

Expression of MMP-13 is associated with invasion and metastasis of papillary thyroid carcinoma

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Abstract. – **BACKGROUND:** Collagenase-3 (MMP-13), a matrix metalloproteinase, is a recently identified member of the matrix metalloproteinases (MMPs) with broad substrate specificity, and a potential role in tumor metastasis and invasion has been proposed. Collagenase-3 expression has been reported in many carcinomas. However, the presence and possible implication of MMP-13 in the progression of papillary thyroid carcinomas are unknown.

MATERIALS AND METHODS: In the present study, we examined MMP-13 gene expression in 208 papillary thyroid carcinomas who underwent surgery without preoperative treatment and 100 matched samples of adjacent normal thyroid tissue (Paraffin-embedded tissue samples) by immunohistochemistry analysis. *In vitro* and *in vivo* studies were done in order to investigate the effect of MMP-13 overexpression or silencing on cancer cells invasion and metastasis.

RESULTS: We found MMP-13 expression was significantly increased in the tumours from local regional lymph node metastases patients. The MMP-13 was stained more intensely in invading fronts than in central portions of local regional lymph node. No MMP-13 staining was observed in matched samples of adjacent normal thyroid tissue. MMP-13 expression was significantly related with TNM and recurrent disease, no relation was found with age extent of tumour and size of tumour. Studies with cell and mice models indicated that overexpression of MMP-13 increased cell migration and promoted metastasis, and MMP-13 silencing decreased cell migration.

CONCLUSIONS: The data suggest that MMP-13 is associated with thyroid tumour invasion and metastasis and it may be a potential target for therapeutic intervention.

Key Words:

Papillary thyroid carcinomas, Metastasis, Matrix metalloproteinase-13.

counts for more deaths than all other endocrine cancers combined. Additionally, PTC has a rapidly growing incidence worldwide¹. PTC has a marked propensity for early invasion of the surrounding neck tissue, as well as metastasis to central lymph nodes (and other regional lymph nodes). In fact, the number of PTC metastases to the central lymph nodes has been correlated with numerous negative prognostic factors, including tumor size, extrathyroidal extension and lateral neck lymph node metastasis³. Additionally, 10-15% of PTC patients present with distant metastases, which have the greatest impact on patient survival rates (40% over 10 years)⁴. Currently, the only treatment for inoperable metastatic PTC is radioactive iodine (RAI). In cases where distant metastases have lost the ability to trap or retain iodine, the 10 year survival rate drops to 10%⁵. Furthermore, high cumulative activity of RAI has been associated with various unfavorable side effects, including an increased risk of subsequent development of leukemia and other secondary cancers. The clinical importance of invasive processes in PTC, as well as the limited availability of tools to combat such disease, has led to the investigation of molecular mediators of invasion in hopes of developing safer, more effective inhibitors of metastatic PTC.

Tumour invasion and metastasis involve degradation of different components of the extracellular matrix and require the actions of proteolytic enzymes, such as matrix metalloproteinases (MMPs), produced either by the tumour cells themselves or by surrounding stromal cells. It therefore seems evident that MMPs play an important role in tumour invasion and metastasis⁶⁻⁸. Kummer and Bauerle et al has recently reported that MMP-9 was related to thyroid cancer cell growth and invasion⁹⁻¹⁰.

Human collagenase-3 (MMP-13) was identified first in human breast carcinomas¹¹, and was correlated with poor prognoses of invasive

Introduction

Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy, and annually ac-

breast cancer¹². Expression of MMP-13 has also been detected in colorectal cancer with liver metastasis¹³, oral tongue¹⁴, renal cell carcinoma bone metastasis¹⁵, non-small cell lung cancer¹⁶, cutaneous malignant melanoma¹⁷ and head and neck cancer¹⁸.

Overexpression of MMP-13 have been suggested as important predictive factors for relapse or nodal metastasis of many carcinomas. However, to our knowledge, there has no studies of the predictive factors for lymph node metastasis in papillary carcinomas. Recent study has shown knockdown of MMP-13 by shRNA suppressed the migration and invasion of glioblastomas cells¹⁹. Bauerle et al¹⁰ has found MMP-13 has some effect on regulating thyroid cancer cell growth and invasion. We, therefore, thought that MMP-13 might be a potential therapeutic target for papillary carcinomas.

