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Tumor progression and expression of matrix metalloproteinase-2 (MMP-2) mRNA by human urinary bladder cancer cells

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Abstract Tumor cells at the stage of tumor progression build up a high tolerance to intrinsic and extrinsic defence systems and/or therapeutic procedures, and the cells deeply infiltrate the adjacent tissue, which is followed by tumor metastasis to remote organs and tissues. This study was designed to investigate the relationship between expression of matrix metalloproteinase-2 (MMP-2) and invasiveness of human bladder cancer cells, using cell lines derived from a parental human urinary bladder tumor cell line, T24. Two subpopulations of the human bladder cancer cell line T24, Hi-T24 and Lo-T24, were selected using an invasion assay and then expression of MMP-2 mRNA and protein was analyzed by reverse-transcription polymerase chain reaction (RT-PCR) and enzyme immunoassay (EIA). The gross morphology, cell growth rate, and adhesion activity to a basement membrane extract (matrigel) of the high-invasive Hi-T24 cells were similar to those of the low-invasive Lo-T24 cells, but the Hi-T24 cells were 3.8-fold more haptotactic through matrigel than the Lo-T24 cells. The haptotactic activity of the Hi-T24 cells was suppressed by the addition of an anti-MMP-2 antibody, and the amounts of MMP-2 protein secreted into the spent medium by the Hi-T24 and Lo-T24 cells were 7.8 ± 0.2 and 3.8 ± 0.3 ng/ml ($P < 0.05$), respectively. The quantities of tissue inhibitor of metalloproteinase-2 (TIMP-2) protein secreted by Hi-T24 and Lo-T24 cells were 133.2 ± 4.3 and 168.7 ± 5.6 ng/ml, respectively ($P < 0.05$). The levels of transcription of the genes encoding MMP-2 and the transmembrane MMP, MT-MMP, evaluated by RT-PCR, were higher in the Hi-T24 cells than in the Lo-T24 cells. Expression of the TIMP-2 gene was slightly lower in the Hi-T24 cells than in the Lo-T24 cells. These results indicate that expression of the metalloproteinases are imbalanced at the gene level in human urinary bladder cancer cells at the stage of tumor progression.

Key words MMP-2 · MT-MMP · TIMP-2 · Urinary bladder carcinoma · RT-PCR · EIA

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Introduction

Bladder cancer is considered to be one of the most common urological tumors and 95% of bladder cancers are classified as transitional cell carcinoma. In approximately 20% of transitional carcinomas of the bladder muscular invasion and/or metastasis of the tumor cells is detected.

Tumor progression, according to the multi-stage theory of carcinogenesis, is illustrated simply as a change in the clinical features of a tumor from the ''bad'' to the ''worse'' stage. The tumor cells build up a high tolerance to intrinsic and extrinsic defence systems and/or therapeutic procedures, and the cells infiltrate deep into the adjacent tissues, which is followed by tumor metastasis to remote organs and tissues [8].

Early events that take place during invasion by the tumor cells are the adhesion of the cells to the basement membrane and the proteolytic modification of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs). [15]. The MMPs are a family of matrix-degrading enzymes that require zinc, and include the collagenases, gelatinases, and the stromelysins, all of which have been implicated in invasive cell behavior. Recent studies have shown that matrix metalloproteinase-2 (MMP-2), which is a type-IV collagenase with a molecular mass of 72 kDa, is activated either by a biochemical interaction with a transmembrane MMP, called MT-MMP [14], or by binding with an alphaV-betal integrin cell surface adhesion receptor [3]. A tissue inhibitor of MMP-2 (TIMP-2) has been reported to be a negative modifier or MMP-2 that restrains the invasive activity of tumor cells [10]. Kossakowska et al. [11] have

suggested that the balance between MMPs and TIMPs is altered in neoplasia, contributing to the invasive and metastatic properties of malignant tumors. Tumor progression, therefore can be defined as a process that results in an imbalance in the expression of metalloproteinases and modifier proteins.

In this study we characterized two subpopulations of the human urinary bladder cancer cell line T24: one a highly invasive subpopulation and the other a less invasive subpopulation. We identified differences in the expression of mRNA and proteins of MMPs by these cell lines.

Materials and methods

Cell culture

The human urinary bladder cancer cell line T24 was obtained from the American Type Culture Collection, and the cells were cultured in RPMI-1640 medium (Gibco-BRL-Life Technologies, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Xavier Investments, Wacol, Australia) and antibiotics. Cells were plated in wells or in vessels in triplicate for every measurements and assays.

