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Development of a Monoclonal Antibody That Specifically Detects Tissue Inhibitor of Metalloproteinase-4 (TIMP-4) in Formalin-Fixed, Paraffin-Embedded Human Tissues

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ABSTRACT

Overexpression of the extracellular metalloproteinase inhibitor TIMP-4 in estrogen receptor-negative breast cancers was found recently to be associated with a poor prognosis for survival. To pursue exploration of the theranostic applications of TIMP-4, specific antibodies with favorable properties for immunohistochemical use and other clinical assays are needed. Here we report the characterization of a monoclonal antibody (clone 9:4–7) specific for full-length human TIMP-4 with suitable qualities. The antibody was determined to be an IgG_{2b} immunoglobulin. In enzyme-linked immunosorbent assay (ELISA) and immunoblotting assays, it did not exhibit any detectable cross-reactivity with recombinant forms of the other human TIMPs 1, 2, and 3. In contrast, the antibody displayed high specificity and sensitivity for TIMP-4 including in formalin-fixed and paraffin-embedded specimens of human breast specimens. An analysis of tissue microarrays of human cancer and corresponding normal tissues revealed specific staining patterns with excellent signal-to-noise ratios. This study documents TIMP-4 monoclonal antibody clone 9:4–7 as an effective tool for preclinical and clinical investigations. J. Cell. Biochem. 110: 1255–1261, 2010.

KEY WORDS: TIMP-4; BIOMARKER; BREAST CANCER; IMMUNOHISTOCHEMISTRY

The family of tissue inhibitor of metalloproteinases (TIMPs) consists of four known members that differ in their structure, biochemical properties, and expression, suggesting that they have distinct physiological roles [Gomez et al., 1997; Brew et al., 2000]. Though viewed as "fail-safe" devices that prevent excessive tissue destruction by matrix metalloproteinases (MMPs), TIMPs have additional and as yet poorly understood effects such as acting as mitogens for a number of cell types [reviewed in Gomez et al., 1997; Brew et al., 2000]. This was originally demonstrated for TIMP-1 as an erythroid-potentiating activity that stimulated the growth of erythroid precursors in vitro and in vivo [Denhardt et al., 1993]. Since then, reports in the literature have shown that members of the TIMP family can affect proliferation, angiogenesis, and apoptosis [Stetler-Stevenson, 2008].

Under normal physiological conditions TIMP-4 expression is seen in heart, brain, pancreas, kidney, colon, testes, and adipose tissue [Greene et al., 1996]. Elevated TIMP-4 levels have been demonstrated in malignancies of the breast, ovary, cervix, prostate, brain, colon, endometrium, and kidney (papillary renal tumors), while pancreatic and clear cell renal tumors are associated with decreased levels of TIMP-4 [Melendez-Zajgla et al., 2008]. The role of TIMP-4 in tumor formation and progression was less clear until recently. Early reports in the literature attributed TIMP-4 with conflicting functions in breast cancer including anti-tumor effects and stimulation of tumorigenesis [Wang et al., 1997; Jiang et al., 2001]. However, a large retrospective study of clinical breast cancer specimens that was performed recently defined TIMP-4 as a strong prognostic biomarker for early disease progression and poor

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survival prognosis [Liss et al., 2009]. To pursue studies of TIMP-4 in tumor formation and progression, we have developed and characterized a novel monoclonal antibody, in particular to identify an antibody with specificity and sensitivity for TIMP-4 in human formalin-fixed, paraffin-embedded (FFPE) clinical material of greatest interest for further biomarker studies.

MATERIALS AND METHODS

MONOCLONAL ANTIBODY GENERATION

A glutathione-*S*-transferase (GST) chimeric protein that encoded the human full-length 224 amino acids of TIMP-4 (NM_003256.2) was generated in *E. coli*. The generated chimeric protein was purified by GST chromatography and the purity was verified by SDS–PAGE. The purified chimeric protein was used to raise monoclonal antibodies in mice. Mice that exhibited a positive immune response in primary bleeds were boosted an additional two times to increase titer before spleen isolation and myeloma fusion essentially as described by Koprowski et al. [1979].

MONOCLONAL ANTIBODY PURIFICATION

The clone determined to have the highest signal-to-noise ratio and titer was selected and adopted to growth in serum-free medium (SFM medium, Gibco). Supernatant of large-scale cultures grown to confluency were pooled, clarified, and added to Protein A columns (GE Healthcare) according to the manufacturer's instructions. Elution fractions with high concentrations of IgG (as determined by absorbance measurement) were pooled and dialyzed at +4°C overnight against phosphate-buffered saline (PBS) containing 0.02% bovine serum albumin (pH 7.4). Aliquots of the purified antibody were tested for antibody concentration and subtype.

