Epitope mapping of an anti-alpha thalassemia/mental retardation syndrome X-linked monoclonal antibody AMab-6

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A B S T R A C T

The alpha-thalassemia/mental-retardation-syndrome-X-linked (ATRX) gene is located on the q arm of the X chromosome. ATRX gene mutations were first discovered in pancreatic neuroendocrine tumors, and subsequently in other cancer subtypes, including gliomas. Molecular subgrouping of gliomas has been more important than conventional histological classifications. Mutations in the isocitrate dehydrogenase (IDH), telomerase reverse transcriptase (TERT) promoter, and ATRX and the codeletion of chromosomes 1p/19q are used as biomarkers for diagnosing the subtypes of diffuse gliomas. We recently developed a sensitive monoclonal antibody (mAb) AMab-6 against ATRX by immunizing mice with recombinant human ATRX. AMab-6 can help to detect ATRX mutations via Western blotting and immunohistochemical analyses. In this study, we characterized the binding epitope of AMab-6 using enzyme-linked immunosorbent assay (ELISA), Western blotting, and immunohistochemical analysis, and found that Gln2368 of ATRX is critical for AMab-6 binding to ATRX. Our findings could be applied to the production of more functional anti-ATRX mAbs.

1. Introduction

The alpha-thalassemia/mental-retardation-syndrome-X-linked (ATRX) gene is located on the q arm of the X chromosome. ATRX gene mutations were first discovered in pancreatic neuroendocrine tumors [1], and subsequently in other cancer subtypes, including gliomas. Gliomas are the most frequently occurring brain tumors and have a heterogeneous molecular background [2]. Molecular subgrouping of gliomas using mutations in isocitrate dehydrogenase (IDH) 1/2, TERT promoter, and ATRX and codeletion of 1p/19q as biomarkers stratifies patients into distinct groups that are more prognostically assessed compared with conventional histological classifications [1,3–5]. These molecular subtypes are clinically important because treatment strategies can be planned in accordance with molecular subtype along with the World Health Organization (WHO) tumor grading system. The 2016 WHO Classification of Tumors of the Central Nervous System (2016 WHO CNS) is both conceptually and practically more advanced than the 2007 WHO CNS [6,7]. The 2016 WHO CNS uses molecular parameters, in addition to histological assessments to define many tumor entities, presents major restructuring of diffuse gliomas, medulloblastomas, and other embryonal tumors; and incorporates new entities that are defined using both histology results and molecular features.

The loss of ATRX mRNA and protein in gliomas is caused by an ATRX mutation. Loss of the ATRX protein can be diagnosed by immunohistochemistry using anti-ATRX antibodies [8,9]; however, nearly all studies on ATRX protein have used polyclonal antibodies [10] because highly sensitive monoclonal antibodies (mAbs) against human ATRX protein had not been established. Recently, we established a novel anti-ATRX mAb, AMab-6, which is very useful in enzyme-linked immunosorbent assay (ELISA), Western blot, and immunohistochemical analysis, and found that Gln2368 of ATRX is critical for AMab-6 binding to ATRX. Our findings could be applied to the production of more functional anti-ATRX mAbs.
2. Materials and methods

2.1. Plasmid preparation

Human ATRX cDNA (Accession No. AB102641) encoding amino acids 2273–2413, 2273–2387, 2273–2308, 2309–2413, 2344–2413, and 2379–2413 were obtained using polymerase chain reaction (PCR) with cDNA derived from human lung cDNA as a template. The primer sets for ATRX are summarized in Supplementary Table 1. ATRXs were subcloned into the expression vector pMAL-c2 (New England Biolabs Inc., Beverly, MA, USA) with MAP (GDGMVP-GIEDK) [12] and PA (GVAMPGAEDDVV) [13] tags using the In-Fusion PCR Cloning Kit (Takara Bio., Inc., Shiga, Japan). Substitution of ATRX amino acids 2273–2413 with either alanine or glycine was performed using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA, USA). This construct was verified by direct DNA sequencing.

2.2. Enzyme-linked immunosorbent assay

Synthesized ATRX peptides using PEPScreen (Sigma-Aldrich Corp., St. Louis, MO, USA) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 10 μg/mL for 30 min at 37 °C. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific Inc.), the plates were incubated with 10 μg/mL purified AMab-6 followed by a 1:2000 dilution of peroxidase-conjugated mouse or rat antibody. Color was developed using the Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific Inc.) or the ImmunoStar LD Chemiluminescence Reagent (FUJIFILM Wako Pure Chemical Corporation) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

2.3. Western blot analyses

Competent Escherichia coli TOP-10 cells (Thermo Fisher Scientific Inc.) were transformed and cultured overnight at 37 °C in LB medium (Thermo Fisher Scientific Inc.) containing 100 μg/mL ampicillin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Cell pellets were resuspended in phosphate buffered solution with 1% Triton X-100 and 50 μg/mL aprotinin (Sigma-Aldrich Corp.). Lysates (10 μg) were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc., Kyoto, Japan). The proteins were electrophoresed on 5–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), the membrane was first incubated with AMab-6 [11] or NZ-1 (anti-PA tag) [13] and then with peroxidase-conjugated anti-mouse or anti-rat antibody (1:1000 diluted; Agilent Technologies Inc.) and developed using the Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific Inc.) or the ImmunoStar LD Chemiluminescence Reagent (FUJIFILM Wako Pure Chemical Corporation) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

2.4. Immunohistochemical analyses

This study examined one patient with oral cancer who underwent surgery at Tokyo Medical and Dental University. The Tokyo Medical and Dental University Institutional Review Board reviewed and approved the use of the human cancer tissues, and written informed consent was obtained from the patient. Histological sections (4-μm thick) were mounted and treated using an EnVision+ Kit (Agilent Technologies Inc.) for 30 min. Color was...
developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies Inc.) for 2 min, and counterstained with hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

