Establishment of Anti-Human ATRX Monoclonal Antibody AMab-6

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Gliomas are the most frequently occurring brain tumors with a heterogeneous molecular background. The molecular subgrouping of gliomas more prognostically stratifies patients into distinct groups compared with conventional histological classification. The most important molecules for the subtype diagnosis of diffuse gliomas are mutations of isocitrate dehydrogenase (IDH), TERT promoter, and α-thalassemia/mental-retardation-syndrome-X-linked (ATRX) and the codeletion of 1p/19q. Among them, IDH and ATRX mutations can be diagnosed using specific monoclonal antibodies (mAbs). We have developed many mAbs against IDH mutants, including HMab-1/HMab-2 against IDH1-R132H and multispecific mAbs MsMab-1/MsMab-2 against IDH1/2 mutations. In contrast, highly sensitive mAbs against ATRX remain to be established. In this study, we immunized mice with recombinant human ATRX and developed a novel mAb, AMab-6. The dissociation constant of AMab-6 was determined to be 9.7×10^{-10} M, indicating that the binding affinity of AMab-6 is very high. Furthermore, AMab-6 sensitively detects ATRX in Western blot and immunohistochemical analyses, indicating that AMab-6 could become the standard marker to determine the ATRX mutation status of gliomas in immunohistochemical analyses.

Keywords: ATRX, glioma, immunohistochemistry

Introduction

THE MOLECULAR SUBGROUPING of gliomas using mutations of isocitrate dehydrogenase (IDH), α-thalassemia/mental-retardation-syndrome-X-linked (ATRX), and TERT promoter and the status of 1p/19q more prognostically stratify patients into distinct groups compared with conventional histological classification.[1–4] Treatment strategies can be planned with respect to molecular subtype along with the WHO grade; therefore, these molecular subtypes are clinically important. The inclusion of molecular parameters in the WHO definition of brain tumors was forwarded as the "ISN-Haarlem" consensus.[5] The 2016 WHO Classification of Tumors of the Central Nervous System (2016 CNS WHO) is both a conceptual and practical advance over the 2007 CNS WHO. The 2016 CNS WHO uses molecular parameters in addition to histology to define many tumor entities; presents major restructuring of the diffuse gliomas, medulloblastomas, and other embryonal tumors; and incorporates new entities, which are defined by both histology and molecular features.[6]

ATRX mutation in gliomas results in the loss of ATRX protein expression, which can be diagnosed by immunohistochemistry using anti-ATRX antibodies.[7] Almost all studies of ATRX have used polyclonal antibodies[8] because highly sensitive monoclonal antibodies (mAbs) against human ATRX remain to be established. Herein, we report a novel anti-ATRX mAb, AMab-6, which is very useful in enzyme-linked immunosorbent assay (ELISA), Western blot, and immunohistochemical analyses.

Materials and Methods

Cell lines and animals

P3 U1 was obtained from the American Type Culture Collection (Manassas, VA) and was cultured in the RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Antibiotics, including 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of amphotericin B (Nacalai Tesque, Inc.),

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were added to the media. Female MRL/lpr mice (4 weeks old) were purchased from SLC Japan (Shizuoka, Japan). Animals were housed under pathogen-free conditions. The ''Animal Care and Use Committee of Tohoku University'' approved the animal experiments described herein.

Hybridoma production

Plasmid preparation and ATRX recombinant protein production are described in the Supplementary Data. MRL/lpr mice were immunized using an intraperitoneal (i.p.) injection of PA-ATRX-RAP-MAP (100 μg) together with Imject Alum (Thermo Fisher Scientific, Inc.). After three additional immunizations, a booster injection was administered by i.p. 2 days before the spleen cells were harvested. The spleen cells were fused with P3 U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The fused cells were grown in the RPMI medium with hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific, Inc.). Culture supernatants were screened using ELISA for the binding to recombinant MBP-ATRX-MAP-PA or GST-ATRX-MAP-PA.

