MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 37, Number 4, 2018

DOI: 10.1089/mab.2018.0019

Epitope Mapping of Anti-Telomerase Reverse Transcriptase Monoclonal Antibodies

Yoshikazu Furusawa,[1,2] Shunsuke Itai,[1] Shinji Yamada,[1] Mika K. Kaneko,[1] and Yukinari Kato[1,2]

Telomerase reverse transcriptase (TERT) is the main catalytic subunit of telomerase-associated protein machinery. Upregulation of TERT at the transcriptional level results in immortal cell phenotype associated with cancer. To date we have developed eight anti-TERT monoclonal antibodies (mAbs) (TMab-4, TMab-5, TMab-6, TMab-7, TMab-9, TMab-10, TMab-11, and TMab-12) by immunizing mice with synthetic peptides ($_{302}$-QHHAGPPSTSRPPRPWDTPC-$_{321}$) of TERT. We further characterized those epitopes using enzyme-linked immunosorbent assay, and here we discuss the critical epitope of an anti-TERT mAb, which is applicable for immunohistochemical analysis.

Keywords: TERT, monoclonal antibody, epitope mapping

Introduction

Telomerase reverse transcriptase (TERT) is the main catalytic subunit of telomerase-associated protein machinery. Mutation hotspots in the TERT promoter have been identified in human melanomas[1,2] and gliomas.[3,4] Diffuse gliomas in adults are separated into three comprehensive tumor groups with distinctive prognoses based on the mutations of isocitrate dehydrogenase (IDH) 1 and 2 and 1p/19q-codeletion.[3] TERT promoter mutations were frequently and selectively observed in IDH wild type glioblastomas and 1p/19q-codeleted oligodendrogliomas.[4] TERT promoter mutations are associated with three distinctive glioma groups, which indicates the involvement of TERT in the pathogenesis of diffuse gliomas.[3] Mutations in the TERT promoter have also been reported to occur frequently in patients with oral squamous cell carcinoma (OSCC)[5]; therefore, somatic TERT promoter mutations could play a vital role in the pathogenesis and progression of OSCC. Therefore, a sensitive and specific anti-human TERT monoclonal antibody (mAb) is very important for the detection of TERT in those tumors.

To date, we have developed eight anti-TERT mAbs (TMab-4, TMab-5, TMab-6, TMab-7, TMab-9, TMab-10, TMab-11, and TMab-12) by immunizing mice with synthetic peptides of TERT. We recently determined the epitope of TMab-6, which is useful for immunohistochemistry in human tissues,[6] using enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry.[7] In this study, we further characterized epitopes of the other anti-TERT mAbs using ELISA, and here we discuss the critical epitope of anti-TERT mAbs, which is applicable for immunohistochemical analysis.

Materials and Methods

Cell lines

P3U1 was obtained from American Type Culture Collection (ATCC, Manassas, VA).[8] P3U1 cells were grown in Roswell Park Memorial Institute medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), 100 U/mL penicillin, 100 μg/mL streptomycin, 25 μg/mL amphotericin B (Nacalai Tesque, Inc.).

The hybridoma cells were grown in Roswell Park Memorial Institute medium with hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific, Inc.), 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, 25 μg/mL amphotericin B (Nacalai Tesque, Inc.), and 5% BriClone Hybridoma Cloning Medium (QED Bioscience, Inc., San Diego, CA). Plasmocin (5 μg/mL; InvivoGen, San Diego, CA) was also used to prevent *Mycoplasma* contamination. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Plasmids of TERT

Human TERT cDNA (Accession No. NM_001193376.1) encoding a specific peptide composed of Glu281-Ala436 amino acid residues was obtained by PCR using a cDNA derived from the HT1080 cell line (ATCC) as a template. The

[1]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
[2]New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

Table 1. Epitope Mapping of Anti-Telomerase Reverse Transcriptase Monoclonal Antibodies by Enzyme-Linked Immunosorbent Assay

