108 (Leica Microsystems GmbH, Wetzlar, Germany) was used to examine the sections and obtain images.

Results

Epitope mapping of C_{44}Mab-108 with alanine (or glycine)-substituted CD44v4 peptides

We previously established an anti-CD44v4 mAb (C_{44}Mab-108) by peptide immunization of CD44v4 region ($_{273}$-DHTKQNQDWTQWNPSHSNP-$_{291}$).[19] We confirmed that C_{44}Mab-108 recognizes only the variant 4-encoded region peptide (aa 271–290), but not other regions of CD44v3-10 extracellular domain.[19] To identify the binding epitope of C_{44}Mab-108, we synthesized 20 alanine (or glycine)-substituted peptides of the CD44v4 (Table 1). C_{44}Mab-108 exhibited reaction with A271G, F272A, D273A, H274A, T275A, K276A, Q277A, N278A, Q279A, T282A, Q283A, W284A, N285A, P286A, S287A, H288A, S289A, N290A, and wild type (WT) (Fig. 1A). In contrast, C_{44}Mab-108 did not react with D280A and W281A (Fig. 1A). This result indicated that Asp280 and Trp281 are included in the critical epitope of C_{44}Mab-108. The results are summarized in Table 1. Figure 1B shows the schematic illustration of CD44s, CD44v3-10, and the critical aa (Asp280 and Trp281) recognized by C_{44}Mab-108.

Flow cytometry using C_{44}Mab-108 with alanine-substituted CD44v4 peptides

We next performed a peptide-blocking assay using flow cytometry to confirm the importance of the C_{44}Mab-108 epitope. As shown in Figure 2, C_{44}Mab-108 reacted with the CHO/CD44v3-10 cells. This reaction was completely neutralized by the WT peptide. In contrast, D280A and W281A

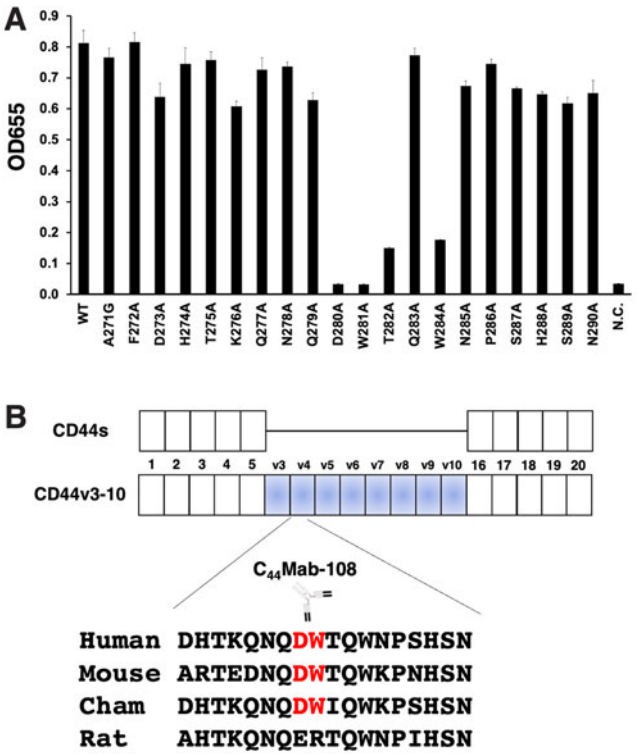

FIG. 1. Determination of the C_{44}Mab-108 epitope by ELISA using alanine (or glycine)-substituted CD44v4 peptides. **(A)** The alanine (or glycine)-substituted CD44v4 peptides in PBS or PBS alone were immobilized on immunoplates. The plates were incubated with C_{44}Mab-108 (10 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. Error bars represent means ± SDs. N.C. (PBS). **(B)** Schematic illustration of CD44s, CD44v3-10, and the C_{44}Mab-108 epitope. The C_{44}Mab-108 epitope includes Asp280 and Trp281 of CD44v3-10. The epitope is conserved in mouse and Chinese hamster (Cham), but not in Rat. CD44s, CD44 standard; CD44v4, CD44 variant 4; ELISA, enzyme-linked immunosorbent assay; N.C., negative control; PBS, phosphate-buffered saline; SD, standard deviation; WT, wild type.

peptides did not block the reaction of C_{44}Mab-108 with CHO/CD44v3-10. The result confirmed that Asp280 and Trp281 of CD44v3-10 are critical for detection by C_{44}Mab-108 using flow cytometry.

Immunohistochemistry using C_{44}Mab-108 with alanine-substituted CD44v4 peptides

We also performed a peptide-blocking assay using immunohistochemical analysis. As shown in Figure 3, C_{44}Mab-108 stained the FFPE section of OSCC, which was completely neutralized by the WT peptide. In contrast, D280A and W281A peptides did not neutralize the reaction. These results were corresponding to that of Figure 2.