ANTIBODY SPECIFICITY AND SUBCLASSIFICATION BY ELISA ANALYSIS

Initial screening was performed in Immulon II plates coated with either 0.1 μg purified recombinant TIMP-4/GST or control GST proteins in a carbonate buffer (pH 9.6) at +4°C overnight [Wallon and Overall, 1997]. The following day, plates were rinsed with PBS/0.02% Tween-20 prior to a blocking step using bovine serum albumin in wash buffer. Plates were then incubated for 1 h at room temperature with supernatants from hybridoma cultures. After extensive washing, secondary HRP-conjugated goat-anti mouse IgG (H + L) and IgG Fc fragment-specific secondary antibodies (GE Healthcare) were added (1:1,000 dilution) for 1 h. After extensive washing, immunoreactive monoclonal antibodies were detected using ABTS substrate (2,2′-azino-di-(ethylbenzthiazoline sulfonate) di-ammonium salt; Southern Biotechnology Associates) and absorbance readings at 405 nm were obtained.

Immunoglobulin subclassification for the monoclonal antibody was performed by plating antibodies in triplicate on Immulon II plates with unlabeled donkey anti-mouse total Ig (Jackson Immunoresearch). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, or IgG_3 (all from Southern Biotechnology Associates) were used as secondary antibodies. Detection using ABTS substrate demonstrated that the anti-TIMP-4 antibody is of IgG_{2b} subtype.

Specific reactivity towards TIMP-4 of the subcloned antibody was tested using Immulon II plates coated with 2, 5, and 10 μg of GST, TIMP-4/GST, commercial recombinant (r) TIMP-4 (R&D Systems), or a single amount (2 μg/well) of rTIMP-1, rTIMP-2, rTIMP-3, rTIMP-4 (all from R&D Systems) in carbonate buffer as before. As controls, two amounts (2 and 10 μg) of rTIMP-1 and rTIMP-2 was added and detected with mouse anti-human TIMP-1 (wells w. TIMP-1) or mouse anti-human TIMP-2 (wells w. TIMP-2). IgG_{2b} isotype goat anti-mouse and polyclonal TIMP-4 antibody were added to wells with 20 μg of GST and TIMP-4/GST as controls.

WESTERN BLOTTING ANALYSIS

Equal amounts (1 μg) of GST, TIMP-4/GST and rTIMP 1, 2, 3, or 4 (R&D Systems) were mixed with Native Protein Sample buffer (Invitrogen) and loaded onto pre-cast 4–16% native-PAGE Bis–Tris gels (Invitrogen). A cell extract of the human breast cancer cell-line MDA-MB-231 obtained from the American Type Culture Collection (ATCC) was prepared using M-Per™ Mammalian Protein Extraction Reagent (Pierce) in the presence of protease inhibitors (Halt™, Thermo Scientific). Protein concentration was determined using BCA™ Protein Assay Reagent (Pierce) and 10 μg of cell extract was mixed with native sample buffer. Conditioned medium [Overall et al., 2000] from the same culture used for protein extraction was also mixed with native sample buffer and loaded onto gels. Samples were separated at constant 80 V at room temperature and then transferred to PVDF membranes according to the manufacturer's instructions (Invitrogen). Membranes were blocked in 5% fat-free dry milk in PBS, pH 7.4, containing 0.06% Tween-20 for 1 h at room temperature. The purified antibody was then added at 1 μg/ml and incubated over night at +4°C. After washes as previously described [Wallon et al., 1994], an HRP-conjugated secondary goat anti-mouse antibody (dilution 1:25,000) (GE Healthcare) was added for 1 h. Membranes were developed using a commercial chemiluminescence kit (GE Healthcare).

IMMUNOHISTOCHEMICAL STAINING

Sections of human breast cancer specimens from cases previously determined as TIMP-4 positive or TIMP-4 negative using a commercial polyclonal antibody [Liss et al., 2009] were used to screen 18 clones exhibiting specific TIMP-4 reactivity. Tissue sections were incubated with supernatants from confluent clones, supernatants from non-reactive hybridomas at 1:20 dilution or 10 μg/ml polyclonal antibody. Immunohistochemical staining was performed as previously described [Liss et al., 2009] with the exception when TIMP-4 monoclonal antibodies were used the secondary reagent kit was VectaStain Elite for mouse antibodies (Vector). Control experiments included formalin-fixed and paraffin-embedded cell pellets of MDA-MB-231 and MDA-MB-435S (ATCC) incubated with the previously used polyclonal antibody, secondary antibody alone or with an isotype specific (IgG_{2b}, BD Biosciences). Presence of TIMP-4 was visualized by incubating sections for 8 min in 3,3′-diamino-benzidine (DAB) followed by counterstaining with hematoxylin for 8 s and cover slips permanently mounted using Permamount (Vector).