Mutation	*Sequence*	*TMab-4*	*TMab-5*	*TMab-6*	*TMab-7*	*TMab-9*	*TMab-10*	*TMab-11*	*TMab-12*
Q302A	AHHAGPPSTSRPPRPWDTP	3+	3+	3+	3+	2+	3+	3+	3+
H303A	QAHAGPPSTSRPPRPWDTP	3+	3+	3+	–	–	–	–	3+
H304A	QHAAGPPSTSRPPRPWDTP	3+	3+	3+	–	–	–	–	3+
A305G	QHHGGPPSTSRPPRPWDTP	3+	3+	3+	–	–	–	–	3+
G306A	QHHAAPPSTSRPPRPWDTP	3+	3+	3+	–	–	–	–	3+
P307A	QHHAGAPSTSRPPRPWDTP	3+	3+	3+	–	–	–	–	3+
P308A	QHHAGPASTSRPPRPWDTP	3+	3+	2+	3+	1+	3+	3+	3+
S309A	QHHAGPPATSRPPRPWDTP	3+	3+	2+	3+	3+	3+	3+	3+
T310A	QHHAGPPSASRPPRPWDTP	3+	3+	–	3+	2+	3+	3+	3+
S311A	QHHAGPPSTARPPRPWDTP	3+	3+	–	3+	2+	3+	3+	3+
R312A	QHHAGPPSTSAPPRPWDTP	3+	3+	3+	3+	3+	3+	3+	3+
P313A	QHHAGPPSTSRAPRPWDTP	3+	3+	2+	3+	3+	3+	3+	3+
P314A	QHHAGPPSTSRPARPWDTP	3+	3+	3+	3+	3+	3+	3+	3+
R315A	QHHAGPPSTSRPPAPWDTP	–	3+	3+	3+	3+	3+	3+	3+
P316A	QHHAGPPSTSRPPRAWDTP	3+	–	3+	3+	3+	3+	3+	3+
W317A	QHHAGPPSTSRPPRPADTP	–	–	1+	3+	2+	3+	3+	1+
D318A	QHHAGPPSTSRPPRPWATP	–	3+	2+	3+	3+	3+	3+	3+
T319A	QHHAGPPSTSRPPRPWDAP	3+	3+	3+	3+	2+	3+	3+	3+
P320A	QHHAGPPSTSRPPRPWDTA	3+	1+	3+	3+	3+	3+	3+	3+

3+, OD655 ≧ 0.7; 2+, 0.4 ≦ OD655 < 0.7; 1+, 0.1 ≦ OD655 < 0.4; –, OD655 < 0.1.
TMab, anti-telomerase reverse transcriptase monoclonal antibodies.

primer set for TERT was as follows: 5′-AAGGATTTCAGA ATTCGAAGCCACCTCTTTGGA-3′ (forward) and 5′-TGC CGTCTCCGAATTCCGCCACAGAGCCCTGGG-3′ (reverse). The PCR product was subcloned into an expression vector, pMAL-c2 (New England Biolabs, Beverly, MA) with MAP tag (GDGMVPPGIEDK)[9] and PA tag (GVAMPG AEDDVV)[10] using In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan).

Production of recombinant TERT

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific, Inc.) were transformed with the plasmid pMAL-c2MAPhPAter/TERTepi and cultured overnight at 37°C in LB medium (Thermo Fisher Scientific, Inc.) containing 100 μg/mL ampicillin (Sigma-Aldrich Corp., St. Louis, MO). Cell pellets were resuspended in phosphate-buffered saline (PBS; Nacalai Tesque, Inc.) with 1% Triton X-100 with 50 μg/mL aprotinin (Sigma-Aldrich Corp.). After sonication, the crude extracts were collected by centrifugation (9000 *g*, 30 min, 4°C). The supernatants were loaded onto amylose resin. The loaded resins were washed extensively with column buffer consisting of 20 mM Tris hydrochloride (pH 7.4), 200 mM sodium chloride and 1 mM ethylenediaminetetraacetic acid, and the fusion proteins were eluted by column buffer with 10 mM maltose.