Discussion

In this study, the critical epitope of C_{44}Mab-108 was determined as Asp280 and Trp281 in the CD44v4 region. Since the reactivity of C_{44}Mab-108 to T282A and W284A was also reduced (Fig. 1A), Thr282 and Trp284 may contribute to the

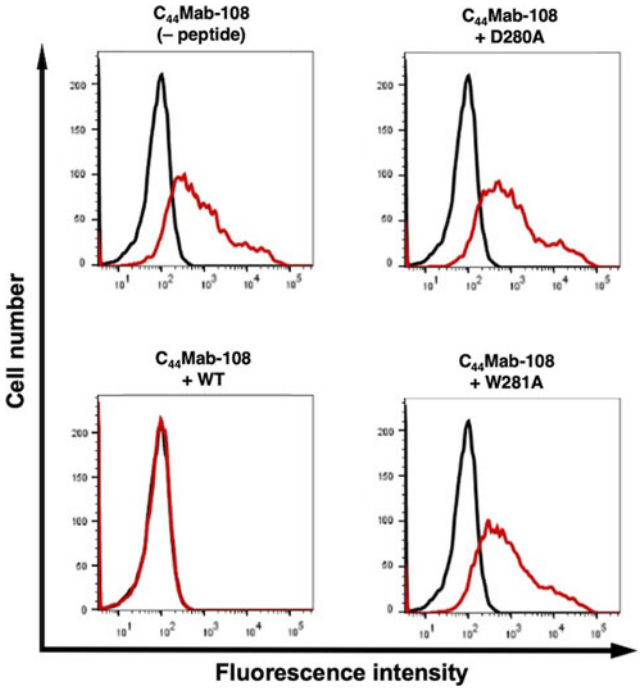

FIG. 2. Flow cytometry using C_{44}Mab-108 and CD44v4 peptides. C_{44}Mab-108 (10 μg/mL) plus WT, the alanine-substituted peptides (10 μg/mL), or control (0.1% DMSO in blocking buffer, –peptide) were reacted with CHO/CD44v3-10 cells for 30 minutes at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. The black line represents the control. DMSO, dimethyl sulfoxide; WT, wild type.

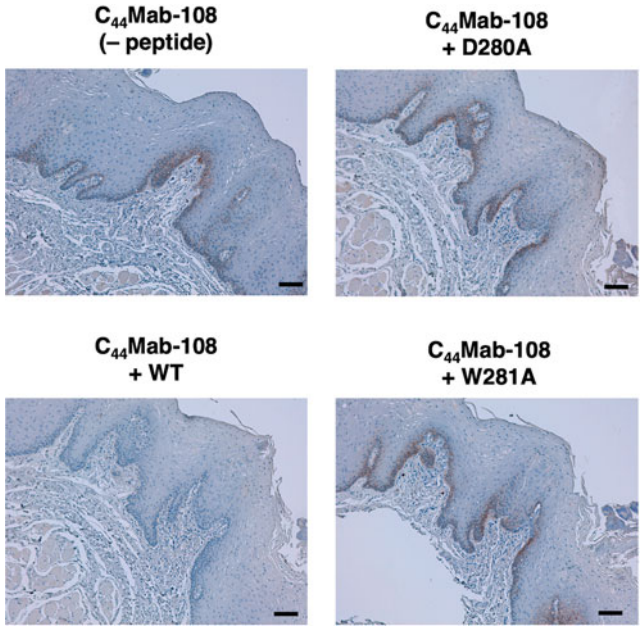

FIG. 3. Immunohistochemistry using C_{44}Mab-108 and CD44v4 peptides. The FFPE sections of OSCC were incubated with C_{44}Mab-108 (10 μg/mL) plus WT, the alanine-substituted peptides (10 μg/mL), or control (0.1% DMSO in blocking buffer, –peptide), followed by that with the Envision + Kit. Scale bar = 100 μm. FFPE, formalin-fixed paraffin-embedded; OSCC, oral squamous cell carcinoma.

recognition partially. Figure 1B shows the homology of the v4 region among human, mouse, Chinese hamster, and rat sequences. The Asp280 and Trp281 are conserved in human, mouse, Chinese hamster, but not in rat. Furthermore, Thr282 and Trp284 are also conserved in human and mouse. Although the result suggests that C_{44}Mab-108 might recognize both human and mouse CD44v4, it did not react with mouse CD44v4 using flow cytometry (data not shown).

Since C_{44}Mab-108 was established by the peptide immunization, C_{44}Mab-108 can recognize the definite peptide structure of the variant 4-encoded region. In contrast, CD44 is predicted to carry 146 *O*-glycosylation sites in the variant region. Among them, 41 of these sites have already been experimentally confirmed.[29] Thr282 is also a confirmed *O*-glycosylation site.[30] Further studies are required to reveal whether the *O*-glycan at Thr282 affects the recognition by C_{44}Mab-108.

In our previous study, C_{44}Mab-108 could detect CD44v3-10-overexpressed cells such as CHO/CD44v3-10, but not detect endogenous CD44v4 in several cancer cell lines in flow cytometry.[19] In contrast, C_{44}Mab-108 could detect endogenous and membranous CD44v4 in immunohistochemistry.[19] These results suggest that the variant 4-encoded region is folded into the inside of protein in living cells, but exposed by antigen retrieval in immunohistochemistry. Recently, we provided a potential strategy for developing cancer-specific antibodies that target locally misfolded cell surface receptors such as human epidermal growth factor receptor 2.[31,32] Further studies are needed to investigate whether C_{44}Mab-108 is involved in the recognition of specific CD44v4 type and/or specific condition of cells.

Authors' Contributions

H.S., M.T., A.H., N.G., and T.T. performed the experiments. M.K.K. and Y.K. designed the experiments. H.S. and M.K.K. analyzed the data. H.S. and Y.K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Author Disclosure Statement

No competing financial interests exist.

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