Enzyme-linked immunosorbent assay

Recombinant MBP-ATRX-MAP-PA or GST-ATRX-MAP-PA was immobilized on Nunc MaxiSorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 5 μg/mL for 30 minutes. After blocking with 1% bovine serum albumin in 0.05% Tween 20/phosphate-buffered saline, the plates were incubated with culture supernatant followed by 1:3000-diluted peroxidase-conjugated anti-mouse IgG (Dako; Agilent Technologies, Inc., Glostrup, Denmark). The enzymatic reaction was performed with a 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). The optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Determination of binding affinity using ELISA

Purified recombinant protein was immobilized at 5 μg/mL. The plates were incubated with serially diluted antibodies (26 pg/mL–10 μg/mL) followed by 1:1000-diluted peroxidase-conjugated anti-mouse IgG (Dako). The dissociation constant (K_D) was obtained by fitting the binding isotherms using the built-in one-site binding models in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Western blot analyses

Recombinant protein (0.1 μg) was boiled in SDS sample buffer (Nacalai Tesque, Inc.). The proteins were electrophoresed on 5%–20% polyacrylamide gels (Wako Pure Chemical Industries Ltd., Osaka, Japan) and were transferred onto a PVDF membrane (EMD Millipore Corp., Billerica, MA). After blocking with 4% skim milk (Nacalai Tesque, Inc.), the membrane was incubated with AMab-6 or anti-MBP (clone: TMab-2; Wako Pure Chemical Industries Ltd.) and then with peroxidase-conjugated anti-mouse or anti-rat antibodies (1:1000 diluted; Dako) and developed with the Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific, Inc.) or the ImmunoStar LD Chemiluminescence Reagent (Wako Pure Chemical Industries Ltd.) using the Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Immunohistochemical analysis

Surgical specimens of oligodendroglioma and diffuse astrocytoma were fixed in 4% paraformaldehyde and embedded in paraffin for routine histopathological and immunohistochemical analysis. Immunohistochemical analysis was performed using avidin-biotin immunoperoxidase technique. In brief, 4-μm-thick tissue sections were deparaffinized using xylene and rehydrated. After antigen retrieval using Target Retrieval Solution, pH 9.0 (Dako), the sections were autoclaved for 10 minutes and then maintained at room temperature for 40 minutes. Sections were quenched using 3% hydrogen peroxide (H_2O_2) in methanol for 20 minutes and blocked with 5% skim milk in TBST for 30 minutes. Sections were incubated with 3 μg/mL AMab-6 overnight at 4°C. Immunocomplexes were treated with an Envision+ Kit (Dako) for 1 hour. Color was developed using 3, 3-diaminobenzidine tetrahydrochloride (DAB Substrate Kit SK-4100, Vector, Japan) for 5 minutes. Sections were then counterstained with hematoxylin (Wako Pure Chemical Industries Ltd.). Images were acquired with a BZ-X700 microscope (Keyence, Osaka, Japan).

Results and Discussion

The two major methods for identifying these subtypes of gliomas are direct DNA sequencing and immunohistochemical analyses using antibodies.[9] Immunohistochemistry is a
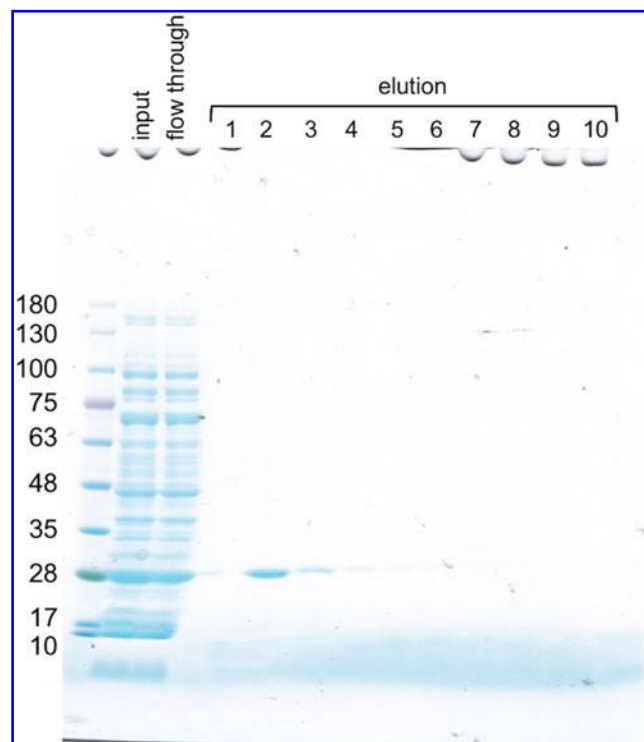