Establishment of TERT-specific antibodies

BALB/c mice were immunized by intraperitoneal (i.p.) injection of the synthetic peptide of TERT ($_{302}$-QHHAG PPSTSRPPRPWDTPC-$_{321}$; Sigma-Aldrich Corp.) conjugated with keyhole limpet hemocyanin together with Imject Alum (Thermo Fisher Scientific, Inc.). After several additional immunizations, a booster injection was given i.p. 2 days before splenocytes were harvested. The splenocytes of immunized mice were fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The Animal Care and Use Committee of Tohoku University approved the animal experiments about hybridoma production in this study.

Enzyme-linked immunosorbent assay

Synthesized TERT peptides (PEPScreen; Sigma-Aldrich Corp.) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at 10 μg/mL for

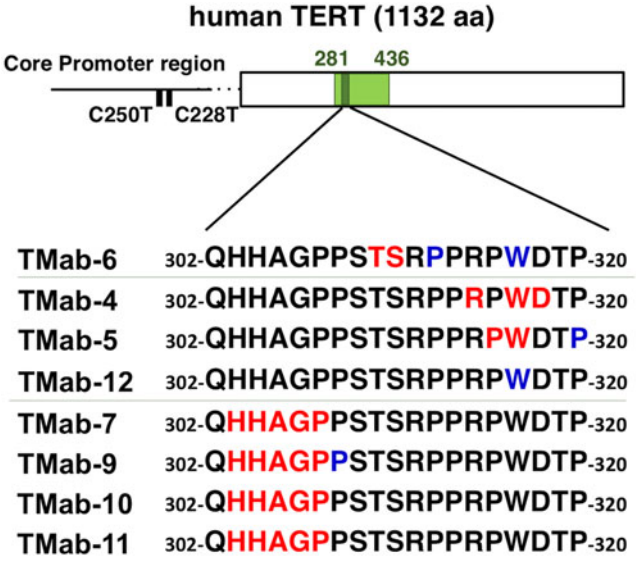

FIG. 1. Epitope mapping of anti-TERT mAbs. Red characters, the most important amino acids detected by anti-TERT mAbs; blue characters, the second most important amino acids detected by anti-TERT mAbs. aa, amino acids; mAbs, monoclonal antibodies; TERT, telomerase reverse transcriptase.

30 min. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), the plates were incubated with purified anti-TERT mAbs (10 μg/mL) or culture supernatant, followed by peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (Agilent Technologies, Inc., Santa Clara, CA) diluted 1:2000. The enzymatic reaction was conducted using 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). These reactions were performed in a volume of 50–100 μL at 37°C.

Results and Discussion

In this study, BALB/c mice were immunized by the i.p. injection of human TERT synthetic peptide ($_{302}$-QHHAGPP STSRPPRPWDTPC-$_{321}$). ELISA was utilized for screening culture supernatants for binding to synthetic peptides and recombinant proteins of TERT. We herein developed seven clones: TMab-4 (IgM, kappa), TMab-5 (IgM, kappa), TMab-7 (IgG_{2b}, kappa), TMab-9 (IgG_{2a}, kappa), TMab-10 (IgG_{2a}, kappa), TMab-11 (IgG_{2a}, kappa), and TMab-12 (IgG_1, kappa).

We next investigated whether the seven clones are applicable for Western blot and immunohistochemical analyses. Unfortunately, none of the seven clones showed a specific band of TERT using glioblastoma cell lines, although they showed a specific band using recombinant protein purified from *E. coli* in Western blot analysis (data not shown). In immunohistochemical analysis, specific staining was not observed in any of the seven clones (data not shown). In contrast, we recently developed clone TMab-6 (IgM, kappa), which is useful for immunohistochemistry in human tissues.[6] Because we also determined the epitope of TMab-6 in a subsequent study,[7] we herein compare the epitope of TMab-6 with those of the other anti-TERT mAbs.

