

COMMUNICATION

Development and characterization of Ea₇Mab-10: A novel monoclonal antibody targeting ephrin type-A receptor 7

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Abstract

The Eph receptor-ephrin system is essential for embryonic, neural, and vascular development and contributes to tumor promotion or suppression. Ephrin type-A receptor 7 (EphA7) has been identified as a tumor suppressor often lost or downregulated in lymphomas. In contrast, *EPHA7* mutations have been suggested as potential driver genes in other cancers. However, the detailed molecular function has not been clarified. Therefore, a monoclonal antibody (mAb) against EphA7 is essential for basic research and clinical applications. In this study, we developed a novel anti-human EphA7 mAb, Ea₇Mab-10, using the Cell-Based Immunization and Screening (CBIS) method. Ea₇Mab-10 reacted with EphA7-overexpressed Chinese hamster ovary-K1 (CHO/EphA7) and EphA7-overexpressed LN229 (LN229/EphA7) in flow cytometry. The binding affinities were determined as 3.1×10^{-9} M for CHO/EphA7 and 6.9×10^{-9} M for LN229/EphA7. Furthermore, Ea₇Mab-10 did not show cross-reactivity with other Eph family members and was suitable for Western blotting and immunohistochemistry. These results demonstrate that Ea₇Mab-10, developed using the CBIS method, supports basic research on EphA7 and could be used for mAb-based targeted therapies.

Keywords: Eph receptor; Ephrin type-A receptor 7; Cell-Based Immunization and Screening method; Monoclonal antibody; Flow cytometry; Western blotting; Immunohistochemistry

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1. Introduction

The Eph receptor-ephrin system has 14 tyrosine kinase receptors (EphA1-A8, A10, and EphB1-B4, and B6) and eight ephrin ligands—five glycosylphosphatidylinositol-anchored (ephrin-A1 to A5) and three transmembrane types (ephrin-B1 to B3).¹ EphA receptors mainly interact with ephrin-As, whereas EphB receptors bind to ephrin-Bs; however, EphA4 is capable of binding to all ephrins, and EphB2 can bind to ephrin-A5.¹ The interaction between Eph receptors and ephrins at intercellular junctions triggers bidirectional signaling: Forward and reverse signaling, respectively. This process involves the dimerization and higher-order oligomerization of Eph receptors and ephrins, the tyrosine phosphorylation of both Eph receptors and ephrin-Bs, and the recruitment of

cytoplasmic effectors containing Src homology 2, PDZ, and other signaling domains.²

The Eph receptor-ephrin system is essential for embryonic,^{3,4} neural,^{5,6} and vascular development,^{7,8} with a growing body of evidence implicating critical roles in cancers.^{9,10} The expression levels of Eph receptors and ephrins are often altered in tumors, upregulated or downregulated compared to normal tissues, suggesting dual roles: Tumor promotion or suppression. In some tumors, Eph receptors are upregulated during the early stages but are downregulated during the tumor progression, indicating that they may play distinct roles in tumor initiation and progression.¹

Ephrin type-A receptor 7 (EphA7) was first cloned as a member of Eph homologous kinase, EHK-3. Its expression was restricted to the adult nervous system and was more widely expressed in the embryo.¹¹ In neural stem cells, the EphA7-ephrin-A5 complex is associated with tumor necrosis factor receptor superfamily member 1A, which results in the induction of apoptotic cell death.¹² EphA7 forward signaling can cause apoptosis in prostate cancer cells through inhibition of protein kinase B-mediated cell survival signaling.^{13,14} EphA7 phosphorylation was positively related to ephrin-A5 expression in human prostate tissue, suggesting the tumor suppressive function of the EphA7-ephrin-A5 axis in prostate cancer. Moreover, loss or downregulation of EphA7 is frequently observed in lymphomas.¹⁵

EPHA7 mutations have been suggested to be a driver in small-cell lung cancer.¹⁶ However, the underlying molecular mechanism remains unclear. *EPHA7* mutations were observed in various tumor types and were strongly associated with reduced patient survival.¹ Furthermore, *EPHA7* mutation in multiple cancers has been implicated as a predictive biomarker for immune checkpoint inhibitors. Among the 14 Eph receptors, *EPHA7* mutations were enriched in patients responding to immune checkpoint inhibitor therapy.¹⁷

A study revealed that EphA7 is associated with the immune inhibitory receptor leukocyte immunoglobulin-like receptor family B5 (LILRB5), which activates LILRB5 signaling. LILRB5 plays a critical role in supporting immunosuppressive myeloid cells, which are essential obstacles to checkpoint inhibitor therapies.¹⁸

Several anti-EphA7 monoclonal antibodies (mAbs) have been developed for Western blotting and immunohistochemistry (IHC).¹⁴ Generating mAbs suitable for flow cytometry is essential to developing mAbs for therapeutic purposes. However, no commercially available anti-EphA7 mAb is currently

ideal for flow cytometry. Previously, we have developed various mAbs against receptor tyrosine kinases, such as the epidermal growth factor receptor family¹⁹⁻²¹ and the Eph family,²²⁻²⁴ using the Cell-Based Immunization and Screening (CBIS) method. This method includes immunization of antigen-overexpressed cells and flow cytometry-based high-throughput screening. This study employs the CBIS method to generate highly versatile anti-EphA7 mAbs.

