Generation of Med28 Specific Monoclonal Antibodies

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Med28 plays a role in transcription, signal transduction, and cell proliferation. The overexpression of med28 is associated with tumor progression in *in vitro* and *in vivo* models. Recently it has been reported that the elevated expression of med28 is associated with poor outcome in women with breast cancer. The expression level of med28 in *in vitro* and *in vivo* was examined by using anti-rabbit polyclonal antibody in previous reports. In this study, we report for the first time the generation and characterization of four monoclonal antibodies against med28 through immunoblotting, immunofluorescence microscopy, immunoprecipitation, and immunohistochemical analyses. These antibodies will be useful in detecting med28 in *in vitro* and *in vivo*.

Introduction

MED28 WAS INITIALLY IDENTIFIED as an endothelial-derived gene-1 (EG-1) in endothelial cells stimulated with tumor-conditioned medium.⁽¹⁾ In addition, the expression of med28 was increased by stimulation of tumor necrosis factor α and basic fibroblast growth factor. The Northern blot using human tissues showed that med28 expression is high in liver, placenta, and testis. Med28 expression has been shown to be increased in malignant cancers, including breast, colon, and prostate.⁽²⁾ Interestingly, the overexpression of med28 increased proliferation via activation of extracellular signal-regulated kinase (ERK), and vice versa, upon knockdown of med28.⁽³⁾ Furthermore, injection of med28-overexpressing HEK293 cell line into nude mice led to tumor formation. Recently, targeted inhibition of med28 using polyclonal antibody or si-RNA resulted in the reduced growth of breast cancer.⁽⁴⁾ The assessment of breast cancer patients showed that the elevated med28 expression could predict poor outcome in women.⁽⁵⁾

Even though the elevated level of med28 is significantly correlated with cancer development, its exact function of med28 is unknown. It has been identified as one of the subunits within the mammalian mediator complex, which regulates activation and repression of RNA polymerase II transcribed genes.⁽⁶⁻⁹⁾ In addition, med28 associates with merlin, a cytoskeleton-related tumor suppressor critical for neurofibromatosis 2 development.^(9,10) Further studies related to molecular mechanism are needed.

Previous studies have been performed with rabbit polyclonal antibody because the generation of monoclonal anti-

body using recombinant med28 protein was not generated. However, application into immunofluorescence staining and immunohistochemical analysis of polyclonal antibody might be limited due to non-specific signal. Thus, in this study, our goal was to generate monoclonal antibodies against med28.

Materials and Methods

Generation of recombinant med28 protein

Med28 genes encoding 178 amino acids was amplified from the HeLa cDNA library and inserted into EcoRI/XhoI site of pET28a (Novagen, Darmstadt, Germany). The plasmid encoding med28 protein was transformed into the *Escherichia coli* strain BL21-DE3. The expression of the med28 protein was induced by 0.1 mM IPTG at 30°C for 4 h. Protein purification method has been described previously.⁽¹¹⁾ The cells were harvested, resuspended in 1X PBS, and then lysed by ultrasonication. After centrifugation of the lysate at 8000 g, the pellet was recovered and further solubilized using 1X PBS containing 6 M guanidine-HCl. After centrifugation of the lysate at 25,000 g for 30 min, the supernatants were collected and diluted with 0.5X PBS containing 200 mM NaCl and 2 mM β -mercaptoethanol. Then proteins were loaded to nickel affinity chromatography and washed with 0.5X PBS containing 4 M urea, 0.1 M imidazole, 100 mM NaCl, 2 mM β -mercaptoethanol, and eluted with 0.5X PBS containing 50 mM imidazole, 500 mM NaCl, 10% glycerol, and 2 mM β -mercaptoethanol. Proteins were dialyzed against 1X PBS containing 300 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 20% glycerol. Proteins were

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quantified with Bradford solution (Bio-Rad, Hercules, CA) and stored at -70°C until use.