In the present study, we first investigated the predictive factors of lymph node metastasis in a large series of papillary carcinomas by analyzing clinicopathological variables, including patient age, tumor size, extrathyroidal extension, TNM and recurrent disease, expression of MMP-13. Then we investigated whether overexpression or Silencing of MMP-13 may promotes or inhibits invasion and metastasis of PTC *in vivo* and *in vitro*.

Materials and Methods

Materials

During a 10-year period (1993 through 2002), 208 patients with papillary thyroid cancer (PTC) (primary treatment) were selected at the Department of Surgery of the Affiliated Hospital of Qingdao Medical College, Qingdao University. Papillary thyroid carcinomas were staged according to the TNM classification of 1997²⁰. Of 208 PTC patients, 98 underwent total thyroidectomy (TT) plus bilateral central neck dissection (ND) with/without lateral ND, while 110 underwent TT or lobectomy. Of the 98 node dissection patients, metastatic nodes were present in the central neck of 60 (61.2%) and the lateral neck of 38 (38.8%) patients, most frequently in the ipsilateral and pretracheal central nodes and lateral jugular nodes. Histologically, of the 208 tumours, 200 (96%) were of the usual type, 8 were the diffuse sclerosing variant. No follicular variant or tall cell variant was included in our series. The typical papillary carcinoma is referred to as the usual type. The diagnosis of PTC is based on nuclear

features, which should be present in a significant proportion of the neoplasm²¹. At the time of initial surgery, no patients presented with distant metastases. Tumours recurred in 52 patients (25%), defined as evidence of disease (local or distant) appearing more than 6 months after the primary operation. Death was related to PTC in 13 of the 208 patients (6.25%). The patients' clinical characteristics are shown in Table I. Clinical staging was based on the TNM classification of the International Union Against Cancer²¹.

Cell Lines and Culture

The invasive papillary thyroid carcinoma PTC) cell line BCPAP was purchased from DSMZ (Braunschweig, Germany), and was cultured and maintained in RPMI 1640* (Mediatech, Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS) as previously described²². A human PTC cell line, TPC1, was obtained from Dr Yuri Nikiforov (University of Cincinnati Medical Center, OH, USA). Cells were cultured in DMEM (Dulbecco's modified eagle medium), supplemented with 10% FBS (fetal bovine serum), 1 microg/ml insulin, 100 U/ml penicillin, 100 microg/ml streptomycin, and 0.25 microg/ml fungizone (Invitrogen Life Technologies, Carlsbad, CA, USA) using cell culture conditions as previously reported²³.

Antibodies and Agents

Mouse anti-human MMP-13 (catalogue #MAB13442), was purchased from Chemicon (Chemicon International Inc., Temecula, CA, USA); Mouse anti-human tubulin (catalogue #T 6074) was obtained from Sigma (Milan, Italy). Monoclonal antibodies to β -actin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). MMP-13 specific inhibitor CL-82198 was purchased from Calbiochem (La Jolla, CA, USA), Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). The enhanced chemiluminescence detection kit was from Amersham (Piscataway, NJ, USA).

Immunohistochemical Procedures

The most representative block of tumor tissue was chosen in each case, and 5 μ m sections were obtained and mounted on poly-L-lysine-coated

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Table I. Clinical characteristics of patients with papillary thyroid cancer and MMP-13 expression.

Patient characteristics	MMP-13 expression			p value
	No. of patients	High	Low	
Age				0.547
< 45	126	63 (50%)	63 (50%)	
> 45	82	35 (42.7%)	47 (57.3%)	
Extent of tumour				0.063
T1 (< 10 mm)	31	14 (45.2%)	17 (54.8%)	
T2 (> 10 mm, < 40 mm)	140	65 (46.4%)	75 (53.6%)	
T3 (> 40 mm)	19	9 (47.4%)	10 (52.6%)	
T4 (beyond the capsule)	18	10 (55.5%)	8 (44.5%)	
Lymph node metastases				0.006
Absent	110	25 (22.7%)	85 (77.3%)	
Present	98	73 (74.5%)	25 (25.5%)	
TNM stagey				0.027
I	142	52 (36.6%)	90 (63.4%)	
II	34	20 (58.8%)	14 (41.2%)	
III	32	26 (81.2%)	6 (18.8%)	
IV				
Recurrent disease				0.032
Absent	156	58 (37.2%)	98 (62.8%)	
Present	52	40 (76.9%)	12 (23.1%)	