2. Materials and methods

2.1. Cell lines

Cell lines, including Chinese hamster ovary (CHO)-K1, LN229 glioblastoma, and P3X63Ag8U.1 (P3U1) myeloma, were obtained from the American Type Culture Collection (United States). The complementary DNA (cDNA) of *EPHA7* (Accession no.: NM_004440; catalog no.: RC226293, OriGene Technologies Inc., United States) with an N-terminal MAP16²⁵ and an N-terminal PA16 tag²⁶—recognized respectively by the anti-MAP16 tag mAb (PMab-1) and an anti-PA16 tag mAb (NZ-1)—was subcloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Japan). Stable transfectants (CHO/PA16-EphA7 [CHO/EphA7] and LN229/MAP16-EphA7 [LN229/EphA7]) were subsequently selected by a cell sorter (SH800, Sony Corp., Japan) using anti-tag mAbs, PMab-1 and NZ-1, respectively. These cell lines and other Eph receptor-expressed CHO-K1 cells (e.g., CHO/EphA2) were cultured as previously reported.²²

2.2. Development of hybridoma

Animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Approval number: 2022Mda-001). Two 5-week-old female BALB/cAJcl mice (CLEA Japan, Japan) were immunized with LN229/EphA7 (1×10^8 cells/mouse) and Alhydrogel adjuvant 2% (InvivoGen, United States) via the intraperitoneal route. Three additional injections of LN229/EphA7 (1×10^8 cells/mouse) were conducted weekly. A last booster injection of LN229/EphA7 (1×10^8 cells/mouse) was given 2 days before harvesting spleen cells from the mice. Hybridomas were generated and cultured as described previously.²²

2.3. Production of recombinant antibodies

Variable (V_H) and constant (C_H) regions of heavy chain cDNAs of Ea₇Mab-10 were subcloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation, Japan). Variable (V_L) and constant (C_L) regions of light chain cDNAs of Ea₇Mab-10 were subcloned into the

pCAG-Ble vector. NZ-33 was produced using the V_H of NZ-1, the C_H of mouse immunoglobulin G (IgG)_{2a}, and the light chain of NZ-1. The V_H of NZ-1 and the C_H of mouse IgG_{2a} were subcloned into the pCAG-Neo vector. V_L and C_L cDNAs of NZ-1 were subcloned into the pCAG-Ble vector (Wako). These vectors were transfected into ExpiCHO-S cells using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc., United States). Ea₇Mab-10 and NZ-33 were purified using Ab-Capcher (ProteNova, Japan).

2.4. Flow cytometry

Cells were washed gently with phosphate-buffered saline containing 0.1% bovine serum albumin and incubated with primary mAbs for 30 min at 4°C. Subsequently, they were stained with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; catalog no: #4408, Cell Signaling Technology Inc., United States) before fluorescence analysis using the SA3800 Cell Analyzer (Sony Corp., Japan).

2.5. Determination of the binding affinity using flow cytometry

CHO/EphA7 and LN229/EphA7 cells were suspended in 100 μ L serially diluted Ea₇Mab-10 (100 μ g/mL to 0.006 μ g/mL), and treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:200). Fluorescence data were subsequently collected using the BD FACSLyric (BD Biosciences, United States), followed by the dissociation constant (K_D) analysis, conducted by fitting the binding isotherms into the built-in one-site binding model in GraphPad PRISM 6 (GraphPad Software, Inc., United States).

2.6. Western blot analysis

Western blot analysis was conducted as described previously.²⁴ Ea₇Mab-10 (1 μ g/mL), anti-isocitrate dehydrogenase 1 mAb (1 μ g/mL; clone RcMab-1, previously developed in our lab),²⁷ and anti-PA16 tag mAb (1 μ g/mL; clone NZ-1, previously developed in our lab) were used as primary mAbs.