Generation of monoclonal antibodies

The CHA Animal Care and Use Committee approved all animal studies, and the investigation conformed to the Guide for the Care and use of Laboratory Animals (National Institutes of Health, Bethesda, MD). Methods were followed as described previously.⁽¹¹⁾ To generate mouse monoclonal antibody, female BALB/C mice (13 weeks old) were immunized subcutaneously. The emulsion was produced by complete mixing of med28 protein (200 μg /200 μL) with equal volume of complete Freund adjuvant (Sigma-Aldrich, St. Louis, MO). Boosting injections were conducted during week 3 or 4. The mouse serum antibody titers were assessed by an indirect ELISA kit that was coated with 0.1 μg /well of med28 protein. The mouse showing positive immune response activity was subjected to a final boost injection during week 7. The mouse harboring the highest reactivity against protein antigen was sacrificed and splenocytes were isolated from the spleen. The splenocytes were fused to SP2/0 cells, and the resulting hybridomas were screened by culturing in HAT medium as described previously.⁽¹²⁾ Hybridomas showing positive reactivity in ELISA were subcloned by standard limiting dilution method. The hybridomas producing monoclonal antibody were grown in a 25T flask, and the supernatant was harvested. The isotyping was performed using a Beadlyte-Mouse Immunoglobulin Isotyping Kit (Upstate, Lake Placid, NY).

ELISA

Indirect ELISA was performed as described previously.⁽¹¹⁾ The plates were coated overnight at 4°C with 100 ng/well of antigen in 50 μL of 1X PBS, washed three times with 1X PBS, and blocked with 5% bovine serum albumin (BSA) in 1X PBS. Test samples were added (100 μL /well) and incubated at 25°C for 1 h, and the wells were washed three times with 1X PBS containing 0.1% Tween-20 (PBST). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was diluted in 1X PBS containing 1% BSA and added to each well (100 μL /well), and then incubated at 25°C for 1 h. The plates were washed three times with PBST, and incubated with 100 μL /well of tetramethylbenzidine (TMB) peroxidase substrate (Sigma-Aldrich) without light at 25°C for 15 min. The reaction was stopped by adding 50 μL of H_2SO_4 to each well and incubated for 5 min. The absorbance was determined at 450 nm using a Benchmark plus plate reader (Bio-Rad, Hercules, CA) using RPMI 1640 media as a blank. Positive ELISA results were defined as those yielding A450 values greater than O.D 1.0.

Immunofluorescence microscopy

MCF-7 cells were maintained with Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified chamber. MCF-7 cells were seeded onto sterile coverslips the day before transfection in 12-well plates. MCF-7 cells were transfected with pcDNA3 vector or pcDNA3 containing myc-med28 for 24 h. MCF-7 cells were washed three times with 1X PBS and fixed with 4% para-

formaldehyde (Sigma-Aldrich) in 1X PBS at 25°C for 10 min. Cells were washed three times with 1X PBS and permeabilized with 0.1% Triton X-100 for 5 min. The cells were blocked with 5% BSA in 1X PBS for 1 h, and incubated with purified med28 antibody (5 $\mu\text{g}/\text{mL}$) at room temperature for 1 h. The cells were washed three times with 1X PBS and incubated with 1:100 diluted FITC-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes, Carlsbad, CA). The coverslips were mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and cell images were captured under the fluorescence microscope (Nikon Eclipse).

Immunoblotting

Raw264.7 cells (mouse macrophage cell line), HEK293 (human embryonic kidney cell line) cells were maintained in DMEM (Gibco-BRL, Rockville, MD) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were seeded onto 6-well plates and transfected with 0.5 μg plasmids expressing human med28 genes for 24 h. Cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 10 mM NaF, 0.1 mM NaVO_3). Thirty μg of whole cell lysates were loaded to 13% reducing SDS-PAGE. The gels were transferred to Immobilon P, a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) using the semi-dry method. The membrane was blocked with TBS (20 mM Tris-HCl [pH 7.6], 150 mM NaCl) containing 5% skim milk and 0.5% Tween-20 at room temperature for 1 h, incubated with primary antibody (ascites 1:10,000 diluted in TBS containing 0.27% Tween-20 [TBST]) at room temperature for 2 h, and washed two times with TBST. The membrane was incubated with 1:20,000 diluted HRP-conjugated goat anti-mouse (Thermo Scientific, Waltham, MA) at room temperature for 1 h. The membrane was washed three times with TBST and developed using enhanced chemiluminescence (ECL) solution (Santa Cruz Biotechnology, Santa Cruz, CA) to detect their specific signals.

Immunohistochemical staining

Formalin-fixed paraffin-embedded human tissues were applied to 3-aminopropyltriethoxysilane (APES)-coated slides (Sigma). Sections (5 μm) were stained for 30 min at 37°C with four different anti-med28 mouse monoclonal antibodies diluted with 1/50. Staining was detected with the UltraView Universal DAB detection kit (Ventana) according to the manufacturer's protocol. The nuclei were counterstained with Mayer's haematoxylin (Dako, Glostrup, Denmark).