slides for immunohistochemical staining. A standard streptavidin-biotin immunoperoxidase method was used for immunostaining with MMP-13 [7 ml, ready-to-use, Boste, China] antibodies. The tissue sections were deparaffinized in xylene, rehydrated in an alcohol series, and immersed in distilled water. The sections were then boiled in a citrate buffer solution [10 mmol/L, pH = 6.0] in a microwave oven X3 for 10 minutes for antigen retrieval of the MMP-13 antibodies. Endogenous peroxidase activity was blocked by exposing sections to a 0.3% solution of hydrogen peroxidase in phosphate-buffered saline [PBS] for 10 minutes at room temperature. After the sections had been rinsed with TRIS buffer, primary antibodies were applied for 60 minutes at room temperature followed by TRIS buffer. Linking antibody and streptavidin peroxidase complex were then added consecutively for 10 minutes at room temperature, and sections were washed again in TRIS buffer. After applying AEC (aminoethyl carbazol) chromogen, the sections were washed in deionized water, counterstained and mounted. Breast carcinoma tissue (which showed positive staining) was used as positive control during the evaluation of MMP-13 immunostaining.

Evaluation/scoring of the Staining

One pathologist [Wang JG] evaluated the stained slides associated with each case. The de-

gree of staining for MMP-13 was scored as follows¹²: The intensity of the immunostaining was defined by the negative and positive controls as four categories. No brown particle staining: 0; light brown particle in cytoplasm: 1; moderate brown particle: 2; and 3: dark brown particle. The percentage of positive cells, as the extent of immunostaining, was quantified under microscope and classified into four groups. 1: < 25% positive cells; 2: 25% to 50% positive cells; 3, 51% to 75% positive cells and 4, > 75% positive cells. The staining index (SI), the product of the intensity and the percentage of positive staining, was used to define high (SI ≥ 6) or low (SI < 6) expression of MMP-13. Discordant scores were re-evaluated by the investigators and the consensus scores were used for further analyses.

pcDNA3.1-MMP-13 cDNA Plasmid Construction and Transfection

The entire coding sequence of the human MMP-13 cDNA was amplified by PCR (polymerase chain reaction) in the human breast tumor as previously described (24). After digestion with Hind III and BamH I, the PCR product was subcloned into the pcDNA3.1 expression vector (Sigma, Milan, Italy) at the Hind III and BamH I restriction sites to generate the pcDNA3.1-MMP-13. cDNA plasmid.pcDNA3.1 empty plasmid and

pcDNA3.1-MMP-13 cDNA plasmid was transfected into the cells using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Since preliminary dose response studies showed that 4 µg/well of MMP-13 cDNA produced optimal overexpression of MMP-13, all transfections were done with these concentrations of cDNA. After transient transfection for 24 hours, the cells were washed with PBS, and the medium was replaced with serum-free medium. Stable transfectants were selected by using medium containing G418 (GeneticinTM, Gibco BRL, Life Technologies Inc., Rockville, MD, USA) (400 µg/ml) for 2 weeks. Cells were routinely maintained in selection media containing 200 µg/ml of G418-sulfate to avoid overgrowth of nontransfected cells.

Construction of MMP-13 siRNA Expression Plasmid and Transfection

For MMP-13 silencing, the plasmid pcDNA3.1-MMP-13 siRNA was generated as follows. Synthetic oligonucleotide primers MMP-13 siRNA as followed: MMP-13 siRNA1: AACAGCUGCACUUAUCUUCUUAACU; MMP-13 siRNA2: AUUUCUCGGAGCCUCUCAGUCAUGG; MMP-13 siRNA3: GGUUCCUGAUGUGGGUGAAUACAAU; Target sequence 5'-CGGCAAGCUGACCUGAA GUUCAU-3' was as a non-silencing control. They were annealed and ligated into Hind III and BamH I digested pcDNA3.1. The resulting plasmid expresses siRNAs (pcDNA3.1-MMP-13 siRNA) was constructed. The plasmid vector carrying the MMP-13 siRNA cassettes were transformed into thyroid cancer cells using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA). The plasmid vector carrying the MMP-13 siRNA cassettes were transformed into thyroid cancer cells using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA) for 48 hours. Stable transfectants were selected by using medium containing G418 as described above.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the transfected cells using the Qiagen RNeasy kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. cDNA synthesis from total RNA and subsequent PCR were performed using an RNA LA (long and accurate) PCR kit (Takara Bio Inc., Mountain View, CA, USA). The procedure was performed according to the manufacturer's proto-