2.7. IHC

Formalin-fixed paraffin-embedded (FFPE) CHO/EphA7 and CHO-K1 cell blocks were prepared using iPGell (Genostaff Co., Ltd., Japan). The FFPE cell sections were stained with Ea₇Mab-10 (0.1 μ g/mL) or NZ-33 (0.1 μ g/mL) using *BenchMark* ULTRA PLUS with the ultraView Universal DAB Detection Kit (Roche Diagnostics, United States).

3. Results

3.1. Development of Ea₇Mab-10

To establish mAbs targeting EphA7, we employed the CBIS method using EphA7-overexpressed cells. Anti-EphA7 mAbs-producing hybridoma were screened using flow cytometry (Figure 1). Two female BALB/cAJcl mice were immunized 5 times with LN229/EphA7. Subsequently, splenocytes were removed from immunized mice and fused with P3U1 cells. After confirming hybridoma formation, flow cytometric high-throughput screening was conducted to select CHO/EphA7 reactive and parental CHO-K1 non-reactive supernatants of hybridomas. After limiting dilution and additional analysis, we established 13 clones of anti-EphA7 mAbs (http://www.med-tohoku-antibody.com/topics/001_paper_antibody_PDIS.htm#EphA7). Among them, we established and selected Ea₇Mab-10 (mouse IgG₁, kappa) based on reactivity and specificity. As shown in Figure 2, Ea₇Mab-10 recognized CHO/EphA7. Importantly, Ea₇Mab-10 never reacted with other Eph receptors (EphA1 to A6, A8, A10, B1 to B4, and B6)-overexpressed CHO-K1. This result indicates that Ea₇Mab-10 is an EphA7-specific mAb.

3.2. Reactivity of Ea₇Mab-10

We cloned cDNAs of V_H and V_L regions of Ea₇Mab-10 and produced recombinant Ea₇Mab-10 for further validation. We first checked the reactivity of Ea₇Mab-10 using flow cytometry. Results showed that Ea₇Mab-10 recognized CHO/EphA7 in a dose-dependent manner (Figure 3A) and did not react with parental CHO-K1 cells (Figure 3A). Furthermore, Ea₇Mab-10 exhibited a dose-dependent reaction to LN229/EphA7 cells (Figure 3B) and recognized parental LN229 cells at 1 μ g/mL and 0.1 μ g/mL (Figure 3B). These results indicate that Ea₇Mab-10 can detect EphA7 in flow cytometry.

3.3. Binding affinity of Ea₇Mab-10

To evaluate the binding affinity of Ea₇Mab-10, flow cytometry was performed using CHO/EphA7 and LN229/EphA7 cells. The K_D values of Ea₇Mab-10 for CHO/EphA7 and LN229/EphA7 were 3.1×10^{-9} M and 6.9×10^{-9} M, respectively (Figure 4). These results demonstrate that Ea₇Mab-10 has a high affinity to EphA7-overexpressed cells.

3.4. Western blot using Ea₇Mab-10

We investigated the suitability of Ea₇Mab-10 for Western blot analysis by analyzing CHO-K1 and CHO/EphA7 cell lysates. As shown in Figure 5 and Figure A1, Ea₇Mab-10

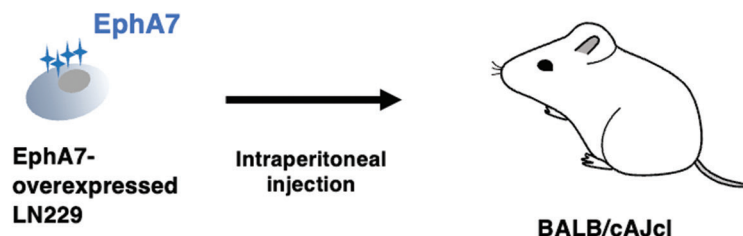
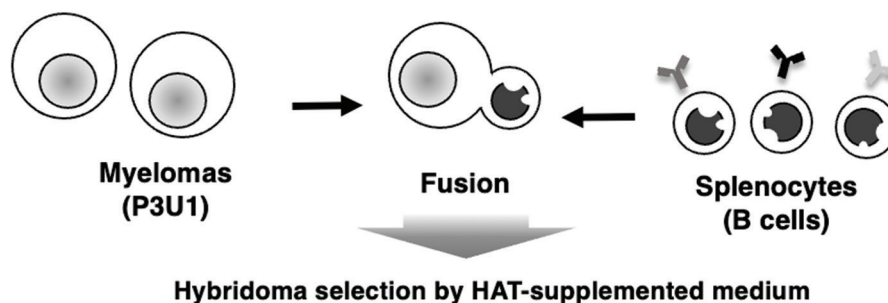
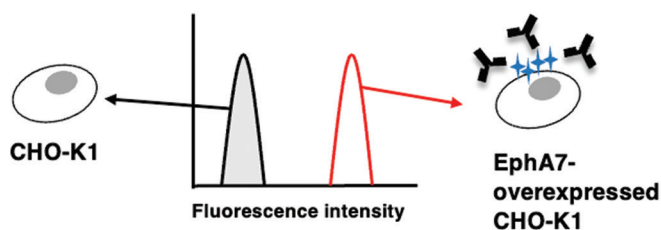
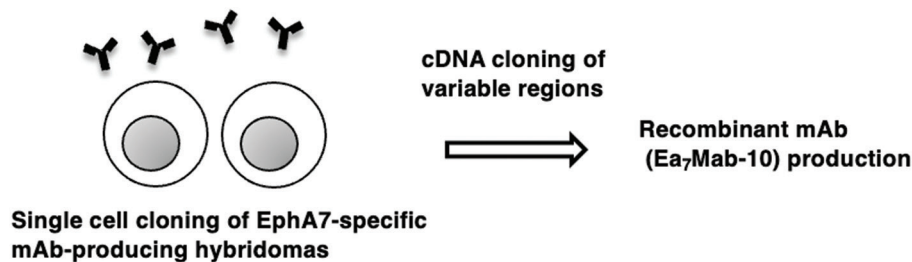
A Immunization of EphA7-overexpressed LN229 cells**B Hybridomas production****C High-throughput hybridoma screening using flow cytometry****D Cloning of mAb-producing hybridomas**

