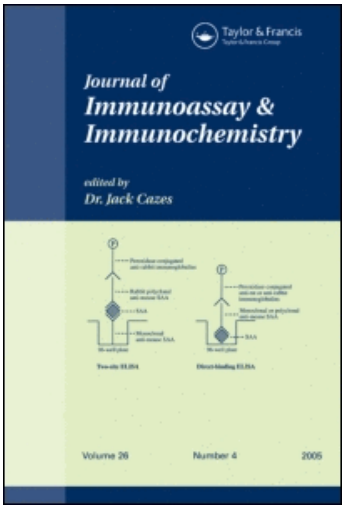


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# TWO-STEP SANDWICH ENZYME IMMUNOASSAY USING MONOCLONAL ANTIBODIES FOR DETECTION OF SOLUBLE AND MEMBRANE- ASSOCIATED HUMAN MEMBRANE TYPE 1-MATRIX METALLOPROTEINASE

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[a] Biopharmaceutical Department, Fuji Chemical Industries, Ltd., Toyama, Japan [b] Department of Otolaryngology, School of Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan [c] Department of Pathology, School of Medicine, Keio University, Japan [d] Department of Cancer Cell Research, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

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# TWO-STEP SANDWICH ENZYME IMMUNOASSAY USING MONOCLONAL ANTIBODIES FOR DETECTION OF SOLUBLE AND MEMBRANE-ASSOCIATED HUMAN MEMBRANE TYPE 1-MATRIX METALLOPROTEINASE

**Takanori Aoki,[1,*] Kayoko Yonezawa,[1] Eiko Ohuchi,[1] Noboru Fujimoto,[1] Kazushi Iwata,[1] Taketoshi Shimada,[2] Takayuki Shiomi,[3] Yasunori Okada,[3] and Motoharu Seiki[4]**

[1]Biopharmaceutical Department, Fuji Chemical Industries, Ltd., 530 Chokeiji, Takaoka, Toyama 933-8511, Japan
[2]Department of Otolaryngology, School of Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan
[3]Department of Pathology, School of Medicine, Keio University, Japan
[4]Department of Cancer Cell Research, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

*Corresponding author. E-mail: t-aoki@fuji-chemi.co.jp

## ABSTRACT

A two-step sandwich enzyme immunoassay (EIA) system for the detection of human membrane Type 1-matrix metalloproteinase (MT1-MMP) was established by using two monoclonal antibodies against recombinant MT1-MMP. MT1-MMP in which samples were reacted with solid-phase antibody and then detected with peroxidase-labeled second antibody. At least 1.25 ng/mL was detected by the EIA system, and linearity was obtained between 1.25 and 160 ng/mL. This EIA system is specific for MT1-MMP and did not show cross-reactivity against several other MMP's examined. Shedding of soluble MT1-MMP into the medium by some cancer cell lines was also detected by this system. However, soluble MT1-MMP in serum from normal and cancer patients was under the detection limit. Membrane-associated MT1-MMP of cancer cell lines was also detected after solubilization of the membranes with extraction buffer containing detergent. Additionally, MT1-MMP in clinical samples was examined. Elevated levels of MT1-MMP were detected in homogenate of cancer tissue compared with the levels for normal tissue and the level of MT1-MMP in tumors correlated with the rate of metastasis to the regional lymph nodes. Thus, we demonstrated that this EIA system is the first to measure MT1-MMP in clinical specimens, thus suggesting its useful for diagnosis of cancer or prediction of malignancy.


## INTRODUCTION

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade the extracellular matrix (ECM) and contribute to both physiological and pathological connective tissue remodeling.(1) In the MMP family, some members are soluble enzymes secreted into tissue cavities, but others are anchored to the plasma membrane by having either a transmembrane domain or a signal for glycosylphosphatidylinositol (GPI) anchoring at their C-terminus.(8) To date, six MMP members are known as membrane-type MMPs (MT-MMPs).(2–7) Most of the soluble MMPs are secreted as a latent form (proMMPs) and activated through proteolytic processing by serine proteinases including trypsin, plasmin, plasma kallikrein, and neutrophil elastase.(9) However, proMMP-2 (pro-gelatinase A), which has been implicated in the through invasion of the basement





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membrane by malignant tumors, cannot be activated by these serine proteinases, but is activated by the MT1-MMP that is frequently expressed in cancer cells.(9–11) It is known that expression levels of MT1-MMP correlate with the activation of proMMP-2 in cancer tissue.(12) In addition, since MT1-MMP has the ability to digest ECM components such as collagen, fibronectin, vitronectin, and cartilage proteoglycan, it is presumed to play a key role in tumor invasion.(13–15)

MT1-MMP localizes on the surface of invasive tumor cells, especially on invadopodia as an integral membrane protein.(16) However, some fraction of MT1-MMP may be released from the cell surface, as demonstrated with a human breast carcinoma cell line, MDA-MB-231, which sheds MT1-MMP into the culture medium upon treatment with concanavalin A (Con A).(17,18) If MT1-MMP, localized on the cancer cell surface, is processed and shed into the circulation, it would be a marker for malignancy of tumors. MT1-MMP shed from tumor cells may be a target for enzyme immunoassay (EIA), like other soluble MMPs.(19–26) In addition, measurement of MT1-MMP levels in tumor tissue may also help to establish a prognosis. However, a quantitative and sensitive method to measure soluble and membrane-bound MT1-MMP has not been available.

In the present study, we developed monoclonal antibodies against MT1-MMP and used them to establish a two-step sandwich EIA system for detection of spontaneously solubilized MT1-MMP and extracted MT1-MMP from cells and tissues. The system was applied for determination of MT1-MMP in culture media, cells in culture, sera, and tumor tissue homogenates in order to evaluate the correlation between MT1-MMP production and metastatic ability of cancers.