col. The primer sets used in this study were sense 5'-CGC CAG AAG AAT CTG TCT TTA AA-3' and antisense 5'-CCA AAT TAT GGA GGA GAT GC-3' for MMP-13, and sense 5'-ACCACAGTC-CATGCCATCAC-3' and antisense 5'-TCCAC-CACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR-amplified products were electrophoretically separated on 2% agarose gel. All samples were analysed in duplicate, and the average value of the duplicates was used for quantification. The variation in the two measurements for each sample was generally in a range of 0.1-10%. If the variation of a sample exceeded 10%, a novel triplicate assay was carried out for this sample. The data were expressed as the ratio of the levels of the target gene mRNA on that of the housekeeping gene GAPDH or MMP-13 mRNA copies/GAPDH copies), which was used as an internal control.

Western Blots

For Western blot analysis, cells were rinsed in ice-cold PBS twice and lysed in cell lysis buffer. The extracts, standardized by total protein, were electrophoretically resolved on 10% SDS-PAGE (polyacrylamide gel electrophoresis) gels, and the proteins transferred to PVDF (polyvinylidene difluoride) membranes. Non-specific binding was blocked with 2.5% dry non-fat milk in tris-buffered saline with 0.1% Tween 20 (TBST) overnight, and membranes were washed twice with TBST and incubated for 2 hours with rabbit anti-human MMP-13 primary antibodies. After further washes with TBST, the membranes were incubated with a 1:1000 dilution of a peroxidase-conjugated goat anti-rabbit antibody (Sigma-Aldrich Corp., St. Louis, MO, USA) in TBST for 1 hour. The membranes were washed again and the bands were visualized by enhanced chemiluminescence.

Migration Assay

BCPAP/MMP-13 siRNA or TPC1/MMP-13 cDNA cells were plated at 10⁵ per well in a six-well plate coated with fibronectin. Once the cells reached 90% confluency, they were serum starved for 12 h. Following serum starvation, 10 µL/mL mitomycin C were added to the medium for 2 h in complete medium and a "wound" was created using a sterile 200 µL pipette tip. Photographs of the wounded area were taken at the time of wounding and thereafter every 24 h for 2 d to determine the rate of wound closure. Percent migration was calculated by measuring the length and width of the cell-free area. The width was measured at five

points along the scratch area and then averaged to get an accurate representation of the entire scratch. Percent migration was determined by using the following formula:

$$[\Delta \text{ area/area (day 0)}] \times 100.$$

Invasion Assay

To determine invasive ability, BCPAP/MMP-13 siRNA or TPC1/MMP-13 cDNA cells were plated on a cell culture insert coated with fibronectin (8 m pore size, 24-well format; Becton Dickinson Labware, Franklin Lakes, NJ, USA) in serum-free medium and a chemoattractant (5% fetal bovine serum) was added to the lower chamber. To determine the amount of invasion, cells were incubated for 24 h and then removed from the upper chamber using a cotton swab. The cells on the underside of the chamber were fixed to the membrane using methanol for 5 min. Once fixed, the cells were stained with crystal violet for 10 min and rinsed with PBS. The chambers were then photographed to compare the amount of invasive cells on the underside of the membrane. Quantitation of the invasive cells was done using a semiquantitative colorimetric analysis of the crystal violet-stained cells. To remove the crystal violet dye from the cells, the membranes were submerged in 0.01% SDS solution for 20 min. Fifty microliters of the solution were then pipetted into a 96-well plate and absorbance measured at 560 nm. Values were then normalized to the unmodified BCPAP or TPC1 cells.

Animals and Spontaneous Metastases

BCPAP/MMP-13 siRNA or TPC1/MMP-13 cDNA cells were washed twice with PBS and de-

tached with trypsin. After serum inactivation, cells were again washed and resuspended in PBS. Female mice 6-8 weeks old, were injected in the tail vein with 5×10^6 cells in 400 μ l. After 18 days, mice were euthanized by standard carbon dioxide asphyxiation. The lungs were removed and rinsed, and lungs were fixed in formalin. Lung sections were stained with hematoxylin and eosin. Micro-metastasis were counted through the whole lung section on slides using a microscope and quantified. All animal studies were approved by the Committee of the Affiliated Hospital of Qingdao Medical College at Qingdao University.