Figure 1. A schematic depiction of anti-ephrin type-A receptor 7 (EphA7) monoclonal antibodies (mAbs) development using the Cell-Based Immunization and Screening method. (A) LN229/EphA7 cells were injected into two female mice via the intraperitoneal route. (B) The spleen cells isolated from antigen-immunized mice were fused with mouse myeloma cells, P3U1. (C) The culture supernatants of hybridoma were screened via flow cytometry using Chinese hamster ovary (CHO)-K1 and CHO/EphA7 to select EphA7-specific mAb-producing hybridomas. (D) Single hybridoma clones were obtained by limiting dilution, followed by additional screening. Finally, Ea₇Mab-10 (mouse IgG₁, kappa) was established. Furthermore, we cloned complementary DNA (cDNA) of the heavy chain and light chain variable regions of Ea₇Mab-10 and produced recombinant Ea₇Mab-10. Abbreviation: HAT: Hypoxanthine-aminopterin-thymidine.

could clearly detect exogenously expressed EphA7 as a 110 kDa band in CHO/EphA7 cell lysates, whereas no band

was detected in parental CHO-K1 cells. The anti-PA16 tag mAb NZ-1 served as a positive control and detected a

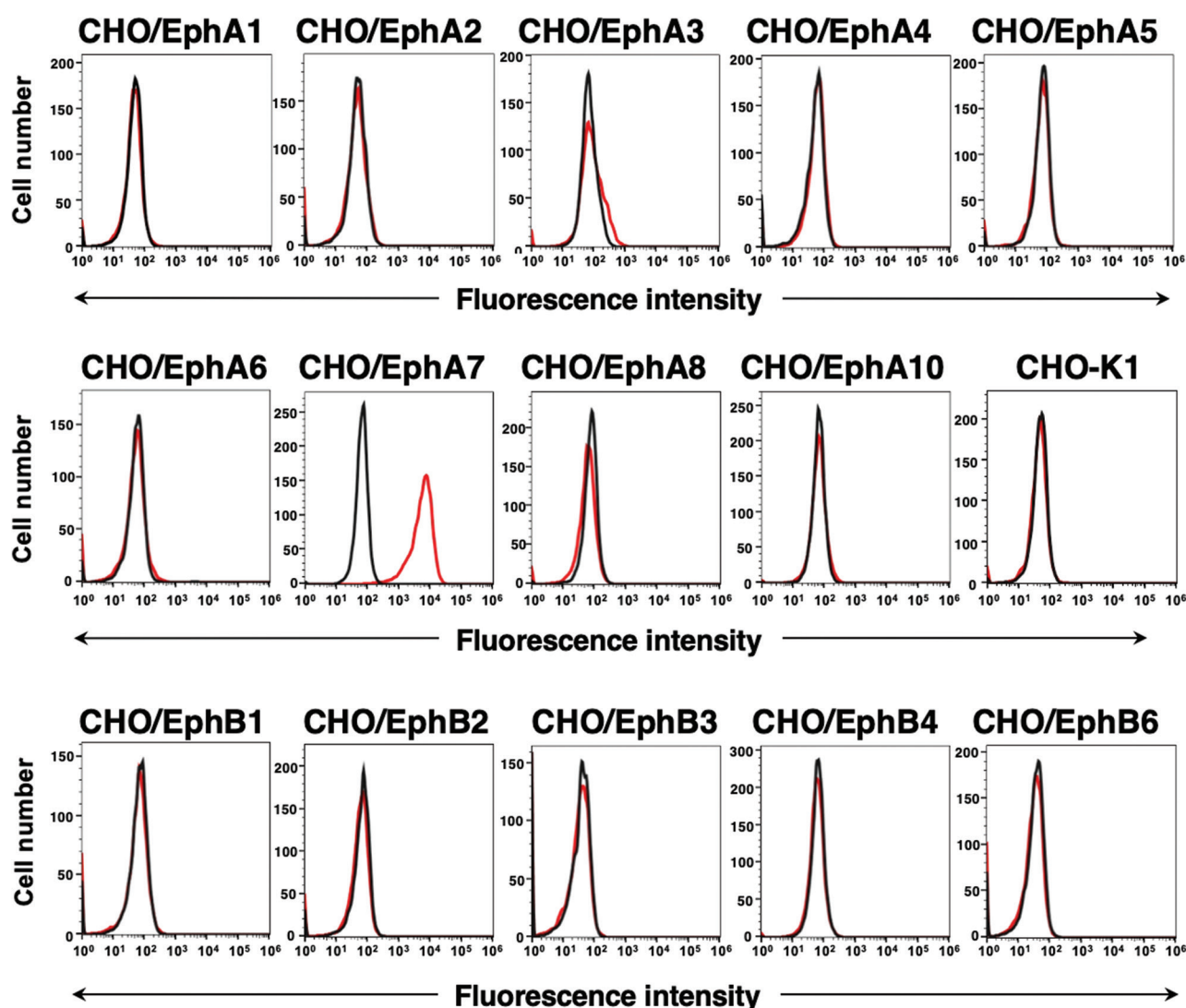