Tissue micro-arrays from multiple human organs consisting of tumor and normal adjacent to tumor tissue (US Biomax, Inc., MC242

and MC244) were first de-waxed by heat treatment at 60°C for 2 h prior to regular IHC [Liss et al., 2009]. Serial arrays were incubated with 10 μg/ml primary monoclonal antibody clone 9:4–7 or isotype specific mouse antibody and detected as described above.

RESULTS

A total of 98 hybridoma clones were tested for specific reactivity to TIMP-4 using an ELISA assay. Wells were coated with either TIMP-4/GST or GST alone to identify antibodies with specific reactivity to epitopes within TIMP-4 of the chimeric protein used for induction of immune response. Supernatants from confluent hybridoma clones were tested with mouse anti-IgG (H + L) and mouse anti-IgG Fc fragment specific secondary antibodies to identify antibodies of IgG monomer class. Through this first screen we identified 18 clones with TIMP-4 specific reactivity. Figure 1 shows clones 7–18 demonstrating no reactivity to GST and varying levels of reactivity to TIMP-4. No significant difference in result was obtain with the two types of secondary antibodies.

The 18 clones with specific TIMP-4 reactivity were then tested in immunohistochemical staining (IHC) using FFPE sections of human breast cancer specimens. Of the clones tested, four clones produced varying degree of detectable immunoreactivity as compared to polyclonal TIMP-4 antibody and medium from hybridoma cultures having no detectable immunoreactivity in ELISA assays (Fig. 2A). Staining conditions were according to the manufacturer of the polyclonal antibody and for times previously determined using this antibody. As shown in Figure 2A, clone 9:4 exhibited a strong, specific tumor cell-associated staining pattern with weak staining of surrounding tissue. Three other clones also produced a strong intensity but were accompanied by a similar staining intensity of non-tumor structures in the tissue (data not shown).

After adapting the clones to serum-free growth, the four clones that were positive for staining of FFPE sections were tested for antibody synthesis. The results demonstrated that clone 9:4 was the better clone with respect to growth and titer. Therefore, together with the results demonstrating higher specificity in IHC, clone 9:4 was further subcloned and purified. From this effort, clone 9:4–7 was purified by Protein A chromatography providing a pure antibody solution of higher concentration. In an ELISA assay only the secondary antibody specific for mouse IgG_{2b} demonstrated reactivity to the monoclonal antibody (data not shown).

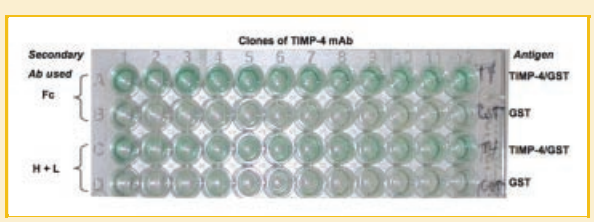

Fig. 1. Screening of hybridoma clones. Shown are clones 7 through 18 (wells 1–12) of the original positive hybridoma cultures. All clones were tested for reactivity to the chimeric protein (TIMP-4/GST) and to the tag domain alone (GST). Secondary antibodies specific for mouse Fc-domain and H + L were used to detect reactivity. Clones 7, 9, 12, and 13 (corresponding to wells number 1, 3, 6, and 7) were further characterized.

The purified, dialyzed, and isotyped clone 9:4–7 was re-tested on new sections of FFPE breast cancer specimens and compared to isotype-specific and polyclonal TIMP-4 antibodies. Sets of three sections for each case were stained using all three antibodies at one concentration and identical staining conditions. As seen in Figure 2B, incubation with isotype-specific antibody resulted in no detectable immunoreactivity, while the polyclonal antibody, used at the manufacturer's concentration, demonstrated low level reactivity in case B and mostly non-specific staining in case A. The new monoclonal 9:4–7 demonstrated strong reactivity associated with tumor cells in case B but limited reactivity in A. The subcloned monoclonal 9:4–7 was re-tested against various amounts of GST, TIMP-4/GST, and rTIMP-4. As seen in Figure 3A, the subcloned and purified clone 9:4–7 retained specific reactivity to TIMP-4/GST (Im) and rTIMP-4 but with lower sensitivity.