## EXPERIMENTAL

### Materials

The following materials were obtained commercially: bovine serum albumin (BSA, Fraction V) from Sigma Chem. Co. (St. Louis, MO, USA); horseradish peroxidase (HRP, grade I) from Roche Diagnostics, GmbH (Mannheim, Germany); MonoAb-ID EIA kit from Zymed Lab., Inc. (San Francisco, CA, USA); PD-10, chelating Sepharose FF, CNBr-activated Sepharose 4B, and Sephacryl S-300HR from Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, UK); Ultrogel AcA 44 from LKB (Villeneuve-la-Garenne, France); protein A-Cellulofine from Seikagaku Corp. (Tokyo, Japan); UK-10 membrane filters from Advantec (Tokyo, Japan); nitrocellulose filters (Trans-Blot transfer medium, 0.45 m)





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and prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards (low range) from Bio-Rad Lab. (Richmond, CA, USA); 1 × 8-wells Micro Well Module in frame from Nunc (Roskilde, Denmark); fetal bovine serum (FBS) from JRH Bioscience, (Lenexa, KS, USA); Dulbecco's modified Eagle medium (DMEM) from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); skim milk from Difco Laboratories (Detroit, MI, USA); *N*-(6-maleimidocaproyloxy) succinimide (EMCS) and 3,3′-diaminobenzidine (DAB) from Dojindo Laboratories (Kumamoto, Japan); S-acetylmercaptosuccinic anhydride from Aldrich Chem. Co. (Milwaukee, WI, USA); 2-mercaptoethanol, SDS, and Tween 20 from Nacalai Tesque, Inc. (Kyoto, Japan); 3,3′,5,5′-tetramethylbenzidine (TMB) solution from Calbiochem (La Jolla, CA, USA); dotMatric protein assay kit from Geno Technology, Inc. (St. Louis, MO, USA); BCA protein assay kit from Pierce (Rockford, IL, USA); pTrcHis from Invitrogen (Carlsbad, CA, USA); Con A, isopropyl-D-thiogalacto-pyranoside (IPTG), and other chemicals from Wako Pure Chem. Ind., Ltd. (Osaka, Japan).

MMP-1, -2, -3, -7, -8, -9, -13, -19 and -20 were purified as described in detail elsewhere.(19–26)

The following human cell lines were also purchased: MDA-MB-231 from the American Type Culture Collection (Rockville, MD, USA) and HT1080, SCC-25, and HeLa from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan).

The expression vector pSG△MT1-MMP for a deletion mutant of MT1-MMP(△MT1-MMP) lacking the transmembrane domain and cytoplasmic tail was prepared as described previously.(27)

Serum from normal subjects and cancer patients were used for the assay. All serum samples were stored at −40°C and usually assayed within 3 months.

## Preparation of Recombinant MT1-MMP in *E. coli*

Expression vector for *E. coli* (pUC△MT1-MMP) was constructed with pUC19 and △MT1-MMP by digestion with Sma I and Hind III of pSG△MT1-MMP.(27) JM109 cells transformed with the plasmid were grown at 37°C to log-phase, and protein expression was induced by adding 0.5 mM IPTG. Growth of the cells was continued for 4 h, and then the cells were harvested. The pellet from a 400 mL culture was suspended in 20 mL of 50 mM *tris*-HCl buffer, pH 8.0, and incubated with 20 mg of egg-white lysozyme. After storage on ice for 20 min, the cells were disrupted by sonication; and then insoluble recombinant protein, in the form of inclusion bodies, was collected by centrifugation.





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The pellet was washed twice with 50 mM *tris*-HCl buffer, pH 7.0, then solubilized with 2 mL of 50 mM *tris*-HCl buffer, pH 7.0, containing 8 M urea, 1% SDS, and 1% 2-mercaptoethanol, and thereafter incubated for 30 min at room temperature. The solubilized inclusion bodies were subjected to SDS-PAGE. The gels were sliced and recombinant MT1-MMP was recovered by electroelution done with a homemade apparatus. Buffer for the protein was exchanged for 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 10 mM ethylenediaminetetraacetic acid (EDTA) by using a PD-10 column; and the recombinant protein was used for immunization or as the EIA standard protein. The protein concentration of the recombinant MT1-MMP in the SDS buffer was determined by means of the dotMetric protein assay kit used according to the instructions of Geno Technology, Inc.

Inclusion bodies expressed in *E. coli* bearing pTrcHisMT1-MMP were dissolved in 50 mM phosphate buffer, pH 7.0, containing 8 M urea, 0.5 M NaCl, and 10 mM imidazole (binding buffer) and applied to a nickel-chelating Sepharose FF gel column. The recombinant protein (HisMT1-MMP) was eluted with the binding buffer containing 0.5 M imidazole. Refolding of the recombinant protein was done in 50 mM *tris*-HCl buffer, pH 8.6, containing 0.15 M NaCl. Soluble fraction was concentrated by UK-10, and applied to a Sephacryl S-300HR gel column, which was equilibrated with 50 mM *tris*-HCl buffer, pH 7.0, containing 0.15 M NaCl. Refolded HisMT1-MMP was digested with 0.1 μg/mL trypsin for 1 h at 37°C and then used for epitope mapping.



## Preparation of Monoclonal Antibodies Against MT1-MMP

Two 6-week-old female BALB/c mice were immunized intraperitoneally with 58 μg of purified recombinant MT1-MMP emulsified with an equal volume of Freund's complete adjuvant. Subsequent booster injections of 66 μg of immunogen in saline were administered intraperitoneally after 20 and 35 days and then intravenously after 70 days. Three days after the intravenous injection, the spleens were removed, and the splenocytes were isolated for fusion with mouse myeloma cells (SP-2/0-Ag14). The hybridization, as well as subsequent culturing and cloning of the hybrids, was carried out as described by Oi and Herzenberg.(28) Antibodies (IgG1) were purified from ascitic fluids by 40% saturated ammonium sulfate fractionation, followed by protein A-Cellulofine column chromatography. Clones 222-1D8 and 222-2D12 obtained were of the IgG1/$\kappa$ family, and used in this study.