FIG. 1. Purification of the recombinant ATRX protein. Cleared lysate (5 μL) from the transformed *Escherichia coli* before (input) and after (flow through) NZ-1-Sepharose capture, as well as 10 elution fractions (lanes 1–10) during column chromatography, was subjected to 5%–20% gradient SDS-PAGE under a reducing condition and stained with CBB. ATRX, α-thalassemia/mental-retardation-syndrome-X-linked; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

robust and widely available method, and immunohistochemical approaches with defined protocols and materials have become essential for assessing molecular genetic changes.[10] Among many molecular parameters, the important molecules for subtype diagnosis of diffuse gliomas are mutations of IDH, TERT promoter, and ATRX and the co-deletion of 1p/19q.[11] In particular, mutations of IDH[9] and ATRX[7,8] can be accurately detected by immunohistochemical analysis. We developed many mAbs against IDH mutants, including HMab-1/HMab-2 against IDH1-R132H and multispecific mAbs MsMab-1/MsMab-2 against IDH1/2 mutations.[7,9,12–28] However, highly sensitive mAbs against human ATRX remain to be established.

In this study, the recombinant PA-ATRX-RAP-MAP was captured using NZ-1-Sepharose and efficiently eluted with MES buffer (pH 6.0), containing $3 M$ $MgCl_2$ (Fig. 1). Mice were then immunized with PA-ATRX-RAP-MAP to develop novel anti-ATRX mAbs. We utilized the C-terminal region of ATRX as immunogen because commercially available polyclonal antibodies (Sigma-Aldrich) against ATRX were also produced against that region.[7] Using ELISA, the culture supernatants of hybridomas were screened for binding to recombinant MBP-ATRX-MAP-PA or GST-ATRX-MAP-PA that was purified from *Escherichia coli*. As a result, AMab-6 (mouse IgG$_1$, kappa) was established after limiting dilution.

As shown in Figure 2, AMab-6 reacted with MBP-ATRX-MAP-PA in a dose-dependent manner. In contrast, AMab-6 did not react with MBP-IDH1. We further performed a kinetic analysis of the interaction of AMab-6 with MBP-ATRX-MAP-PA (Fig. 2). The K_D of AMab-6 was determined to be 9.7×10^{-10} M, indicating that the apparent binding affinity of AMab-6 is very high.

Western blot analysis demonstrated that AMab-6 detected MBP-ATRX-MAP-PA, but not MBP-IDH1, indicating that AMab-6 does not detect MBP tag in Western blot analysis (Fig. 3). Immunohistochemical analyses revealed that AMab-6 reacted with the nuclei of oligodendroglioma cells without ATRX mutations (Fig. 4A). ATRX mutations in gliomas result
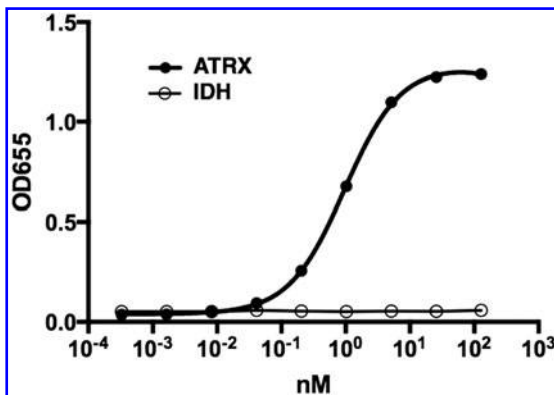

FIG. 3. Western blot analysis. Recombinant proteins $(0.1 \mu g)$ of MBP-ATRX-MAP-PA and MBP-IDH1 were electrophoresed and transferred onto a PVDF membrane. After blocking, the membrane was incubated with AMab-6, RMab-3 (anti-IDH1), or TMab-2 (anti-MBP).