The amino acid sequence for human TIMP-4 is 51% identical, with 70% similarity, to human TIMP-2 and human TIMP-3. The identity and similarity of human TIMP-4 to human TIMP-1 is lower, 37% and 57%, respectively [Melendez-Zajgla et al., 2008]. Using an ELISA assay and commercially available human recombinant (r) TIMP-1, -2, -3, and -4 (2 μg each protein/well) a low level reactivity to rTIMP-1, -2, and -3 (Abs 405 nm 0.125) was observed (Fig. 3B). This reactivity is about twofold over background (Abs 405 nm 0.060) while the reactivity to rTIMP-4 is 4.8-fold over background (Abs 405 nm 0.290). However, when anti-human TIMP-1 and TIMP-2 monoclonal antibodies were added to wells coated with 2 or 10 μg/well of rTIMP-1 and rTIMP-2, respectively, a strong reactivity was observed (Fig. 3C) demonstrating that clone 9:4–7 does not recognize these human TIMPs. As control, the polyclonal anti-TIMP-4 antibody was tested in wells coated with high amounts (20 μg/well) of TIMP-4/GST (Im) and GST. The results seen in Figure 3D demonstrate that the control antibody has no identifiable reactivity to the chimeric tag (GST). Also, an isotype specific primary antibody (IgG_{2b}) resulted in no detectable reactivity demonstrating that a naïve IgG_{2b} is not sufficient for a measurable reaction (Fig. 3D).

Western blotting was performed with cell extracts from MDA-MB-231 and conditioned medium from the same dish. Samples were separated on native-PAGE together with GST, TIMP-4/GST, rTIMP-1, -2, -3, and -4 (Fig. 4). As in the ELISA assay, no reactivity was identifiable for clone 9:4–7 toward GST or rTIMP-1, -2, or -3. The monoclonal antibody demonstrated strong reactivity for the TIMP-4/GST and as previously seen, a lower sensitivity for the recombinant TIMP-4 protein. Longer exposure time (i.e., 30 min) did not reveal any crossreactivity to rTIMP-1, -2, or -3 (data not shown). A high molecular weight band was detected in the cell lysate possibly indicating TIMP-4 bound to MMP-2. No reactivity was identified in the conditioned medium.

The monoclonal 9:4–7 clone was tested for possible reactivity in other normal and malignant human tissues using multi organ tissue microarrays (US Biomax, Inc.). These arrays consisted of normal adjacent tissue and tumor material from the same individual. We were able to identify specific TIMP-4 staining in tissues previously reported to have TIMP-4 expression [Melendez-Zajgla et al., 2008].