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### Preparation of Fab′-HRP Conjugate

$F(ab')_2$ was prepared from the purified monoclonal antibody (clone 222-1D8) by digestion with pepsin. The $F(ab')_2$ obtained was then reduced with 2-aminoethanethiol, and the resulting Fab′ was conjugated with HRP by using EMCS.(29)

### Two-Step Sandwich EIA System for MT1-MMP

Twenty-five microliters of specimen containing MT1-MMP was diluted with 100 μL of 30 mM sodium phosphate buffer, pH 7.0, containing 1% BSA, 3% horse serum, 0.1 M NaCl, 10 mM EDTA, 0.42% SDS, and 0.4% 2-mercaptoethanol (dilution buffer). Aliquots of the diluted samples (100 μL) were transferred to microplate wells previously coated with anti-MT1-MMP IgG, clone 222-2D12, and the plate was then incubated overnight at 4°C without shaking. The plate was next washed three times with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1% (w/v) Tween 20 (washing buffer). One hundred microliters of 0.2 μg/mL anti-MT1-MMP Fab′ (clone 222-1D8)-HRP conjugate in 30 mM sodium phosphate buffer, pH 7.0, containing 1% BSA, 0.1 M NaCl and 10 mM EDTA was added to each well. The plate was then incubated for 1 h at room temperature and thereafter washed three times with the washing buffer. TMB solution (100 μL) was dispensed into each well, and incubation continued for 20 min at room temperature. The reaction was stopped by adding 100 μL of 1 M sulfuric acid, and the absorbance at 450 nm was measured with a microplate reader (Tosoh model MPR-A4, Tokyo).

### Preparation of Samples Containing MT1-MMP from Culture Media and Cell Extracts

HT1080, MDA-MB-231, SCC-25, and HeLa cells were cultured in 7 mL of DMEM, supplemented with 10% FBS in an atmosphere of humidified 5% $CO_2$ in air at 37°C in 10 cm tissue culture dishes ($2 \times 10^6$ cells/dish). The confluent cells were rinsed twice with 5 mL of phosphate-buffered saline (PBS) and then cultured with 7 mL of serum-free medium containing 200 μg/mL of Con A. The conditioned media were harvested 3 days later and used for the EIA. The cells were scraped off into 1 mL of PBS containing 10 mM EDTA and washed in the same buffer. After centrifugation, the cells were resuspended in 0.4 mL of 50 mM *tris*-HCl buffer, pH 7.0, containing 0.15 M NaCl, 10 mM $CaCl_2$, and 0.05% Brij35

(TNCB), and homogenized with a homogenizer or weakly sonicated. The homogenates/sonicates were clarified by centrifugation, and the supernatants were recovered as cell extracts.

### Electrophoresis and Immunoblotting

Samples were subjected to SDS-PAGE (12% total acrylamide) and transferred onto nitrocellulose membranes. Monoclonal antibodies of clones 222-1D8, 222-2D12, 113-15E7, and 114-1F2 were conjugated with HRP by using *S*-acetylmercaptosuccinic anhydride.(29) The membranes reacted with 1 μg/mL of each IgG-HRP overnight at room temperature in 30 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 3% skim milk. After having been washed with 30 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, the membranes were stained by the hydrogen peroxide system with 0.5 mg/mL DAB. Anti-MT1-MMP monoclonal antibodies, clones 113-15E7 and 114-1F2, were used for detection of the hemopexin-like domain and catalytic domain of MT1-MMP, respectively.(2)

### Affinity Concentration of MT1-MMP from Human Serum

Monoclonal antibody against MT1-MMP (clone 222-2D12, 5 mg) in sodium borate buffer, pH 8.0, containing 0.5 M NaCl was reacted with 0.5 g CNBr-activated Sepharose 4B and gently shaken for 2 h at room temperature and then overnight at 4°C. The unreacted sites were blocked with 0.2 M glycine-NaOH buffer, pH 8.0.

5 mL of the serum from a patient with metastatic carcinoma was diluted with 20 mL of the dilution buffer. This sample was applied to a column of IgG (clone 222-2D12) coupled to 1 mL Sepharose 4B gel. The column was washed with PBS, and then the protein was eluted with 0.1 M glycine-HCl buffer, pH 2.0. The eluate was neutralized and concentrated to 100 μL. An aliquot of the concentrated sample was subjected to the EIA system.

### Preparation of Tissue Homogenates for the EIA System

Frozen tissues of head and neck, and lung cancers stored at −40°C were minced and homogenized in 0.4 mL of TNCB with a spindle-type homogenizer. The homogenates were clarified by centrifugation, and the





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supernatants were recovered as tissue extracts and subjected to the EIA. Protein concentrations of the tissue and cell extracts were determined with the Micro BCA protein assay kit, according to the instructions of Pierce. MT1-MMP levels in the samples were calculated and reported as ng/mg protein.

## RESULTS

### Purification of Recombinant MT1-MMP for Use as Immunogen and EIA Standard Protein

A recombinant soluble MT1-MMP (△MT1-MMP) lacking its transmembrane domain and cytoplasmic tail was expressed in *E. coli* by using a bacterial expression plasmid (pUC△MT1-MMP). The recombinant protein was expressed as insoluble inclusion bodies, which were solubilized with 62.5 mM *tris*-HCl buffer, pH 6.8, containing 8 M urea, 1% SDS and 1% 2-mercaptoethanol. Then, △MT1-MMP was purified by preparative SDS-PAGE to a grade showing a single band on SDS-PAGE at the position of about 60 kDa, as shown in Figure 1A. The yield of △MT1-MMP was 2.6 mg from 400 mL of culture medium.

### Specificity of the Monoclonal Antibodies

Using the purified protein as an antigen, we prepared hybridoma cell lines. The cell lines were selected by the ELISA method and immunoblotting using the recombinant protein. Five clones (222-1D8, 222-2D12, 222-3E12, 222-4G5, and 222-9A3) secreting monoclonal antibodies against MT1-MMP were obtained. According to the results of isotype assay for each monoclonal antibody conducted with the MonoAb-ID kit, all of the light chains were the $\kappa$ type, but the heavy chains were $\gamma 1$ for four clones and $\gamma 3$ for one. Various combinations of two monoclonal antibodies were examined to develop sandwich EIA for MT1-MMP, and we chose the combination of clone 222-2D12 for the solid phase and 222-1D8 for the conjugate coupled with HRP.