3. Results and discussion

Immunohistochemistry is a robust and widely available method used to assess genetic changes at the molecular level using defined protocols and materials [14,15]. The important molecules for subtype diagnosis of diffuse gliomas are mutations of IDH1/2, TERT promoter, and ATRX and the codeletion of 1p/19q among the many molecular parameters [16]. Mutations of IDH1/2 [14] and ATRX [8,10] can be accurately detected using this method. We recently developed both anti-mutated IDH mAbs [17] and an anti-ATRX mAb, AMab-6 [11]. Several mAbs against IDH mutants include HMab-1/HMab-2 against IDH1-R132H and multi-specific mAbs MsMab-1/MsMab-2 against IDH1-R132H and multi-specific mAbs MsMab-1/MsMab-2 against IDH1/R132H and multi-specific mAbs MsMab-1/MsMab-2 against IDH1/R132H [18–21].

As shown in Fig. 1, we produced three C-terminal deletion mutants (dc2378, dc2343, and dc2308) and three N-terminal deletion mutants (dn2309, dn2344, and dn2379). Western blot analysis demonstrated that AMab-6 detected dc2378, dn2309, and dn2344 but not dc2343, dc2308, and dn2379 (Fig. 2A), indicating that the N-terminus of the AMab-6-epitope exits between amino acids 2344 and 2379, and the C-terminus of the AMab-6-epitope exits between amino acids 2343 and 2378. Next, we produced the following four peptides: pp2344–2363 (ATRX amino acids 2344–2363), pp2349–2368 (ATRX amino acids 2349–2368), pp2354–2373 (ATRX amino acids 2354–2373), and pp2359–2378 (ATRX amino acids 2359–2378) as depicted in Fig. 1.

ELISA demonstrated that AMab-6 detected pp2354–2373 and pp2359–2378, and did not react with pp2344–2363 or pp2349–2368 (Table 1).

| Peptide Sequence | OD655<br><br> | AMab-6<br><br> |
|-----------------|-----------------|-----------------|
| 2344–2363       | 1.0             | –               |
| 2349–2368       | 0.6             | ++              |
| 2354–2373       | 0.1             | +               |
| 2354–2378       | 1.0             | –               |
| 2359–2378       | 0.6             | ++              |
| 2359–2368       | 0.1             | +               |
| 2364A           | 1.0             | –               |
| 2364B           | 0.6             | ++              |
| 2364C           | 0.1             | +               |
| 2365A           | 1.0             | –               |
| 2365B           | 0.6             | ++              |
| 2365C           | 0.1             | +               |
| 2366A           | 1.0             | –               |
| 2366B           | 0.6             | ++              |
| 2366C           | 0.1             | +               |
| 2367A           | 1.0             | –               |
| 2367B           | 0.6             | ++              |
| 2367C           | 0.1             | +               |
| 2368A           | 1.0             | –               |
| 2368B           | 0.6             | ++              |
| 2368C           | 0.1             | +               |
| 2369A           | 1.0             | –               |
| 2369B           | 0.6             | ++              |
| 2369C           | 0.1             | +               |
| 2370A           | 1.0             | –               |
| 2370B           | 0.6             | ++              |
| 2370C           | 0.1             | +               |
| 2371A           | 1.0             | –               |
| 2371B           | 0.6             | ++              |
| 2371C           | 0.1             | +               |
| 2372A           | 1.0             | –               |
| 2372B           | 0.6             | ++              |
| 2372C           | 0.1             | +               |
| 2373A           | 1.0             | –               |
| 2373B           | 0.6             | ++              |
| 2373C           | 0.1             | +               |

We further synthesized the following 15 peptides of ATRX point mutations: K2359A, E2360A, N2361A, M2362A, N2363A, L2364A, S2365A, E2366A, A2367G, Q2368A, V2369A, Q2370A, A2371G, L2372A, and A2373G (Table 1). ELISA demonstrated that AMab-6 reacted strongly with nearly all point mutants; however, it reacted very weakly with Q2368A, indicating that Gln2368 is important for AMab-6 binding to ATRX protein.

We produced the following 11 ATRX point mutants: N2363A, N2364A, S2365A, E2366A, A2367G, Q2368A, V2369A, Q2370A, A2371G, L2372A, and A2373G (Table 1). ELISA demonstrated that AMab-6 reacted strongly with nearly all point mutants; however, it reacted very weakly with Q2368A, indicating that Gln2368 is important for AMab-6 binding to ATRX protein.

We produced the following 11 ATRX point mutants: N2363A,
L2364A, S2365A, E2366A, A2367G, Q2368A, V2369A, Q2370A, A2371G, L2372A, and A2373G. Western blotting demonstrated that AMab-6 did not neutralize Q2368A (Fig. 3). In contrast, the Q2368A peptide did not block the reaction of AMab-6, thereby confirming the results of epitope mapping using ELISA and Western blotting.

In conclusion, Gln2368 of ATRX is critical for AMab-6 binding. Our findings can be applied for the production of more functional anti-ATRX mAbs.

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Conflict of interest

The authors declare no conflicts of interest involving this article.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.07.003.

Appendix A. Supplementary material

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Fig. 3. Immunohistochemistry using oral cancer tissue. Oral cancer tissues were autoclaved for 20 min in a citrate buffer. Sections were then incubated with 5 μg/mL AMab-6 or 5 μg/mL peptides and stained using an EnVision+ kit. Color development was performed using 3,3′-diaminobenzidine tetrahydrochloride. Sections were then counterstained with hematoxylin. Notes: scale bar = 100 μm; HE, hematoxylin and eosin.

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