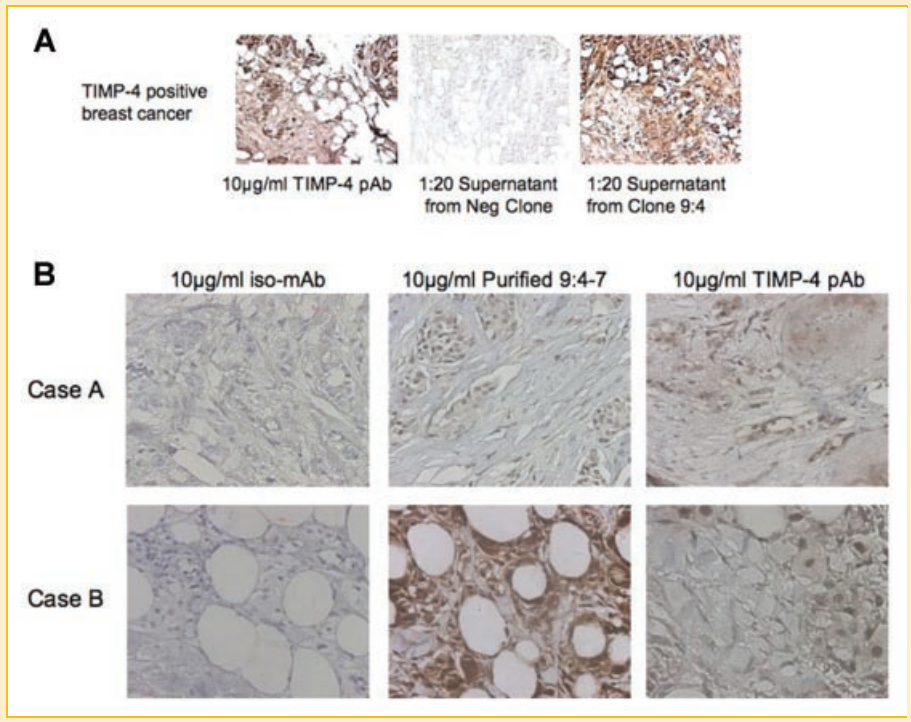

Fig. 2. Test for reactivity in immunohistochemical staining of FFPE breast tissue. Four clones were tested for reactivity in clinical breast cancer specimens. Using a protocol according to instructions for the commercial polyclonal (pAb) TIMP-4 antibody demonstrated that a 1:20 dilution of the supernatant from clone 9:4–7 had tumor specific reactivity (A) comparing staining pattern to that of the pAb and medium alone. After purification of 9:4–7 conditioned supernatants, sections were incubated with equal concentrations of pAb and mAb and reactivity visualized with DAB (B). Less intense staining with pAb is due to reduced incubation time in DAB compared to (A) (from 15 to 5 min) to avoid excess staining with 9:4–7.

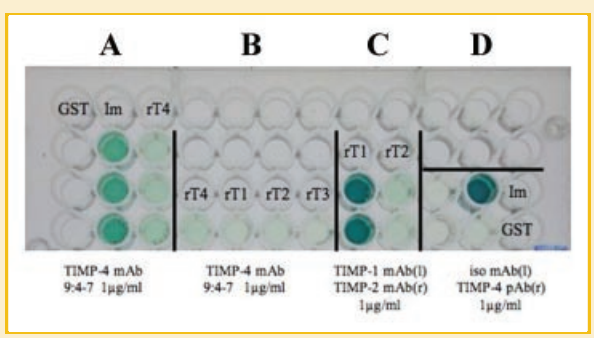

Fig. 3. TIMP-4 specific reactivity of clone 9:4–7. Immulon II plates were coated with purified fusion tag (GST), TIMP-4/GST (Im) or a commercial recombinant human TIMP-4 (rT4) protein and incubated with clone 9:4–7 (A). The three proteins were added at 2, 5, and 10 µg/well (top to bottom) (A). Recombinant human TIMP-1 (rT1), TIMP-2 (rT2), TIMP-3 (rT3), and TIMP-4 (rT4), were added at 2 µg/well and incubated with the TIMP-4 mAb clone 9:4–7 (B). As controls, 2 µg/well (lower row) and 10 µg/well (upper row) of rTIMP-1 (rT1) and rTIMP-2 (rT2) were added and tested with a specific TIMP-1 antibody (left column) or TIMP-2 antibody (right column) (C). As shown in (D), an isotype specific antibody had no reactivity to 20 µg/well of either TIMP-4/GST (Im) or GST (left column) while the previously used commercial pAb had strong specific reactivity to TIMP-4/GST (Im) accompanied by no reactivity to GST (right column).

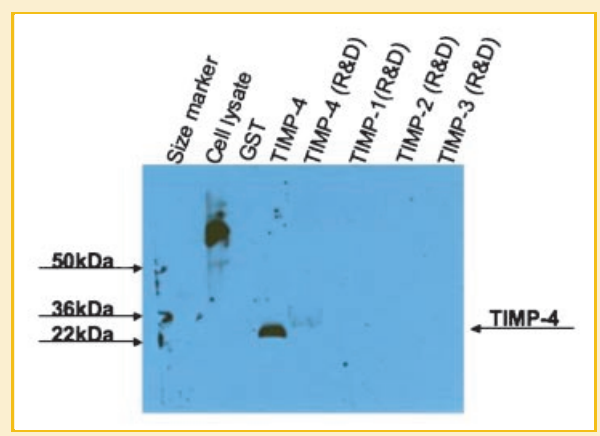

Fig. 4. Native-PAGE analysis of TIMP-4 mAb 9:4–7. Equal amounts of purified GST, TIMP-4/GST (antigen) and a commercial recombinant TIMP-4 (rTIMP-4) were mixed with either native sample buffer alone (1), native sample buffer supplemented with DDM (2), or digitonin (3) to a final concentration of 1% and then separated on native-PAGE gels according to the manufacturers instructions. Blots were incubated with 1:100 dilutions of either TIMP-4 mAb clone 9:4–7 (left) or TIMP-4 pAb (right) and reactive bands visualized by chemiluminescence.