Immunoblot analysis was performed to confirm the specificity of the monoclonal antibodies. △MT1-MMP (0.8 μg) was subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was reacted with 1 μg/mL IgG-HRP (clones 222-1D8 and 222-12D2) overnight at room temperature and was then stained by the hydrogen peroxide system with





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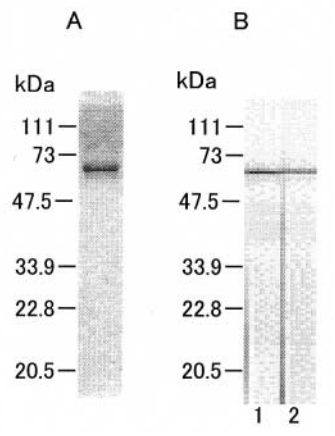


***Figure 1.*** SDS-PAGE and immunoblotting of △MT1-MMP. A, Purified MT1-MMP (8 μg) was subjected to SDS-PAGE (12% total acrylamide) under reducing conditions. The gel was stained with CBB. B, Purified △MT1-MMP (0.8 μg) was subjected to SDS-PAGE (12% total acrylamide) under reducing conditions and then transferred onto a nitrocellulose membrane. The membrane was reacted with 1 μg/mL IgG-HRP (lane 1, clone 222-1D8; and lane 2, 222-12D2) overnight at room temperature in 30 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, and 3% skim milk and was stained by the hydrogen peroxide system with 0.5 mg/mL DAB.

DAB. As shown in Figure 1B, both clones 222-1D8 and 222-12D2 reacted with △MT1-MMP 57 kDa.

In order to determine the epitope for these monoclonal antibodies, epitope mapping was performed by immunoblot analysis using trypsin-treated △MT1-MMP fragments tagged with His 6. The blotted membrane was reacted with the antibodies prepared in the present study (clones 222-1D8 and 222-12D2), anti-hemopexin-like domain (clone 113-15E7), and anti-catalytic domain (clone 114-1F2) of MT1-MMP, and the antibodies were visualized as stated in Experimental. As shown in Figure 2, both clones 222-1D8 and 222-12D2 reacted strongly with the hemopexin-like domain of MT1-MMP (31 and 33 kDa-bands), similarly as clone 113-15E7.(2)

Cross-reactivity of these antibodies with MT2-, MT3-, MT4-, and MT5-MMPs was also tested. Clone 222-1D8 slightly reacted with MT5-MMP, but not with MT2-, MT3-, or MT4-MMP. On the other hand, clone 222-2D12 did not react with any of the MT-MMPs examined by immunoblotting (data not shown).





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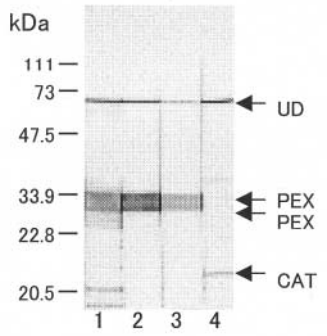


***Figure 2.*** Epitope mapping of the anti-△MT1-MMP monoclonal antibodies. Refolded △MT1-MMP was digested with trypsin (1 μg/lane) for 1 h at 37°C and subjected to SDS-PAGE (12% total acrylamide) under reducing conditions and reacted with anti-MT1-MMP antibodies. The membrane was then stained with 1 μg/mL IgG-HRP (lane 1, clone 222-1D8; lane 2, 222-12D2; lane 3, 113-15E7; lane 4, 114-1F2). The arrows in the figure indicate undigested His△MT1-MMP (UD), hemopexin-like domain (PEX) and catalytic domain (CAT) of MT1-MMP, respectively.



## Standard Assay Curve for MT1-MMP by Sandwich EIA System

Figure 3 shows standard curve obtained with the sandwich EIA for MT1-MMP using the combination of clone 222-2D12 for the solid phase and 222-1D8 for the conjugate coupled with HRP. The sensitivity was 1.25 ng/mL (0.025 ng/well, two SDs above the zero point), and linearity was obtained between 1.25 and 160 ng/mL (0.025–3.2 ng/well). The intra-assay CVs ($n = 8$) at 8 different concentrations of MT1-MMP was 3.9–9.3%.

## Precision and Accuracy

The intra- and inter-assay reproducibility and recoveries of MT1-MMP were examined in serum. When different amounts of MT1-MMP (71.2, 35.6, and 17.8 ng/mL) were added to serum as a standard, recoveries of 89.0–102.5% were obtained. The intra-assay CVs and inter-assay CVs were 2.5–3.4% ($n = 8$) and 3.1–5.0 % ($n = 8$), respectively.

Recovery of MT1-MMP from culture medium or extracts of MDA-MB-231 cells was also examined. Recovery rates of △MT1-MMP (40, 20, and 10 ng/mL) added to the culture medium or cell extract as a standard



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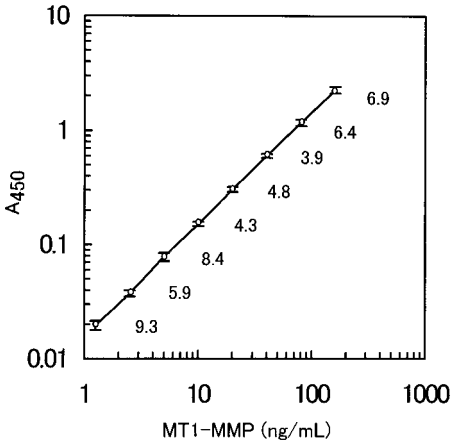


***Figure 3.*** Standard curve determined for MT1-MMP by the sandwich EIA system. Purified △MT1-MMP was analyzed by the sandwich EIA as described in Experimental. Vertical bars show mean value ± SD of 8 independent measurements ($n = 8$) at different concentrations. Numbers in the figure indicate the intra-assay CVs (%).