Figure 2. Cross-reactivity of Ea₇Mab-10 in Eph receptor-expressing Chinese hamster ovary (CHO)-K1 cells. CHO-K1 cells, overexpressed each of the 14 Eph receptors, were treated with 10 µg/mL of Ea₇Mab-10 (red line) followed by anti-mouse IgG conjugated with Alexa Fluor 488. Fluorescence data were collected using the SA3800 Cell Analyzer. The black line indicates the control (no primary antibody treatment). These experiments were performed at least twice.

band at the same position in CHO/EphA7 cell lysates. An anti-IDH 1 mAb (clone RcMab-1) was used as an internal control. These results demonstrate that Ea₇Mab-10 is suitable for detecting EphA7 by Western blotting.

3.5. IHC using Ea₇Mab-10

To evaluate the suitability of Ea₇Mab-10 for IHC, FFPE CHO-K1 and CHO/EphA7 sections were stained with Ea₇Mab-10. Apparent membranous staining by Ea₇Mab-10 was observed in CHO/EphA7 (Figure 6A). The anti-PA16 tag mAb, NZ-33, also showed potent reactivity to CHO/

EphA7 (Figure 6B). These results indicate that Ea₇Mab-10 can be applied to IHC to detect EphA7-positive cells in FFPE cell samples.

4. Discussion

This study demonstrated a novel anti-human EphA7 mAb, Ea₇Mab-10, applicable for various experiments. Ea₇Mab-10 can specifically detect EphA7 in flow cytometry (Figures 2-4), Western blotting (Figure 5), and IHC (Figure 6).