Immunoprecipitation

HEK293 cells were transfected with empty vector or pcDNA3-GFP-med28 for 24 h. Cells were harvested and lysed with lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 12 mM beta-glycerophosphate, 10 mM NaF, 1 mM NaOV_3 , 1 mM PMSF, 2 mM beta-mercaptoethanol), incubated on ice for 30 min, and centrifuged at 25,000 g for 10 min at 4°C . Purified IgG (2 μg) was incubated with protein extracts for 4 h at 4°C and further incubated with protein G agarose for 2 h at 4°C . The

beads were collected by centrifuging at 3000 *g* for 2 min, washed three times with a lysis buffer without beta-mercaptoethanol, and resuspended in 2X SDS-PAGE sample buffer. The samples were boiled and loaded into 15% SDS-PAGE. Immunoprecipitation results were confirmed by Western blotting.

Results

Production of mouse monoclonal antibodies

To generate mouse monoclonal antibodies, med28 encoding 178 aa was cloned into *E. coli* expression vector pET28a harboring 6-histidine tag. Firstly, proteins were expressed in *E. coli* under the stimulation of 0.1 mM IPTG. Most of the proteins were shown to be present in inclusion bodies. Thus, we tried to make med28 protein soluble using an in-chromatography renaturation method after purification using the denaturation method. His-med28 proteins were solubilized using 0.5X PBS containing 8 M urea, bound to nickel affinity chromatography, and washed with 20 bed volume of wash buffer (0.5X PBS containing 100 mM NaCl, 50 mM imidazole, 2 mM β -mercaptoethanol, 4 M urea). His-med28 proteins were eluted with elution buffer (0.5X PBS containing 500 mM NaCl, 300 mM imidazole, 2 mM β -mercaptoethanol), and buffer was replaced with storage buffer (1X PBS containing 300 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol) using G25 gel-filtration chromatography. The amount and purity of med28 protein obtained in-chromatography renaturation method were checked on the SDS-PAGE (Fig. 1). Densitometric analysis showed that protein purity was over 93%. The affinity purified proteins were used to raise mouse monoclonal antibodies. The mice were immunized five times; after four boosting injections, the mouse serum antibody titer was examined by indirect ELISA. Splenocytes showing the highest serum titer against recombinant med28 were fused with SP2/0 myeloma cells to form hybridomas. The culture supernatants were screened using indirect ELISA and Western blotting, and positive

hybridoma cells showing high titer values were sub-cloned by standard limiting dilution. The four stable clones were finalized: 3B2, 4F11, 9G5, and 10D4. Isotyping using a commercial kit showed that all of them were identified as IgG1, kappa (data not shown). The properties were further characterized by Western blot analysis, immunoprecipitation, immunofluorescence staining, and immunohistochemical analyses.

Western blot analysis

The obtained med28 mouse monoclonal antibodies were tested to analyze their specificity for immunoblot application. Raw264.7 cells (mouse macrophage cell line), HEK293 cells (human embryonic kidney cell line) transfected with empty vector or pcDNA3-myc-human med28 were analyzed by performing Western blotting. Mouse monoclonal anti-med28 antibodies readily detected exogenously expressed human med28 proteins (Fig. 2). In addition, mouse monoclonal anti-med28 antibodies were found to detect endogenous med28 protein in both mouse and human cell line without non-specific binding (Fig. 2).

Characterization of antibodies by immunoprecipitation

We investigated whether the generated antibodies against human med28 could be available for the application of immunoprecipitation (IP). For endogenous IP assay, 1 mg of HEK293 protein extracts was used. In addition, HEK293 cells were transfected with GFP-med28 for 24 h because molecular weight of myc-med28 was overlapped with IgG light chain, and 0.2 mg of protein extracts was used for exogenous IP. Proteins were incubated with 2 μ g of purified IgG for 4 h and then further incubated with protein G agarose for 2 h. The eluted proteins were subjected to 15% SDS-PAGE and blotted with 9G5 med28 IgG.

FIG. 1. Purification of recombinant human med28 antigen. Samples were analyzed by Coomassie blue-stained SDS-PAGE. Lane 1, 0.1 mM IPTG induced lysates; lane 2, supernatant after ultrasonication; lane 3, pellet after ultrasonication; lanes 4–6, purified recombinant human med28 protein (1 μ g, 2 μ g, 5 μ g, respectively).

FIG. 2. Western blot analysis using mouse monoclonal antibodies. Raw264.7 cells and HEK293 cells were transiently transfected with pcDNA3 empty vector or pcDNA3-myc-human med28. 30 μ g of protein extracts were loaded to 13% SDS-PAGE and blotted with each indicated purified monoclonal antibody. Lane 1, Raw264.7 cell lysates; lane 2, HEK293 cell lysates with pcDNA3 empty vector; lane 3, HEK293 cell lysates with pcDNA3-myc-human med28.