***Table 1.*** Precision and Accuracy of the EIA System for MT1-MMP

| Specimen | Recovery (%) | Intra-Assay CV (%) | Inter-Assay CV (%) |
|---|---|---|---|
| Normal serum | 89.0–102.5 | 2.5 ($n = 8$) | 3.1 ($n = 8$) |
| Cultured medium* | 92.8–105.8 | 6.9 ($n = 6$) | 7.1 ($n = 6$) |
| Cell homogenate* | 90.0–101.7 | 9.5 ($n = 6$) | 10.2 ($n = 6$) |

*Prepared from Con A-stimulated MDA-MB-231 cells as described in Experimental Section.

were 92.8–105.8 or 90.0–101.7%, respectively. Results of intra- and inter-assay in culture medium and cell extract are shown in Table 1.

## Characterization of the EIA System for MT1-MMP

The cross-reactivity of the EIA for MT1-MMP toward various MMPs was examined by using MMP-1, 2, 3, 7, 8, 9, 13, 19, and 20; but no signal was obtained from these MMPs, as documented in Table 2.





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*Table 2.* Cross-Reactivity of the EIA System for MT1-MMP toward Other MMP's

| MMPs | Concentration (μg/mL) | $A_{450}$ |
|---|---|---|
| MMP-1 | 1 | 0.025 |
| MMP-2 | 1 | 0.024 |
| MMP-3 | 1 | 0.027 |
| MMP-7 | 1 | 0.026 |
| MMP-8 | 1 | 0.028 |
| MMP-9 | 1 | 0.039 |
| MMP-13 | 1 | 0.026 |
| MMP-19 | 1 | 0.027 |
| MMP-20 | 1 | 0.071 |
| MT1-MMP | 0.16 | 2.206 |
| Blank | – | 0.027 |



## Determination of MT1-MMP in Human Cell Lines by the EIA

To measure MT1-MMP expression levels in human cancer cell lines, we lysed HT1080, MDA-MB-231, SCC-25, and HeLa cells and subjected the lysates to the EIA. MT1-MMP was detected in HT1080, MDA-MB-231, and SCC-25 cells, but not in HeLa cells (Table 3). At the same time, MT1-MMP released from the cells was measured. A substantial amount of MT1-MMP was detected in the medium conditioned by HT1080, MDA-MB-231, and SCC-25 cells, and it increased following Con A treatment of the cells (Table 4). As expected, MT1-MMP was not detected in the cell extract of HeLa cells. In a comparison of Tables 3 and 4, the production levels (ng/mg) by these cell lines corresponded well to the levels of MT1-MMP secreted into the medium (ng/mL) by each cell line.

## MT1-MMP in Human Serum

The EIA system was applied to serum samples from 2 normal and 121 various cancer patients, including those having metastasis (Table 5). However, MT1-MMP levels in all the serum samples were under the lower detection limit (1.25 ng/mL) of the assay system. The serum from a patient with metastatic carcinoma was concentrated 50-fold by using anti-MT1-MMP monoclonal antibody (clone 222-2D12) coupled with



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***Table 3.*** Measurement by the EIA of MT1-MMP Extracted from Human Cell Lysates

| Cell Line | MT-MMP* (ng/mg) |
|---|---|
| HT1080 | 36.1 |
| MDA-MB-231 | 26.7 |
| SCC-25 | 14.7 |
| HeLa | UD |

*Detected as MT1-MMP (ng) per protein (mg) in each cell homogenate.

***Table 4.*** Measurement by the EIA of MT1-MMP in Culture Media of Human Cell Lines

| | Concentration of MT1-MMP (ng/mL) | |
|---|---|---|
| Cell Line | Without Con A | With Con A |
| HT1080 | 10.1 | 80.3 |
| MDA-MB-231 | 7.6 | 48.8 |
| SCC-25 | 2.8 | 43.1 |
| HeLa | UD | UD |

UD: under the lower detection limit (< 1.25 ng/mL).

***Table 5.*** List of Human Serum Samples Examined by the EIA

| Serum | *n* |
|---|---|
| Cancer patient | |
| Colon | 31 |
| Breast | 26 |
| Stomach | 25 |
| Prostate (bone metastasis) | 17 |
| Liver (metastasis) | 4 |
| Others | 18 |
| Normal | 2 |
| Total | 123 |





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Sepharose 4B gel, and MT1-MMP in the eluate was determined by the EIA. MT1-MMP level in the serum sample was estimated to be less than 25 pg/mL, because it was undetectable even in the 50-fold concentrated eluate.

## Determination of MT1-MMP in Human Carcinoma Tissue Samples

Tissue homogenates of cancer patients were then examined by using the EIA system. Head and neck cancers (laryngeal, pharyngeal, oral, and salivary gland) and normal tissues adjacent to the cancers were isolated and subjected to the EIA after lysis. The MT1-MMP levels in normal ($n = 26$) and tumor tissues ($n = 80$) were $0.8 \pm 1.1$ and $5.0 \pm 4.3$ ng/mg (mean $\pm$ SD), respectively (Figure 4). The MT1-MMP levels were significantly 6.3-fold higher in tumors than in normal tissues ($P < 0.001$ by Student's $t$ test).

Specimens were also isolated from lung cancer patients ($n = 51$) at different stages (TNM classification) and analyzed similarly.(35) Again, MT1-MMP levels were higher in tumors than in normal tissues (Figure 5A). Furthermore, tumor specimens with regional lymph node metastasis (N1–N3) showed higher levels of MT1-MMP than those designated N0 ($P < 0.05$ by Student's $t$ test), as shown in Figure 5B.

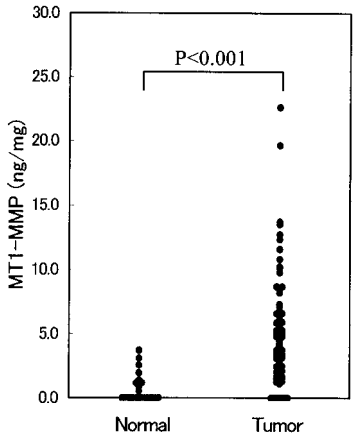


***Figure 4.*** MT1-MMP levels in head and neck cancer tissue homogenates. MT1-MMP levels in homogenates from head and neck cancers ($n = 80$) and from normal tissue adjacent to such cancers ($n = 26$) were determined by the sandwich EIA as described in Experimental. $p < 0.001$ by Student's $t$ test.