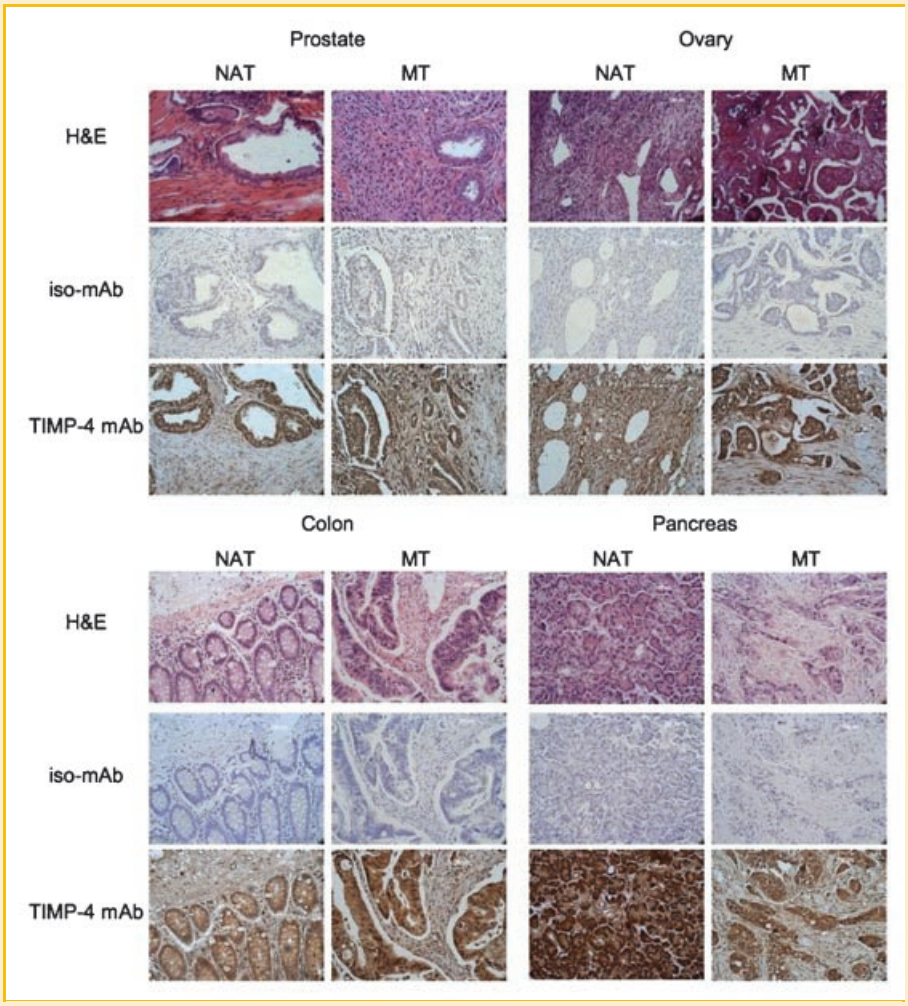

Fig. 5. TIMP-4 immunohistochemical staining of human tissues. Tissue microarrays of human FFPE tissues (MC242 (colon and pancreas, shown) and MC244 (prostate and ovary shown), US Biomax, Inc.) were incubated with 10 μg/ml of either isotype specific mouse IgG_{2b} or TIMP-4 mAb clone 9:4–7. All tissues demonstrated some level of TIMP-4 expression with lower levels in normal adjacent tissue (NAT) compared to malignant tumor (MT) with the exception of pancreas were stronger staining was observed in normal tissue as previously reported.

As seen in Figure 5, apart from breast tissue the monoclonal 9:4–7 antibody detects elevated levels of TIMP-4 in malignancies of the prostate, colon, and ovary while pancreatic tumors have diminished expression. The TIMP-4 protein is localized to tumor epithelial cells as determined by comparison to the H&E staining. Using the isotype IgG_{2b} antibody did not result in any specific staining.

Together these experiments demonstrate that the monoclonal TIMP-4 antibody clone 9:4–7 is specific for human TIMP-4 protein a variety of tissues with no crossreactivity to other TIMPs.

DISCUSSION

TIMPs are expressed in a variety of tissues and regulate tissue remodeling under normal physiological and pathological conditions. Traditionally, TIMPs are deemed "fail-safe" inhibitors of matrix metalloproteinase activities and as such safeguard the integrity of the normal architecture of the tissue microenvironment. In recent years, it has been recognized that TIMPs harbor other functions affecting cellular processes such as proliferation, apoptosis, and angiogenesis [Gomez et al., 1997; Brew et al., 2000].

In earlier work, we identified TIMP-4 as a marker for disease progression among early-stage infiltrating ductal carcinomas [Liss et al., 2009]. The published work was performed using a commercial polyclonal antibody for full-length TIMP-4. In order to adapt our knowledge to clinical practice and for further studies of the role of TIMP-4 in tumor formation and progression, it is necessary to have a highly specific monoclonal antibody that can ensure optimal staining of FFPE specimens and highly reproducible results over time. With a lack of commercially available monoclonal TIMP-4 antibodies, and based on the promising results from the retrospective breast cancer prognostic study, we generated a monoclonal antibody directed against human TIMP-4 protein.