FIG. 3. Immunoprecipitation analysis using mouse monoclonal antibodies. HEK293 cells were transiently transfected with pcDNA3 empty vector or pcDNA3-GFP-human med28. Protein extracts were incubated with 2 μ g of purified anti-med28 monoclonal IgG for 4 h at 4°C, and further incubated with protein G agarose for 2 h at 4°C. Beads were washed with lysis buffer three times and samples were collected by boiling. The proteins were subjected to 15% SDS-PAGE for Western blotting. Normal IgG was used as a negative control.

As shown in Figure 3, each med28 monoclonal antibody showed a clear band at ~45 kDa in exogenous IP, confirming the molecular weight of GFP-med28. In addition, endogenous IP clearly showed that endogenous med28 was located at ~22 kDa. Taken together, all of the clones generated against human med28 showed immunoprecipitation activity in exogenous and endogenous IP.

Immunofluorescence staining analysis

Generated antibodies were tested for their ability in immunofluorescence applications. MCF-7 cells transfected with empty vector or pcDNA3-myc-human med28 were seeded onto coverslips, fixed, and incubated with four different anti-

med28 monoclonal antibodies. Counterstaining was performed with DAPI to visualize nuclei. As a positive control, cells were incubated with an anti-myc-antibody. As a result of immunofluorescence assay, all four purified anti-med28 IgG showed that med28 was located at both cytoplasm and nucleus (Fig. 4A). Additionally, the staining of endogenous med28 showed the same results (Fig. 4B). The results signify that all four monoclonal antibodies efficiently worked in this application.

Characterization of antibodies in application of immunohistochemical staining

Human breast cancer tissues were collected by Bundang CHA Hospital. Paraffin-embedded tissues were sectioned

FIG. 4. Immunofluorescence microscopic analysis in MCF-7 cells. (A) MCF-7 cells were transiently transfected with pcDNA3-myc-human med28. (B) For the detection of endogenous med28, cells were stained with purified mouse anti-med28 monoclonal IgG (1 μ g/mL) as indicated. The nuclei were counterstained with DAPI. Normal IgG and anti-myc antibody were used as negative and positive control, respectively.

FIG. 5. Immunohistochemical staining analysis using human breast cancer tissues. Human breast cancer tissues were embedded into paraffin and sectioned with 5 μ m. The sections were incubated with anti-med28 antibodies and developed with DAB, as described in the Methods section. Normal IgG was used as negative control.

(5 μ m), and the endogenous peroxidase was quenched with methanol/peroxidase solution. The med28 protein was captured with the anti-med28 IgG diluted 1:100. Next, the anti-med28 antibody was captured with ultraView Universal DAB detection kit, and sections were counterstained with Mayer's haematoxylin. As a result of immunohistochemical staining, clones 3B2, 9G5, and 10D4 showed that med28 was mainly localized in the nucleus and partly in cytoplasm (Fig. 5). However, clone 4F11 could not be applicable to immunohistochemical staining.

Discussion

It has been known that med28 overexpression stimulates cell proliferation *in vitro* and *in vivo*, whereas med28 suppression inhibits the growth and tumorigenicity of breast cancer cells.^(3,4) This property suggested that med28 could play a critical role in tumorigenesis. However, its molecular mechanism, including regulatory proteins related to tumorigenesis, is still unknown. In tumorigenesis, med28 might be regulated by a variety of molecules and also regulate its downstream targets to exert its function. Protein-protein interaction studies are needed to identify med28-mediated regulatory network. One of the key materials is high quality antibody. However, generation of med28 specific antibody is not easy to accomplish, even in

the case of rabbit polyclonal antibody, due to non-specific background.

In order to generate high quality anti-med28 monoclonal antibody, we used various immunization protocols, boosting intervals, and screened 76 clones by ELISA and immunoblotting. However, nearly all clones were only reactive to recombinant or exogenous med28, but not to endogenous med28. Only four hybridomas were shown to recognize not only exogenous but also endogenous med28, which did not show non-specific band in immunoblotting. Taken together, we have successfully generated med28 specific monoclonal antibodies that could be applicable to Western blot, immunoprecipitation, immunofluorescence staining, and immunohistochemical analysis. These antibodies will be useful in expanding the med28 research area in *in vitro* and *in vivo* studies.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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