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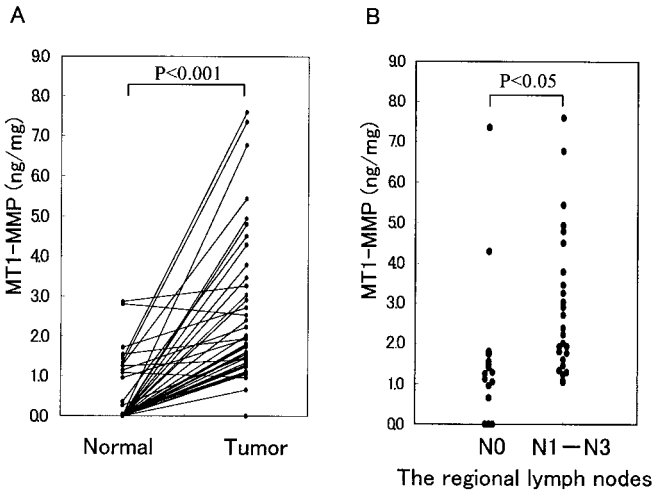


***Figure 5.*** MT1-MMP levels in lung cancer tissue homogenates. MT1-MMP levels in homogenates from lung tissue were determined by the sandwich EIA as described in Experimental. Significance of the difference was evaluated by Student's $t$ test ($p < 0.05$). A: MT1-MMP levels in homogenates from cancer and adjacent normal tissues are shown. B: MT1-MMP levels in the tissue homogenates are separately indicated according to the degree of regional lymph node metastasis.

## DISCUSSION

We established a two-step sandwich EIA system to measure both soluble and membrane-associated MT1-MMP. Monoclonal antibodies used for the EIA were generated against recombinant MT1-MMP that was lacking its transmembrane domain and cytoplasmic tail. The recombinant MT1-MMP expressed in *E. coli* as inclusion bodies was solubilized with *tris*-HCl buffer, pH 7.0, containing urea, SDS and 2-mercaptoethanol, and then purified by preparative SDS-PAGE. The purified MT1-MMP did not show any enzymatic activities, and it was stable after 24 h incubation at 37°C without the presence of EDTA or phenylmethylsulfonyl fluoride. The stable recombinant protein was suitable for use as the standard protein for the EIA.

Five monoclonal antibodies were obtained, and we chose a particular combination of two of them for the two-step sandwich EIA system. The established EIA system was quantitative and specific for MT1-MMP, showing no cross-reactivity against the other MMP's examined. By epitope analysis, we proved that these two antibodies recognized the hemopexin-like domain of MT1-MMP. Therefore, these antibodies cannot distinguish

between pro form and active form of MT1-MMP. Theoretically, this EIA system, using this pair of antibodies against the hemopexin-like domain, could detect all three types of MT1-MMP molecules, i.e., membrane-associated form, shed form, and the 43 kDa form described previously.(2,17,30) This EIA system likely reflects the expression levels of MT1-MMP.

This system was applied to detect MT1-MMP in human cancer cell lines and in clinical samples. A human breast carcinoma cell line MDA-MB-231 is reported to express MT1-MMP and to shed it into culture medium upon treatment with ConA.(17,18) Consistent with these previous reports, shed MT1-MMP was detected in the culture medium by this EIA method. Shed MT1-MMP was also detected in the conditioned medium of HT1080 and SCC-25 cells after ConA treatment (Table 3). Other cancer cell lines (SW837, MG-63, and CaSki) also produced shed MT1-MMP, but it was not detectable in the culture medium of HeLa, MCF7, Burkitt's lymphoma (Raji, Daudi), and leukaemia (K-562, HL-60) cell lines (data not shown). These results agree with the expression of MT1-MMP assessed by Northern blot analysis of the cell lines.(31) MT1-MMP detected in conditioned medium paralleled expression levels of MT1-MMP in the cells and may reflect metastatic ability of the cells.

On the other hand, MT1-MMP levels in sera from two normal adults and from 121 patients with cancer including 21 with metastasis, were below the lower detection limit of this assay system (1.25 ng/mL). It seems that the EIA cannot detect MT1-MMP from serum specimens, because the amount of shed MT1-MMP is very limited. MT1-MMP level in the serum was estimated to be less than 25 pg/mL.

MT1-MMP is usually anchored to the cell surface through its hydrophobic transmembrane domain at the C-terminus and contributes to activation of proMMP-2 and degradation of ECM under physiological or pathological conditions. Thus, it is also important to determine quantity of MT1-MMP in tumors. In order to extract MT1-MMP from the cell surface, we disrupted the cells with a lysis buffer containing SDS and 2-mercaptoethanol, and then subjected the lysates to the EIA. The EIA system effectively detected solubilized MT1-MMP from cancer cell lines as well as the shed MT1-MMP (Tables 3 and 4). These results suggested that the EIA could detect membrane-bound MT1-MMP from tumor tissue after the solubilization step using denaturants.

Then, we applied the method to detect MT1-MMP in clinical specimens. Head and neck cancer tissue and adjacent normal tissue were surgically dissected and subjected to the analysis. MT1-MMP was detected in both normal and cancer tissues, but the levels in tumors (5.0 ng/mL, $n = 80$) were significantly higher than those in the normal tissues (0.8 ng/mL, $n = 26$;





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Figure 4). The same tendency was observed with lung cancer (Figure 5A). Lung tumor specimens with regional lymph node metastasis (N1–N3) showed higher levels of MT1-MMP than those with no metastasis (N0; Figure 5B). As this EIA system requires denaturation of specimens, it could not distinguish MT1-MMP/TIMP complexes from free MT1-MMP. Consequently, this EIA could not demonstrate the biological behavior of MT1-MMP/TIMP complexes or free MT1-MMP in metastasis. This may be reason why there was no clear correlation between the MT1-MMP level and the stage of the primary tumor (data not shown).

