Biochemistry and Biophysics Reports 15 (2018) 76–80

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

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

Mika K. Kaneko[a], Shinji Yamada[a], Shunsuke Itai[a,b], Yoshikazu Furusawa[a,c,d], Takuro Nakamura[a], Miyuki Yanaka[a], Saori Handa[a], Kayo Hisamatsu[a], Yoshimi Nakamura[a], Masato Fukui[d], Hiroyuki Harada[b], Yukinari Kato[a,c,*]

[a] Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
[b] Department of Oral and Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan
[c] New Industry Creation Hatchery Center, Tohoku University, 2-1, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
[d] ZENOAQ RESOURCE CO., LTD., 1-1 Tairanoue, Sasagawa, Asaka-machi, Koriyama, Fukushima 963-0196, Japan

ARTICLE INFO

Keywords:
ATRX
AMab-6
Epitope mapping

ABSTRACT

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 analyses [11]. In this study, we characterized the binding epitope of AMab-6 using ELISA, Western blot analysis, and immunohistochemical analyses.

Abbreviations: ATRX, alpha-thalassemia/mental-retardation-syndrome-X-linked; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DAB, 3,3-diaminobenzidine tetrahydrochloride

* Corresponding author at: New Industry Creation Hatchery Center, Tohoku University, 2-1, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan.
E-mail address: yukinarikato@med.tohoku.ac.jp (Y. Kato).

https://doi.org/10.1016/j.bbrep.2018.07.003
Received 15 June 2018; Received in revised form 1 July 2018; Accepted 2 July 2018

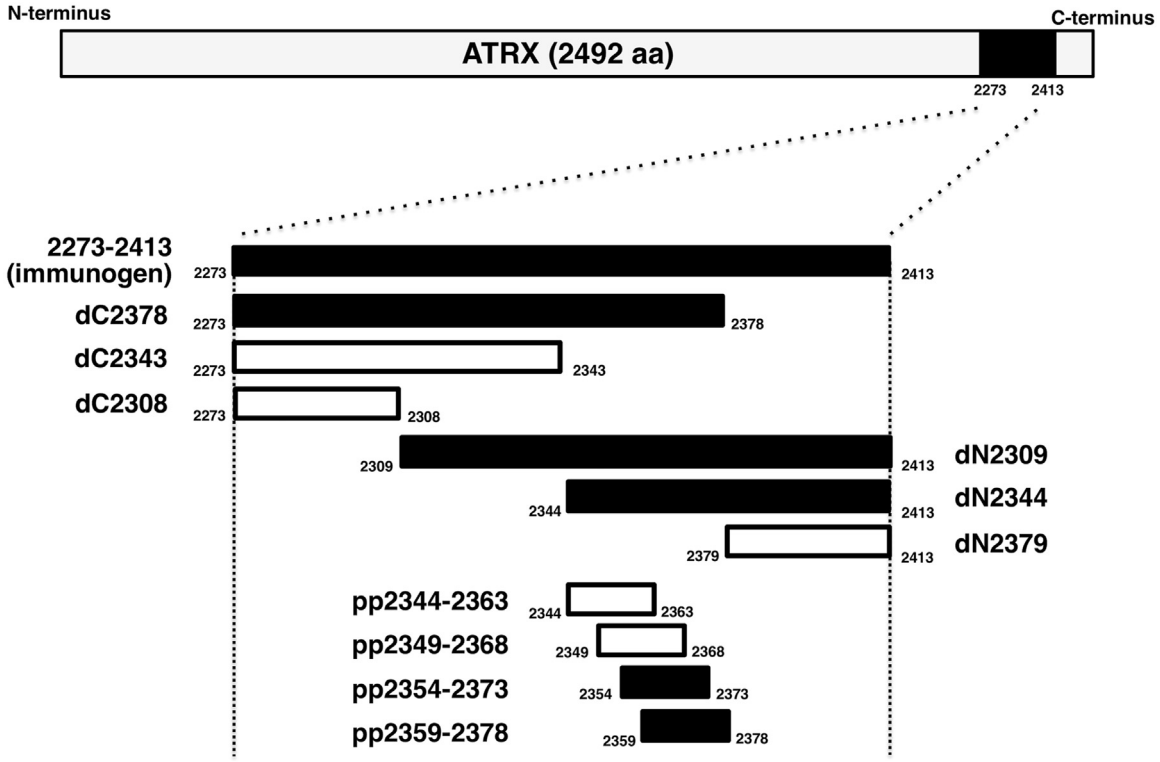

Fig. 1. Production of ATRX deletion mutants. Three ATRX C-terminal deletion mutants and three ATRX N-terminal deletion mutants were produced. Four ATRX peptides were also synthesized. Black bars, the deletion mutants or synthesized peptides, which were detected by AMab-6; white bar, the deletion mutants or synthesized peptides, which were not detected by AMab-6.

2. Materials and methods

2.1. Plasmid preparation

Human ATRX cDNA (Accession No. AB102641) encoding amino acids 2273–2413, 2273–2378, 2273–2343, 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 (GDGMVPPGIEDK) [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 anti-mouse IgG (Agilent Technologies Inc.). The enzymatic reaction was conducted using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific Inc.). Optical density was measured at 655 nm using an iMark Microplate Reader (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). These reactions were performed at 37 °C using a total sample volume of 50–100 μL.

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 directly autoclaved for 20 min in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan). After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific Inc.), the sections were incubated with 5 μg/mL AMab-6 or 5 μg/mL AMab-6 plus 5 μg/mL peptides for 1 h at room temperature and treated using an EnVision + Kit (Agilent Technologies Inc.) for 30 min. Color was