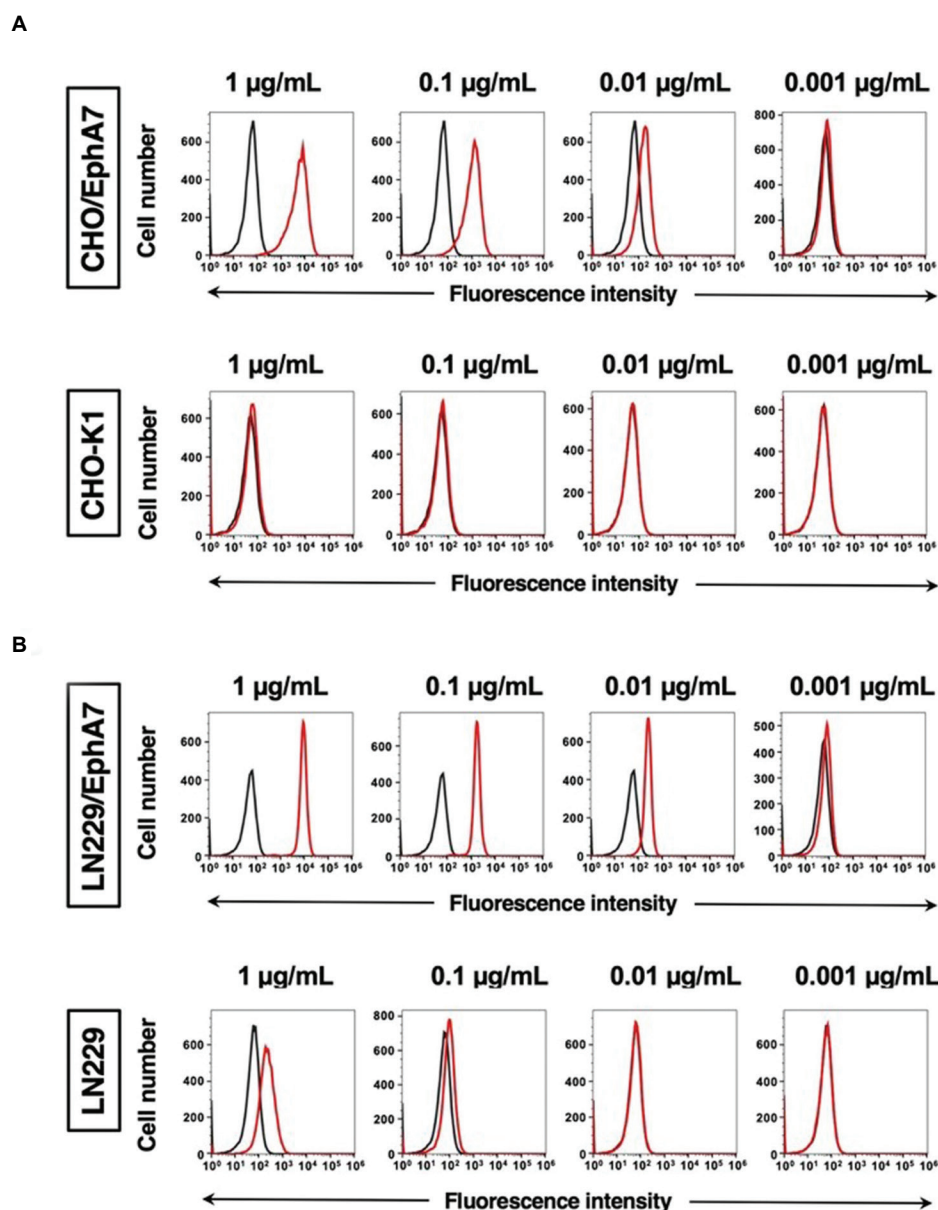


Figure 3. Flow cytometric analysis of E α 7Mab-10 against exogenously expressed EphA7. (A) Chinese hamster ovary (CHO)/EphA7 and CHO-K1 cells were treated with 0.001–1 μ g/mL of E α 7Mab-10 (red line). (B) LN229/EphA7 and LN229 cells were treated with 0.001–1 μ g/mL of E α 7Mab-10 (red line). The cells were then treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. The black line indicates the control (no primary antibody treatment). These experiments were performed at least twice.

Studies have shown that EphA7 shows context-dependent roles in tumor progression and suppression.²⁸ EphA7 is inactivated in 72% of follicular lymphomas.¹⁵ The EphA7 knockdown drove lymphoma development in a murine model. Furthermore, a soluble *EPHA7* splice variant interfered with another Eph-receptor and inhibited tumorigenic signaling in follicular lymphoma cells.¹⁵ In prostate cancer specimens, EphA7 was significantly decreased compared to paired normal tissues.¹⁴ In

esophageal squamous cell carcinoma, low EphA7 expression significantly correlated with lymph node metastases, poor tumor differentiation, and advanced pTNM staging.²⁹ Consequently, patients with a low EphA7 expression have a poorer prognosis compared with those with high expression.²⁹

Conversely, increased EphA7 expression correlated with adverse outcomes and increased tumor vascularity in glioblastoma multiforme.³⁰ Since E α 7Mab-10 recognizes