Statistical Analysis

Statistical analyses were performed using the SPSS software package 11.0 (SPSS, Inc. Chicago, IL, USA). The correlations between MMP-13 expression and clinicopathologic variables were analyzed using Pearson Chi-square analysis. Student's *t*-test was used to compare mean values where appropriate. *p* values < 0.05 were considered significant. All data are expressed as means $\pm$ SD for a series of experiments.

Results

Immunohistochemical Analysis of MMP-13 Expression in Papillary Thyroid Cancer Specimens

To examine the expression of MMP-13 at the cellular level, we performed immunohistochemical analyses using histochemical preparations of formalin-fixed paraffin embedded surgical

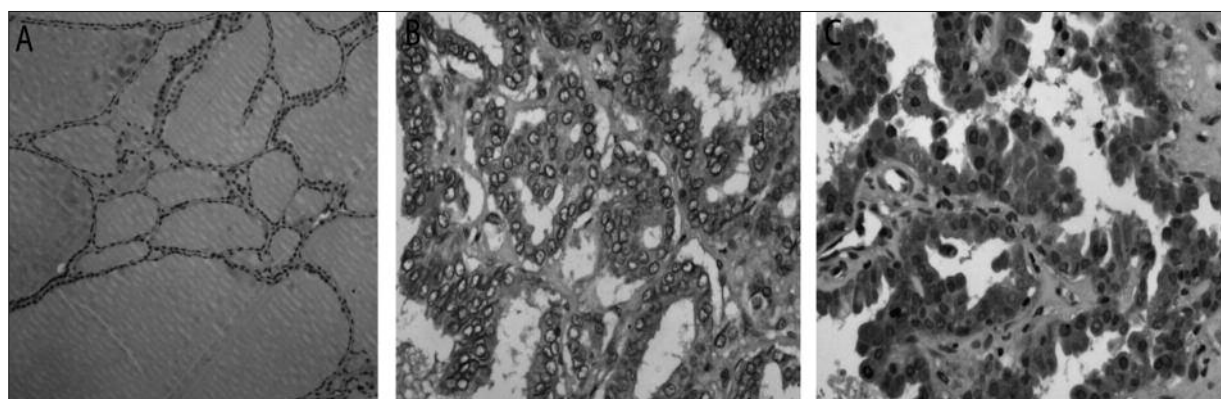


Figure 1. Expression of MMP-13 in normal thyroid, papillary thyroid cancer specimens and its lymph node metastasis. **A**, Negative staining in normal thyroid. **B**, High levels of MMP-13 protein exclusively in the cytoplasm of the metastatic cancer cells. **C**, Low levels of MMP-13 protein exclusively in the cytoplasm of the non-metastatic cancer cells. The brown color represents the IHC staining of MMP-13. The blue color represents the nuclear counterstain. $\times 200$.

specimens. Normal thyroid follicular cells were not stained with the MMP-13 antibody (Figure 1A). Distinct IHC staining of MMP-13 was obtained from all 208 cases. MMP-13 protein expression was detected in cytoplasm of in malignant cells. In these MMP-13-positive PTC samples, stronger staining was often observed at the tumour-invading front (Figure 1B) as compared to the central region. In general, metastatic PTC samples had stronger staining (Figure 1B) when compared with the non metastatic PTC samples (Figure 1C).

Correlation of high MMP-13 Expression with Clinicopathological Parameters

The tumor staining was semi-quantitatively scored by the SI scales and assigned into high and low categories. As shown in Table I, MMP-13 expression was significantly related with local regional lymph node metastasis, TNM and recurrent disease, no relation was found with age, extent of tumour and size of tumour.

Efficient Extinction of MMP-13 Expression in BCPAP Cells by siRNA Strategy

We used a siRNA method to target MMP-13 in the BCPAP cell line, which constitutively expresses high levels of MMP-13 (Figure 1A). The constructs we designed encoded an RNA that targets the MMP-13 mRNA (Figure 1A). In pcDNA3.1-MMP-13 siRNA stably transfected BCPAP cells, MMP-13 mRNA was completely silenced, however, negative pcDNA3.1 was incapable of inhibiting MMP-13 gene expression (Figure 2A). By western blot analysis, MMP-13 protein was also significantly inhibited (Figure 2B).