The expression of MT1-MMP has been detected by immunostaining or Northern blot analysis in a variety of tumor tissues, including head and neck, lung, breast, gastric, colon, and so on (2,12,31,32). Furthermore, a positive correlation between expression levels MT1-MMP and metastasis of breast carcinoma, thyroid carcinoma, and colorectal cancer have reported (12,33,34). Since MT1-MMP expressed in tumors is a marker for invasive and metastatic potential, we believe our EIA system, which reflects the expression level of MT1-MMP, is useful for the prognosis of cancer patients.

## REFERENCES

1. Nagase, H.; Woessner, J.F. Matrix Metalloproteinases. J. Biol. Chem. **1999**, *274* (31), 21491–21494.
2. Sato, H.; Takino, T.; Okada, Y.; Cao, J.; Shinagawa, A.; Yamamoto, E.; Seiki, M. A Matrix Metalloproteinase Expressed on the Surface of Invasive Tumour Cells. Nature **1994**, *370* (6484), 61–65.
3. Will, H.; Hinzmann, B. cDNA Sequence and mRNA Tissue Distribution of a Novel Human Matrix Metalloproteinase With a Potential Transmembrane Segment. Eur. J. Biochem. **1995**, *231* (3), 602–608.
4. Takino, T.; Sato, H.; Shinagawa, A.; Seiki, M. Identification of the Second Membrane-Type Matrix Metalloproteinase (MT-MMP-2) Gene From a Human Placenta cDNA Library. MT-MMPs Form a Unique Membrane-Type Subclass in the MMP Family. J. Biol. Chem. **1995**, *270* (39), 23013–23020.
5. Puente, X.S.; Pendas, A.M.; Llano, E.; Velasco, G.; López-Otín, C. Molecular Cloning of a Novel Membrane-Type Matrix Metalloproteinase From a Human Breast Carcinoma. Cancer Res. **1996**, *56* (5), 944–949.
6. Llano, E.; Pendas, A.M.; Freije, J.P.; Nakano, A.; Knäuper, V.; Murphy, G.; López-Otín, C. Related Articles, Protein, Nucleotide Identification and Characterization of Human MT5-MMP, a New





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Membrane-Bound Activator of Progelatinase A Overexpressed in Brain Tumors. Cancer Res. **1999**, *59* (11), 2570–2576.
7. Pei, D. Leukolysin/MMP25/MT6-MMP: A Novel Matrix Metalloproteinase Specifically Expressed in the Leukocyte Lineage. Cell Res. **1999**, *9* (4), 291–303.
8. Itoh, Y.; Kajita, M.; Kinoh, H.; Mori, H.; Okada, A.; Seiki, M. Membrane Type 4 Matrix Metalloproteinase (MT4-MMP, MMP-17) is a Glycosyl-Phosphatidylinositol-Anchored Proteinase. J. Biol. Chem. **1999**, *274* (48), 34260–34266.
9. Stetler-Stevenson, W.G.; Aznavoorian, S.; Liotta, L.A. Tumor Cell Interactions With the Extracellular Matrix During Invasion and Metastasis. Ann. Rev. Cell Biol. **1993**, *9*, 541–573.
10. Nomura, H.; Sato, H.; Seiki, M.; Mai, M.; Okada, Y. Expression of Membrane-Type Matrix Metalloproteinase in Human Gastric Carcinomas. Cancer Res. **1995**, *55* (15), 3263–3266.
11. Okada, Y.; Morodomi, T.; Enghild, J.J.; Suzuki, K.; Yasui, A.; Nakanishi, I.; Salvesen, G.; Nagase, H. Matrix Metalloproteinase 2 From Human Rheumatoid Synovial Fibroblasts. Purification and Activation of the Precursor and Enzymic Properties. Eur. J. Biochem. **1990**, *194* (3), 721–730.
12. Ueno, H.; Nakamura, H.; Inoue, M.; Imai, K.; Noguchi, M.; Sato, H.; Seiki, M.; Okada, Y. Expression and Tissue Localization of Membrane-Types 1, 2, and 3 Matrix Metalloproteinases in Human Invasive Breast Carcinomas. Cancer Res. **1997**, *57* (10), 2055–2060.
13. Ohuchi, E.; Imai, K.; Fujii, Y.; Sato, H.; Seiki, M.; Okada, Y. Membrane Type 1 Matrix Metalloproteinase Digests Interstitial Collagens and Other Extracellular Matrix Macromolecules. J. Biol. Chem. **1997**, *272* (4), 2446–2451.
14. d'Ortho, M.P.; Will, H.; Atkinson, S.; Butler, G.; Messent, A.; Gavrilovic, J.; Smith, B.; Timpl, R.; Zardi, L.; Murphy, G. Membrane-Type Matrix Metalloproteinases 1 and 2 Exhibit Broad-Spectrum Proteolytic Capacities Comparable to Many Matrix Metalloproteinases. Eur. J. Biochem. **1997**, *250* (3), 751–757.
15. Pei, D.; Weiss, S.J. Transmembrane-Deletion Mutants of the Membrane-Type Matrix Metalloproteinase-1 Process Progelatinase A and Express Intrinsic Matrix-Degrading Activity. J. Biol. Chem. **1996**, *271* (15), 9135–9140.
16. Nakahara, H.; Howard, L.; Thompson, E.W.; Sato, H.; Seiki, M.; Yeh, Y.; Chen, W.T. Transmembrane/Cytoplasmic Domain-Mediated Membrane Type 1-Matrix Metalloprotease Docking to Invadopodia is Required for Cell Invasion. Proc. Natl. Acad. Sci. USA **1997**, *94* (15), 7959–7964.