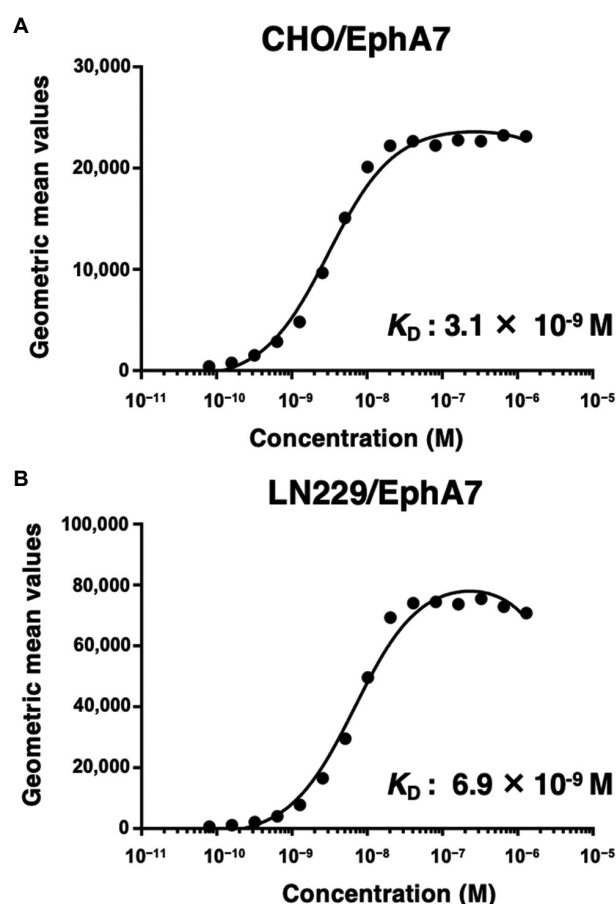


Figure 4. Evaluation of the dissociation constant (K_D) values of Ea₇Mab-10. (A) Chinese hamster ovary (CHO)/ephrin type-A receptor 7 (EphA7) and (B) LN229/EphA7 cells were suspended in 100 μL of serially diluted 100 $\mu\text{g/mL}$ to 0.006 $\mu\text{g/mL}$ of Ea₇Mab-10. After treatments of primary monoclonal antibodies (mAbs), cells were treated with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G. Subsequently, the geometric mean values from fluorescence data were collected, and the K_D values were calculated. These experiments were performed at least twice.

glioblastoma LN229 without exogenous EphA7 expression (Figure 3B), Ea₇Mab-10 can be used to detect endogenous EphA7. Glioblastoma patients with positive EphA7 expression showed reduced median survival compared to those with negative expression, with EphA7 protein expression inversely correlating with overall survival,³⁰ in IHC analyses performed using a rabbit anti-EphA7 polyclonal antibody. Further studies are needed to show the usefulness of Ea₇Mab-10 for IHC of FFPE tumor tissues.

EPHA7 mutation has been implicated as a key driver in small-cell lung cancer.¹⁶ Although EphA7 has been implicated in regulating the actin cytoskeleton, its precise role has not yet been thoroughly investigated. *EPHA7* mutations were also found in various tumors and exhibited a strong association with reduced patient survival.¹ In

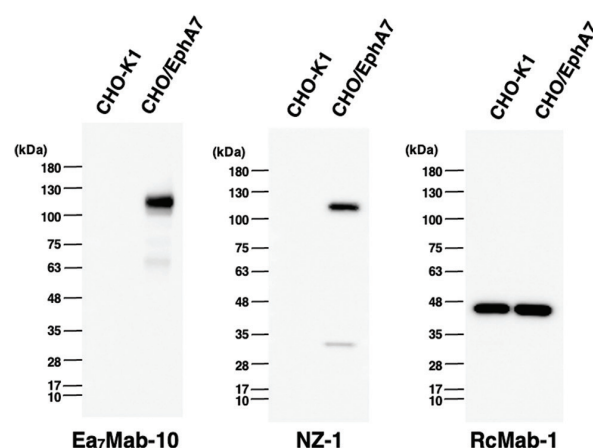


Figure 5. Detection of ephrin type-A receptor 7 (EphA7) protein using Ea₇Mab-10 by Western blotting. Chinese hamster ovary (CHO)-K1 and CHO-K1/EphA7 cell lysates were electrophoresed on polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with Ea₇Mab-10 (1 $\mu\text{g/mL}$), an anti-PA16 tag mAb (clone NZ-1, 1 $\mu\text{g/mL}$), and RcMab-1 (1 $\mu\text{g/mL}$) and subsequently with horseradish peroxidase-conjugated anti-mouse or anti-rat immunoglobulins. These experiments were performed at least twice.