MMP-13 Overexpression in TPC1 Cells by MMP-13 cDNA Transfection

We overexpressed MMP-13 in the TPC1 cells, which expresses low levels of MMP-13 (Figure 3). Low levels of MMP-13 mRNA was also expressed in TPC1 cells (data not shown). In pcDNA3.1-MMP-13 cDNA stably transfected TPC1 cells, MMP-13 protein was significantly increased, however, negative pcDNA3.1 was incapable of increasing MMP-13 protein expression (Figure 3).

Silencing of MMP-13 Affects the Migratory Potential of BCPAP Cells in vitro

BCPAP is a highly metastatic PTC cell line. In vitro studies were done to determine the effects of MMP-13 silencing on both migration and invasion of these cell clones. Cell migration was first determined using a wound-healing assay in which cells were scratched and allowed to migrate into the wound area. The amount of migration or wound closure was enumerated 72 hours after disruption. Compared with the normal BCPAP cells that showed 49% wound closure by 48 hours, clones expressing the MMP-13 siRNA showed 3.6% wound closure in the same period (Figure 4A). Using a Boyden chamber, coated with fibronectin, we determined changes in cell invasiveness after 24 hours. Cells were fixed and stained with crystal violet to determine the number of cells that invaded across the membrane. Compared with unmodified BCPAP cells, the BCPAP/MMP-13 siRNA clone showed a significant decrease in invasion (Figure 4B). Colorimetric analysis of the crystal violet-stained migratory cells indicated a significant decrease in the number of invasive BCPAP /MMP-13 siRNA cells when compared with parental BCPAP cells.

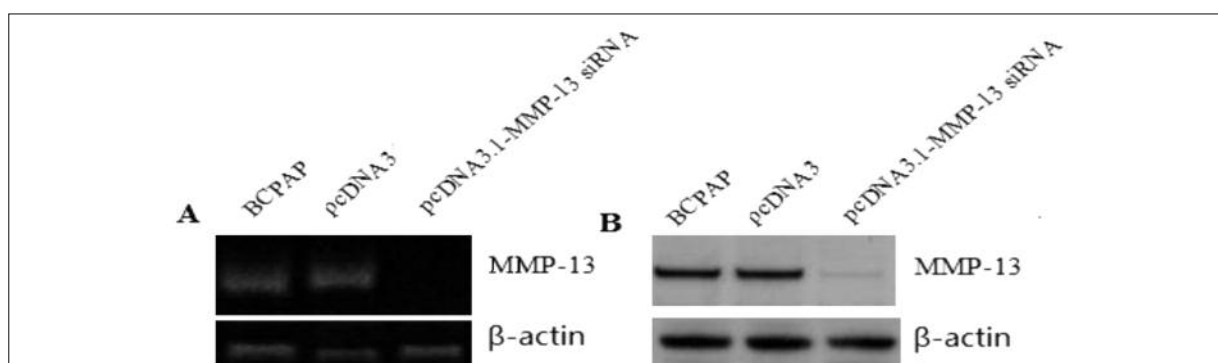


Figure 2. Efficient inhibition of MMP-13 in BCPAP cells using siRNA. **A**, RT-PCR analysis for MMP-13 mRNA in BCPAP, pcDNA3.1 and pcDNA3.1-MMP-13 siRNA cells. MMP-13 mRNA was completely silenced in pcDNA3.1-MMP-13 siRNA cells. **B**, Western blot analysis for MMP-13 protein in BCPAP, pcDNA3.1 and pcDNA3.1-MMP-13 siRNA cells. MMP-13 protein was completely inhibited in pcDNA3.1-MMP-13 siRNA cells.

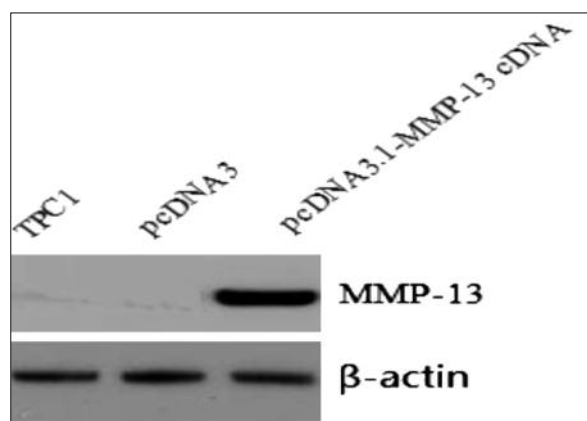


Figure 3. Efficient MMP-13 overexpression in TPC1 cells by MMP-13 cDNA transfection. Western blot analysis for MMP-13 protein in TPC1, pcDNA3.1 and pcDNA3.1-MMP-13 cDNA TPC1 cells. MMP-13 protein was significantly increased in pcDNA3.1-MMP-13 cDNA transfected cells.