As shown here, we have generated an antibody with reactivity to human TIMP-4 in FFPE sections of human tissue specimens. The initial ELISA screen identified clones with specific recognition of

epitopes within the TIMP-4 protein (Fig. 1). Both mouse anti-IgG (H + L) and anti-IgG Fc secondary antibodies were used to favor the recognition of IgG subclass of monoclonal antibody.

With the intent to identify an antibody for IHC, all hybridomas with specific TIMP-4 reactivity in the initial ELISA assay were tested by IHC along with the previously used polyclonal TIMP-4 antibody and conditioned medium from non-reactive cultures as negative controls. This screen resulted in four possible clones with one clone, 9:4, determined to produce the strongest signal-to-noise ratio (Fig. 2A). This clone was further subcloned to ensure a true monoclonal hybridoma culture and adapted to serum-free growth conditions. The resulting clone 9:4–7 was purified by Protein A chromatography and re-tested by ELISA, Western blotting, and IHC.

Of concern was both specificity for TIMP-4 over GST and specificity for TIMP-4 over any of the other human TIMPs. At the amino acid level, TIMP-4 shares 51% identity and 70% similarity to human TIMP-2 and TIMP-3. Even though the identity and similarity to TIMP-1 are lower (31% and 57%, respectively) the epitope recognized by clone 9:4–7 could possibly be found within TIMP-3. Therefore, both ELISA and Western blotting analyses were performed using commercial recombinant proteins of all three human TIMPs to assess the reactivity of clone 9:4–7. Native Western blotting did not indicate any crossreactivity of the monoclonal TIMP-4 antibody to rTIMP-1, -2, or -3 while low level of reactivity was seen in ELISA assays. However, this level of reactivity was lower than the observed reactivity to rTIMP-4 (4.8-fold over background) in ELISA assays using equal amounts of TIMP proteins as antigen. As expected, when TIMP-1 and TIMP-2 monoclonal antibodies were tested against their corresponding TIMP a strong reactivity was observed (Fig. 3). We anticipate that this antibody will have reactivity to TIMP-4 of other species, such as mouse and rat, considering the high degree of identity and similarity between the human TIMP-4 and that of these other species [Melendez-Zajgla et al., 2008].

This new antibody generates a strong and specific reaction that clearly is associated with tumor epithelial cells in several tissues. We have tested over 50 FFPE sections of clinical breast cancer specimens and in all cases this antibody generates a superior signal-to-noise ratio over the polyclonal antibody providing consistent and reproducible results in IHC (e.g., Fig. 2B). This monoclonal antibody is also superior in immunofluorescence compared to the polyclonal antisera in staining para-formaldehyde or methanol fixed human breast cancer cell lines (data not shown).

Reports in the literature have demonstrated a possible role for TIMP-4 as a biomarker for colorectal cancer [Hilska et al., 2007]. Therefore, we tested the purified monoclonal antibody for reactivity in a series of tissues. In all tissues tested, we detected a change in expression level comparable to those previously reported [Tunuguntla et al., 2003; Zhao et al., 2004; Lee et al., 2006; Pilka et al., 2006; Ripley et al., 2006; Bister et al., 2007; Hilska et al., 2007]. For example, we detected an increased expression in colon cancer compared to normal tissue and decreased levels of TIMP-4 in pancreatic cancer specimens as compared to normal pancreatic tissue. Clinical marker testing is performed by computer software, which requires antibodies to generate a strong signal-to-noise ratio for accurate assessment of specimens. Also, such testing requires reliable staining results, and a monoclonal antibody is preferable due to lower variably between batches. This new antibody could therefore be a useful tool for future studies of TIMP-4 and its role in physiological and pathological processes in a variety of tissues as well as a biomarker for disease progression and therapeutic response.

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REFERENCES

Bister V, Skoog T, Virolainen S, Kiviluoto T, Puolakkainen P, Saarialho-Kere U. 2007. Increased expression of matrix metalloproteinases-21 and -26 and TIMP-4 in pancreatic adenocarcinoma. Mod Pathol 20:1128–1140.

Brew K, Dinakarpandian D, Nagase H. 2000. Tissue inhibitors of metalloproteinases: Evolution, structure and function. Biochim Biophys Acta 1477:267–283.