In this study, we utilized a series of TERT peptides from 302 to 320 amino acids in length using point mutations (Table 1). ELISA using an immunohistochemistry-applicable anti-TERT mAb (TMab-6) did not react with T310A and S311A and weakly reacted with P313A and W317A, which indicates that $_{310}$-TSRPPRPW-$_{317}$ is a critical epitope of TMab-6 (Fig. 1) and that Thr310 and Ser311 of TERT are especially significant amino acids for TMab-6-recognition. In contrast, the epitopes of TMab-4, TMab-5, and TMab-12 were determined to be $_{315}$-RPWD-$_{318}$, $_{316}$-PWDTP-$_{320}$, and Trp317 in C-terminus of immunized peptides, respectively. The epitope of TMab-7, TMab-9, TMab-10, and TMab-11 was determined to be $_{303}$-HHAGP-$_{307}$. These results indicate that Thr310 and Ser311 of TERT are very important epitopes for immunohistochemical analysis.

The information regarding the epitopes of anti-TERT mAbs in the present study could be valuable for the development of sensitive and specific mAbs against TERT in immunohistochemical analysis.

Acknowledgments

The authors thank Takuro Nakamura, Miyuki Yanaka, Saori Handa, and Yoshimi Nakamura for excellent technical assistance. This research was supported in part by AMED under Grant Nos.: JP18am0101078 (Y.K.), JP18am0301010 (Y.K.), and JP18ae0101028 (Y.K.), and by JSPS KAKENHI Grant Nos.: 17K07299 (M.K.K.) and 16K10748 (Y.K.).

Author Disclosure Statement

No competing financial interests exist.

References

1. Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, Kadel S, Moll I, Nagore E, Hemminki K, Schadendorf D, and Kumar R: TERT promoter mutations in familial and sporadic melanoma. Science 2013;339:959–961.
2. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, and Garraway LA: Highly recurrent TERT promoter mutations in human melanoma. Science 2013;339:957–959.
3. Cancer Genome Atlas Research Network: Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. N Engl J Med 2015;372:2481–2498.
4. Arita H, Narita Y, Fukushima S, Tateishi K, Matsushita Y, Yoshida A, Miyakita Y, Ohno M, Collins VP, Kawahara N, Shibui S, and Ichimura K: Upregulating mutations in the TERT promoter commonly occur in adult malignant gliomas and are strongly associated with total 1p19q loss. Acta Neuropathol 2013;126:267–276.
5. Chang KP, Wang CI, Pickering CR, Huang Y, Tsai CN, Tsang NM, Kao HK, Cheng MH, and Myers JN: Prevalence of promoter mutations in the TERT gene in oral cavity squamous cell carcinoma. Head Neck 2017;39: 1131–1137.
6. Masui K, Komori T, Kato Y, Masutomi K, Ichimura K, Ogasawara S, Kaneko MK, Oki H, Suzuki H, Nitta M, Maruyama T, Muragaki Y, Kawamata T, Sawada T, and Shibata N: Elevated TERT expression in TERT wild-type adult diffuse gliomas: Histological evaluation with a novel TERT-specific antibody. Biomed Res Int 2018;2018:1–12.
7. Kaneko MK, Yamada S, Itai S, Chang YW, Nakamura T, Yanaka M, Harada H, Suzuki H, and Kato Y: Elucidation of the TMab-6 monoclonal antibody epitope against telomerase reverse transcriptase. Monoclon Antib Immunodiagn Immunother 2018 [Epub ahead of print]; DOI: 10.1089/mab .2018.0007.
8. Kato Y, and Kaneko MK: A cancer-specific monoclonal antibody recognizes the aberrantly glycosylated podoplanin. Sci Rep 2014;4:5924.
9. Fujii Y, Kaneko MK, and Kato Y: MAP tag: A novel tagging system for protein purification and detection. Monoclon Antib Immunodiagn Immunother 2016;35:293–299.
10. Fujii Y, Kaneko M, Neyazaki M, Nogi T, Kato Y, and Takagi J: PA tag: A versatile protein tagging system using a super high affinity antibody against a dodecapeptide derived from human podoplanin. Protein Expr Purif 2014;95: 240–247.

Address correspondence to:
Yukinari Kato
New Industry Creation Hatchery Center
Tohoku University
2-1, Seiryo-machi, Aoba-ku
Sendai, Miyagi 980-8575
Japan

E-mail: yukinari-k@bea.hi-ho.ne.jp;
yukinarikato@med.tohoku.ac.jp

Received: May 2, 2018
Accepted: June 2, 2018