We next investigated whether MMP-13 overexpression promoted migration and invasion in TPC1 cells. The results shown MMP-13 overexpression by MMP-13 cDNA transfection in TPC1 cells promote migration and invasion when compared with parental TPC1 cells (data not shown).

MMP-13 siRNA Clones had Diminished Lung Metastasis

Mice with BCPAP and BCPAP/MMP-13 siRNA cells were sacrificed on the same day of tumor injection (18 days), lungs were harvested and lung sections were examined for cancer cell micrometastasis. All mice (6/6) with BCPAP mice had numerous lung micrometastases, while 1/6 mice with MMP-13 siRNA mice had micrometastasis (Figure 5A).

MMP-13 cDNA Clones had Increased Lung Metastasis

Mice with TPC1 and TPC1/MMP-13 cDNA cells were sacrificed on the same day of tumor injection (18 days), lungs were harvested and lung sections were examined for cancer cell micrometastasis. Although all mice (6/6) with TPC1 mice had numerous lung micrometastases, the TPC1/MMP-13 cDNA mice has more lung micrometastases than the TPC1 mice (Figure 5 B).

Discussion

Expression of proteolytic enzymes during the progression of cancer has been consistently associated with poor prognosis in a variety of cancers. Matrix metalloproteinases are believed to mediate tumor invasion through several mechanisms, including activation of other enzymes, processing of matrix components to modulate cell migration, and release of bioactive factors bound to the ECM²⁵. Matrix metalloproteinase 13 (MMP-13), a member of the matrix metalloproteinase family, is considered to play a role in the tumor cell proliferation and invasion. MMP-13 is overexpressed in many carcinomas¹³⁻¹⁸. In addition, overexpression of MMP-13 have been suggested as important predictive factors for relapse or nodal metastasis of many carcinomas. However, to our knowledge, there has no studies of the predictive factors for lymph node metastasis in papillary carcinomas.

In our series of 208 PTC patients, we have shown increased MMP-13 expression in thyroid tumour cells metastasized to regional lymph nodes or distant organs and at the tumour invasion front

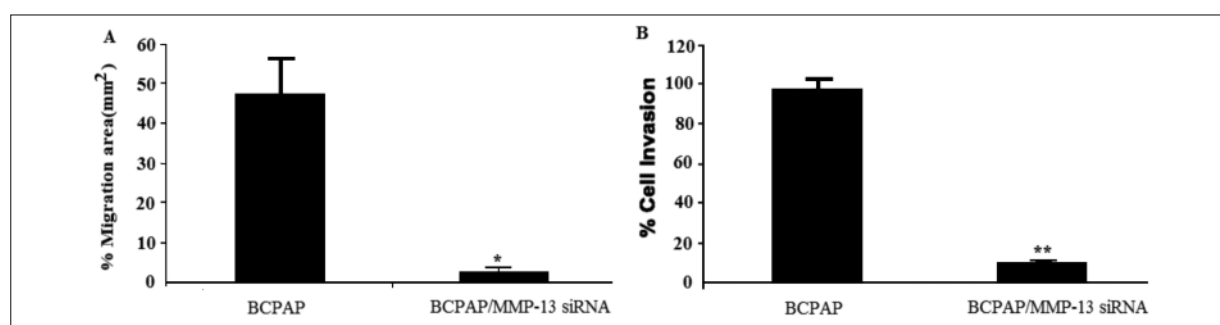


Figure 4. Silencing of MMP-13 affects migration and invasion of BCPAP cells *in vitro*. BCPAP cells and stable clones containing MMP-13 siRNA were analyzed for cell migration using the wound-healing scratch assay. **A**, Cells were “wounded” and monitored every 24 h for 2 d to determine the rate of migration into the scratched area. Invasiveness of cells was determined using a Boyden chamber assay. Cells were plated in the upper chamber of the apparatus and allowed to grow for 24 h in serum-free medium. A chemoattractant (5% fetal bovine serum) was placed in the lower chamber. **B**, cells were fixed to the membrane and stained after 24 h to determine invasion. * $p < 0.0001$; ** $p < 0.001$; all assays were done in triplicate.