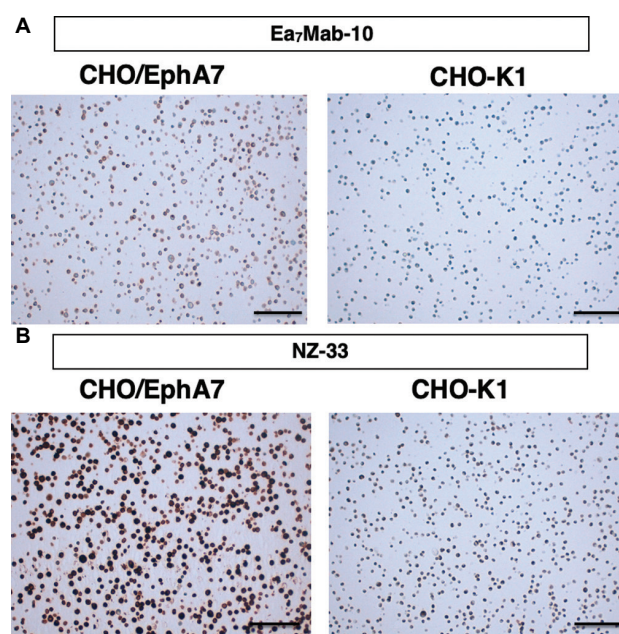


Figure 6. Immunohistochemical staining of paraffin-embedded Chinese hamster ovary (CHO)/ephrin type-A receptor 7 (EphA7) and CHO-K1 sections. The sections of CHO/EphA7 and CHO-K1 cells were treated with (A) Ea₇Mab-10 (0.1 $\mu\text{g/mL}$) or (B) an anti-PA16 tag mAb (clone NZ-33, 0.1 $\mu\text{g/mL}$). The staining was performed using BenchMark ULTRA PLUS with the ultraView Universal DAB Detection Kit. Scale bar: 100 μm ; magnification: $\times 200$. These experiments were performed at least twice.

contrast, a study demonstrated a robust link between *EPHA7* mutations and better clinical outcomes in patients treated with immune checkpoint inhibitors. Notably,

EPHA7 mutant tumor patients without immune checkpoint inhibitor therapy had significantly worse overall survival.¹⁷ Although further studies are essential to determine whether EphA7 in tumors should be targeted, Ea₇Mab-10 may be useful as a future therapeutic and/or diagnostic mAb.

EphA7 is reportedly involved in immunosuppression through interaction with LILRB5 on immunosuppressive myeloid cells. EphA7-binding to LILRB5 stimulates the expression of immunosuppressive markers on myeloid cells from cancer patients.¹⁸ In a transgenic mouse model of myeloid cell-specific LILRB5 expression, the Eph receptor on tumor cells bound to LILRB5 on myeloid cells, resulting in an increased number of immunosuppressive myeloid cells, a decreased number of functional T cells, and elevated tumor growth compared to control.¹⁸ Therefore, the inhibition of EphA7-induced LILRB5 signaling is thought to be important for potentiating immune checkpoint inhibitor therapies. In addition to Ea₇Mab-10, we have also cloned several anti-EphA7 mAbs. Evaluating the neutralization activity of the EphA7–LILRB5 interaction is essential for developing novel tumor immunotherapy.

5. Conclusion

Ea₇Mab-10, generated using the CBIS method, is applicable for various experiments, including flow cytometry, Western blotting, and IHC. Ea₇Mab-10 can be used in basic research on EphA7 and developed for mAb-based targeted therapies.

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Conflict of interest

The authors declare that they have no competing interests.

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Writing—review & editing: Yukinari Kato

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Approval number: 2022MdA-001).

Consent for publication

Not applicable.

Availability of data

The data presented in this study are available in the article.

Further disclosure

The paper has been uploaded to a preprint server (DOI: 10.20944/preprints202504.2395.v1).

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Appendix

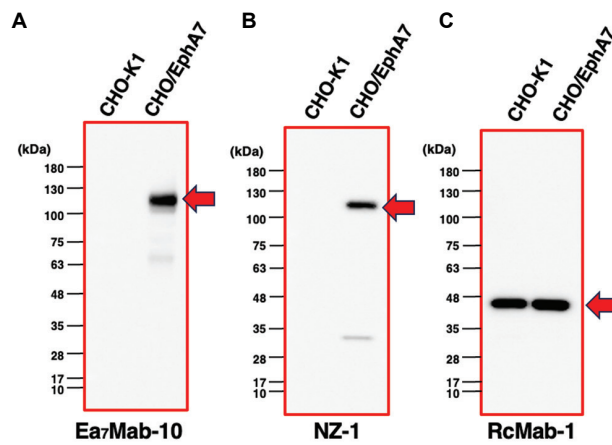


Figure A1. Original images for the Western blots in Figure 5. Western blots for (A) ephrin type-A receptor 7 (EphA7) detected by E_aMab-10 (110 kDa), (B) PA16-tagged EphA7 detected by NZ-1 (110 kDa), and (C) isocitrate dehydrogenase 1 detected by RcMab-1 (45 kDa). Abbreviation: CHO: Chinese hamster ovary.