FIG. 2. Determination of binding affinity using enzyme-linked immunosorbent assay. Purified recombinant protein (MBP-ATRX-MAP-PA or MBP-IDH1) was immobilized at $5 \mu g/mL$. The plates were incubated with serially diluted antibodies (26 pg/mL–10 μg/mL) followed by 1:1000-diluted peroxidase-conjugated anti-mouse IgG. The dissociation constant (K_D) was obtained by fitting the binding isotherms using the built-in one-site binding models in GraphPad Prism 6. IDH, isocitrate dehydrogenase.
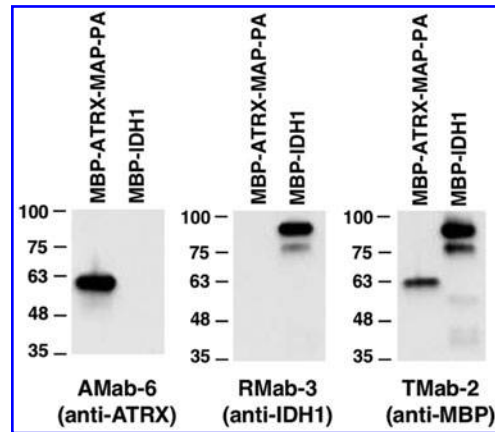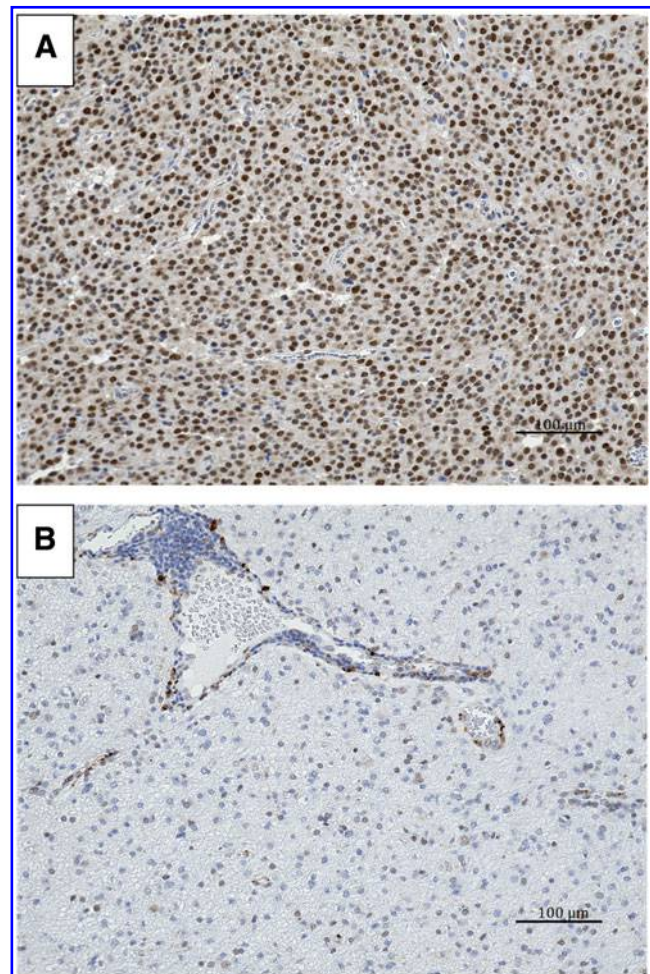

FIG. 4. Immunohistochemical analysis. **(A)** Oligodendroglioma, IDH-mutant (WHO grade II). Note the ATRX immunostaining in the nucleus of tumor cells without ATRX mutation. **(B)** Diffuse astrocytoma, IDH mutant (WHO grade II). Note the negative staining of ATRX in the nucleus of tumor cells with ATRX mutation, whereas positive staining was observed in the vascular endothelial cells without ATRX mutation.

in the loss of ATRX protein expression, which can be diagnosed using immunohistochemical analysis.[7] Likewise, AMab-6 did not recognize the nuclei of diffuse astrocytoma cells, which possess ATRX mutation, but reacted with those of vascular endothelial cells without ATRX mutation (Fig. 4B). These results indicate that AMab-6 is very sensitive against ATRX, which is expressed in gliomas without ATRX mutation.

In conclusion, a novel anti-ATRX mAb, AMab-6, sensitively reacted with human ATRX in ELISA, Western blot, and immunohistochemical analyses. Although many studies have used commercially available polyclonal anti-ATRX antibodies,[7] we confirmed that the reactivity of AMab-6 is compatible with that of polyclonal antibodies (data not shown). Furthermore, we performed immunohistochemical analyses under many other conditions for antigen retrievals (citrate buffer, pH 6.0 or 9.0) or incubation time of AMab-6 (30 minutes, 1 hour, or overnight) and obtained positive staining results as shown in Figure 4. Altogether, AMab-6 could serve as a standard marker to determine the ATRX mutation status of gliomas during immunohistochemical analyses.

Author Disclosure Statement

No competing financial interests exist.

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