Marcel Dekker, Inc.
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17. Imai, K.; Ohuchi, E.; Aoki, T.; Nomura, H.; Fujii, Y.; Sato, H.; Seiki, M.; Okada, Y. Membrane-Type Matrix Metalloproteinase 1 is a Gelatinolytic Enzyme and is Secreted in a Complex With Tissue Inhibitor of Metalloproteinases 2. Cancer Res. **1996**, *56* (12), 2707–2710.
18. Harayama, T.; Ohuchi, E.; Aoki, T.; Sato, H.; Seiki, M.; Okada, Y. Shedding of Membrane Type 1 Matrix Metalloproteinase in a Human Breast Carcinoma Cell Line. Jpn. J. Cancer Res. **1999**, *90* (9), 942–950.
19. Zhang, J.; Fujimoto, N.; Iwata, K.; Sakai, T.; Okada, Y.; Hayakawa, T. A One-Step Sandwich Enzyme Immunoassay for Human Matrix Metalloproteinase-1 (Interstitial Collagenase) Using Monoclonal Antibodies. Clin. Chim. Acta **1993**, *219* (1–2), 1–14.
20. Fujimoto, N.; Mouri, N.; Iwata, K.; Ohuchi, E.; Okada, Y.; Hayakawa, T. A One-Step Sandwich Enzyme Immunoassay for Human Matrix Metalloproteinase 2 (72-kDa Gelatinase Type IV Collagenase) Using Monoclonal Antibodies. Clin. Chim. Acta **1993**, *221* (1–2), 91–103.
21. Obata, K.; Iwata, K.; Okada, Y.; Kohrin, Y.; Ohuchi, E.; Yoshida, S.; Shinmei, M.; Hayakawa, T. A One-Step Sandwich Enzyme Immunoassay for Human Matrix Metalloproteinase-3 (Stromelysin-1) Using Monoclonal Antibodies. Clin. Chim. Acta **1992**, *211* (1–2), 59–72.
22. Ohuchi, E.; Azumano, I.; Yoshida, S.; Iwata, K.; Okada, Y. A One-Step Sandwich Enzyme Immunoassay for Human Matrix Metalloproteinase 7 (Matrilysin) Using Monoclonal Antibodies. Clin. Chim. Acta **1996**, *244* (2), 181–198.
23. Matsuki, H.; Fujimoto, N.; Iwata, K.; Knäuper, V.; Okada, Y.; Hayakawa, T. A One-Step Sandwich Enzyme Immunoassay for Human Matrix Metalloproteinase 8 (Neutrophil Collagenase) Using Monoclonal Antibodies. Clin. Chim. Acta **1996**, *244* (2), 129–143.
24. Okada, Y.; Gonoji, Y.; Naka, K.; Tomita, K.; Nakanishi, I.; Iwata, K.; Yamashita, K.; Hayakawa, T. Matrix Metalloproteinase 9 (92kDa Gelatinase/Type IV Collagenase) From HT 1080 Human Fibrosarcoma Cells. J. Biol. Chem. **1992**, *267* (30), 21712–21719.
25. Tamei, H.; Azumano, I.; Iwata, K.; Yoshihara, Y.; López-Otín, C.; Vizoso, F.; Knäuper, V.; Murphy, G. One-Step Sandwich Enzyme Immunoassays for Human Matrix Metalloproteinase 13 (Collagenase-3) Using Monoclonal Antibodies. Connective Tissue **1998**, *30*, 15–21.
26. Wang, T.; Aoki, T.; Iwata, K.; Takata, T.; Uchida, T.; Knäuper, V.; Llano, E.; Okada, Y.; Bartlett, J.D. One-Step Sandwich Enzyme Immunoassay Using Monoclonal Antibodies for Detection of Human Enamelysin (MMP-20). Eur. J. Oral Sci. **2000**, *108* (6), 530–537.





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27. Cao, J.; Sato, H.; Takino, T.; Seiki, M. The C-Terminal Region of Membrane Type Matrix Metalloproteinase is a Functional Transmembrane Domain Required for Pro-Gelatinase A Activation. J. Biol. Chem. **1995**, *270* (2), 801–805.
28. Oi, V.T.; Herzenberg, L.A. Immunoglobulin-Producing Hybrid Cell Lines. In *Selected Methods in Cellular Immunology;* Mishell, B.B., Shiigi, S.M., Eds.; W. H. Freeman and Company: San Francisco, 1980; 351–372.
29. Ishikawa, E.; Imagawa, M.; Hashida, S.; Yoshitake, S.; Hamaguchi, Y.; Ueno, T. Enzyme-Labeling of Antibodies and Their Fragments for Enzyme Immunoassay and Immunohistochemical Staining. J. Immunoassay **1983**, *4* (3), 209–327.
30. Lehti, K.; Lohi, J.; Valtanen, H.; Keski-Oja, J. Proteolytic Processing of Membrane-Type-1 Matrix Metalloproteinase is Associated With Gelatinase A Activation at the Cell Surface. Biochem. J. **1998**, *334* (pt 2), 345–353.
31. Okada, A.; Bellocq, J.P.; Rouyer, N.; Chenard, M.P.; Rio, M.C.; Chambon, P.; Basset, P. Membrane-Type Matrix Metalloproteinase (MT-MMP) Gene is Expressed in Stromal Cells of Human Colon, Breast, and Head and Neck Carcinomas. Proc. Natl. Acad. Sci. USA **1995**, *92* (7), 2730–2734.
32. Polette, M.; Nawrocki, B.; Gilles, C.; Sato, H.; Seiki, M.; Tournier, J.M.; Birembaut, P. MT-MMP Expression and Localisation in Human Lung and Breast Cancers. Virchows Arch. **1996**, *428* (1), 29–35.
33. Nakamura, H.; Ueno, H.; Yamashita, K.; Shimada, T.; Yamamoto, E.; Noguchi, M.; Fujimoto, N.; Sato, H.; Seiki, M.; Okada, Y. Enhanced Production and Activation of Progelatinase A Mediated by Membrane-Type 1 Matrix Metalloproteinase in Human Papillary Thyroid Carcinomas. Cancer Res. **1999**, *59* (2), 467–473.
34. Sardinha, T.C.; Nogueras, J.J.; Xiong, H.; Weiss, E.G.; Wexner, S.D.; Abramson, S. Membrane-Type 1 Matrix Metalloproteinase mRNA Expression in Colorectal Cancer. Dis. Colon. Rectum. **2000**, *43* (3), 389–395.
35. Sobin, L.H.; Wittekind, C. *TNM Classification of Malignant Tumours*; 5th Ed.; John Wiley & Sons, Inc.: New York, 1997.

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