Denhardt DT, Feng B, Edwards DR, Cocuzzi ET, Malyankar UM. 1993. Tissue inhibitor of metalloproteinases (TIMP, aka EPA): Structure, control of expression and biological functions. Pharmacol Ther 59:329–341.

Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. 1997. Tissue inhibitors of metalloproteinases: Structure, regulation and biological functions. Eur J Cell Biol 74:111–122.

Greene J, Wang M, Liu YE, Raymond LA, Rosen C, Shi YE. 1996. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. J Biol Chem 271:30375–30380.

Hilska M, Roberts PJ, Collan YU, Laine VJ, Kossi J, Hirsimaki P, Rahkonen O, Laato M. 2007. Prognostic significance of matrix metalloproteinases-1, -2, -7 and -13 and tissue inhibitors of metalloproteinases-1, -2, -3 and -4 in colorectal cancer. Int J Cancer 121:714–723.

Jiang Y, Wang M, Celiker MY, Liu YE, Xiang Q, Sang A, Goldberg ID, Shi YE. 2001. Stimulation of mammary tumorigenesis by systemic tissue inhibitor of matrix metalloproteinase 4 gene therapy. Cancer Res 61:2365–2370.

Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. 1979. Colorectal carcinoma antigens detected by hybridoma antibodies. Somatic Cell Genet 5:957–971.

Lee S, Desai KK, Iczkowski KA, Newcomer RG, Wu KJ, Zhao YG, Tan WW, Roycik MD, Sang QX. 2006. Coordinated peak expression of MMP-26 and TIMP-4 in preinvasive human prostate tumor. Cell Res 16:750–758.

Liss M, Sreedhar N, Keshgegian A, Sauter G, Chernick MR, Prendergast GC, Wallon UM. 2009. Tissue inhibitor of metalloproteinase-4 is elevated in early-stage breast cancers with accelerated progression and poor clinical course. Am J Pathol 175:940–946.

Melendez-Zajgla J, Del Pozo L, Ceballos G, Maldonado V. 2008. Tissue inhibitor of metalloproteinases-4. The road less traveled. Mol Cancer 7:85.

Overall CM, Tam E, McQuibban GA, Morrison C, Wallon UM, Bigg HF, King AE, Roberts CR. 2000. Domain interactions in the gelatinase A.TIMP-2.MT1-MMP activation complex. The ectodomain of the 44-kDa form of membrane type-1 matrix metalloproteinase does not modulate gelatinase A activation. J Biol Chem 275:39497–506.

Pilka R, Noskova V, Domanski H, Andersson C, Hansson S, Casslen B. 2006. Endometrial TIMP-4 mRNA is expressed in the stroma, while TIMP-4 protein accumulates in the epithelium and is released to the uterine fluid. Mol Hum Reprod 12:497–503.

Ripley D, Tunuguntla R, Susi L, Chegini N. 2006. Expression of matrix metalloproteinase-26 and tissue inhibitors of metalloproteinase-3 and -4 in normal ovary and ovarian carcinoma. Int J Gynecol Cancer 16: 1794–1800.

Stetler-Stevenson WG. 2008. The tumor microenvironment: Regulation by MMP-independent effects of tissue inhibitor of metalloproteinases-2. Cancer Metastasis Rev 27:57–66.

Tunuguntla R, Ripley D, Sang QX, Chegini N. 2003. Expression of matrix metalloproteinase-26 and tissue inhibitors of metalloproteinases TIMP-3 and -4 in benign endometrium and endometrial cancer. Gynecol Oncol 89:453–459.

Wallon UM, Overall CM. 1997. The hemopexin-like domain (C domain) of human gelatinase A (matrix metalloproteinase-2) requires Ca2+ for fibronectin and heparin binding. Binding properties of recombinant gelatinase A C domain to extracellular matrix and basement membrane components. J Biol Chem 272:7473–7481.

Wallon UM, Shassetz LR, Cress AE, Bowden GT, Gerner EW. 1994. Polyamine-dependent expression of the matrix metalloproteinase matrilysin in a human colon cancer-derived cell line. Mol Carcinog 11:138–144.

Wang M, Liu YE, Greene J, Sheng S, Fuchs A, Rosen EM, Shi YE. 1997. Inhibition of tumor growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4. Oncogene 14:2767–2774.

Zhao YG, Xiao AZ, Park HI, Newcomer RG, Yan M, Man YG, Heffelfinger SC, Sang QX. 2004. Endometase/matrilysin-2 in human breast ductal carcinoma in situ and its inhibition by tissue inhibitors of metalloproteinases-2 and -4: A putative role in the initiation of breast cancer invasion. Cancer Res 64:590–598.