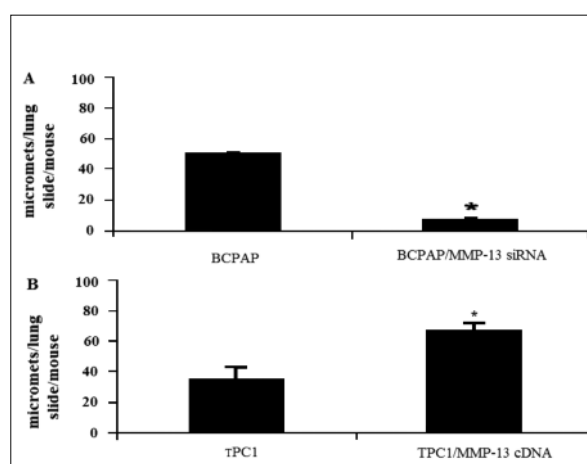


Figure 5. MMP-13 expression regulates lung metastasis. 5×10^6 BCPAP and BCPAP/MMP-13 siRNA cells were injected into tail vein in mice for 18 days. Then the mice were sacrificed and tumor metastases were examined in the lung sections. **A**, Representative images of lung micro-metastases were shown. The bar in each image represents 100 μ m in length. One way ANOVA analysis was performed to compare the number of micro-metastasis in the lung of mice carrying BCPAP and BCPAP/MMP-13 siRNA clones. **B**, 5×10^6 TPC1 and TPC1/MMP-13 cDNA cells were injected into tail vein in mice for 18 days. Then the mice were sacrificed and tumor metastases were examined in the lung sections. Representative images of lung micro-metastases were shown. The bar in each image represents 100 μ m in length. One way ANOVA analysis was performed to compare the number of micro-metastasis in the lung of mice carrying TPC1 and TPC1/MMP-13 cDNA clones. * $p < 0.05$.

in matched primary thyroid tumour and their metastatic specimens. These findings provide further support that MMP-13 is involved in thyroid cancer invasion and metastasis. The prognosis for patients with PTC are based on both histological type and tumour stage. In the present study, we followed patients and recorded whether a higher MMP-13 level predicted recurrence.

We found that the prognosis of patients was significantly associated with MMP-13 expression. In addition, MMP-13 expression was significantly related with TNM, no relation was found with age, extent of tumour and size of tumour. These data suggest that MMP-13 may be able to predict the aggressive behaviour and as a potential target for therapeutic intervention in PTC.

RNA interference is becoming a common application for *in vivo* cancer therapy²⁶⁻²⁷. Although MMP-13 is ubiquitously expressed, the level of expression is much higher in thyroid tumor tissues and their metastatic foci, making it a good candidate for gene silencing. To test whether

MMP-13 may be as a target for TPC therapy, we transfected the MMP-13 siRNA into BCPAP cells to knockdown of MMP-13, only found MMP-13 silencing inhibited invasion *in vitro* in the BCPAP cells. It has previously reported that the systemic administration of an adenovirus expressing the green fluorescent protein has higher efficiency of transgene expression by the tail vein injection²⁸. In the present study, we injected the BCPAP/MMP-13 siRNA clones via tail vein, only found fewer lung metastasis in mice compared to parental untransfected cells. To test the significance of MMP-13 expression in TPC, we transfected the MMP-13 cDNA into TPC1 cells. After transfection, the invasive ability of TPC1 cells increased dramatically. MMP-13-transfected cells implanted into nude mice by tail vein metastasized widely into the lung compared to parental untransfected cells. This corroborated the role of MMP-13 in TPC progression, and indicates that MMP-13 plays an important role in TPC invasion and metastasis. To our knowledge, this is the first demonstration that down-regulation of MMP-13 expression in BCPAP cells prevents invasion *in vitro* and lung metastasis *in vivo*, and *vice versa*. This work adds to a significant body of literature that implicates the MMP-13 in the pathogenesis of lung and other cancers.

Conclusions

MMP-13 was overexpressed in the TPC tissues, and MMP-13 expression was significantly related with local regional lymph node metastasis, TNM and recurrent disease. MMP-13 knockdown on thyroid cancer cell inhibited invasion and lung metastasis, and MMP-13 overexpression on thyroid cancer cell promoted invasion and lung metastasis. The current study provides the proof of concept in its therapeutic potential and warrants further development.

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