Selection of high-invasive and low-invasive subpopulations from T24 cells

Parental T24 cells (3×10^5 cells) suspended in RPMI-1640 supplemented with 0.1% FBS were plated on a prefixed membrane filter of the cell culture insert (pore size 8 μm, diameter 6.5 mm; Transwell cell culture chamber; Costar, Cambridge, Mass.). The membrane filter was precoated with 0.5 ml of an undiluted reconstituted basement membrane extract matrigel (Becton Dickinson Labware, USA). RPMI-1640 supplemented with 10% FBS (complete growth medium) was added to the lower compartments of the cell culture chamber. After incubating the cells for 5 h at 37°C in 5% CO_2, the cells that remained on the surface of the matrigel-precoated filters were collected and designated low-invasive T24 cells, Lo-T24. Cells that had infiltrated and passed through the matrigel membrane were collected from the lower compartment after incubating the plates for 10 more days in a CO_2 incubator. The procedure was repeated twice to ensure the establishment of a high-invasive subpopulation, Hi-T24.

Cell growth

The Hi-T24 and Lo-T24 cells were seeded in complete growth medium in 24-well plates (1×10^4 cells/well) and the number of living cells was counted daily after staining with trypan blue.

Attachment assay

Cell suspensions (100 μl) which contained 2×10^4 cells in serum-free RPMI-1640 were inoculated into a 96-well plate (Corning, Cambridge, Mass.) which had been precoated with various concentrations of matrigel. After a 50-min incubation in a CO_2 incubator, the floating cells were removed, and the cells attached to the matrix were fixed with 10% formalin, stained with 0.2% crystal violet and lysed with 1% sodium dodecylsulfate. The absorbancy at 595 nm of the lysate was measured using a microplate reader (Model 450, Bio-Rad, Richmond, Calif.). The number of the cells attached to the matrigel was expressed as the absorbance reading.

In vitro cell invasion assay

Invasiveness of the cells was determined using a BioCoat matrigel invasion chamber (Becton Dickinson Labware). Although this chamber is similar to the Transwell cell culture chamber, the matrigel filter (pore size 8 μm) of the upper insert was ready made and had been precoated with a thin layer of matrigel. Cells (1.25×10^6) in 0.25 ml of RPMI-1640 with 0.1% FBS were inoculated onto the upper insert, and 1 ml of complete growth medium was added to each well in the lower chamber. The invasion chambers were incubated at 37°C for 10 day, and then the cells that were attached to the lower compartment of the chamber were counted manually under a microscope. Anti-human MMP-2 antibody (FYK, Toyama, Japan) (0.5, 5.0 and 50 μg/ml) was directly added to the cells on the upper insert. The antibody concentrations were determined in preliminary experiments that showed less than 75 μg/ml anti-MMP-2 antibody did not reduce cell survival.

Assay of MMP-2 and TIMP-2 in the spent medium

Cells (1×10^4), which had been cultured for 3 days in complete growth medium, were washed gently with phosphate buffered saline solution with serum and were refreshed with serum-free RPMI-1640 medium. After the cells were incubated for an additional 24 h in a CO_2 incubator, the spent medium was collected and the MMP-2 and TIMP-2 concentrations in the spent medium were measured using a one-step sandwich enzyme immunoassay (EIA) kit (FYK, Japan) following the manufacturer's protocol. The absorbance at 492 nm or 450 nm was measured for MMP-2 or TIMP-2, respectively, using a spectrophotometer (Micro-Flow; Shimadzu Kyoto, Japan).

Messenger RNA extraction and reverse transcription (RT)

Polyadenylated mRNA was isolated from cell extracts using a kit (MicroFast track kit; Invitrogen, Carlsbad, Calif.) and following the manufacturer's instructions. mRNA from each sample (100 ng) and Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, Calif.) was used for first strand cDNA synthesis following the manufacturer's protocol.

Polymerase chain reaction (PCR)

cDNA (5 μl) from the pool generated from the RT reaction was used for the subsequent PCR amplification by adding 5 units of Taq polymerase (Taq Plus Long polymerase; stratagene) and 100 pmol each of 5′- and 3′-sequence-specific oligonucleotide primers (able 1) [13, 17]. The mixture was covered with mineral oil

Table 1 Primer sequences used for polymer chain reaction (PCR) of matrix metalloproteinase-2 (MMP-2), transmembrane MMP (MT-MMP), and tissue inhibitor of metalloproteinase-(TIMP-2)

MMP-2 (product size 480 bp) sense: 5′-ACAAAGAGTGGCAGTGCAA-3′ anti-sense: 5′-CACGAGCAAAGGCATCATCC-3′
MT-MMP (product size 550 bp) sense: 5′-CCCTATGCCTACATCCGTGA-3′ anti-sense: 5′-TCCATCCATCACTTGGTTAT-3′
TIMP-2 (product size 590 bp) sense: 5′-CCGAATTCTGCAGCTGCTCCCCGGTGCACCCG-3′ anti-sense: 5′-GGAAGCTTTTATGGTCCCTCGATGTCGAG-3′

(Sigma, St Louis, Mo.), and amplified in a thermocycler (Perkin-Elmer Cetus; Takara, Kyoto, Japan), which was programmed for 40 cycles of incubation at 93°C for 90 s, 58°C for 90 s, and 72°C for 3 min. The PCR products were subjected to agarose gel electrophoresis and were visualized by ethidium bromide staining.

Statistical analysis

The StatView-J 4.5 application (Abacus Concepts, Berkeley, Calif.) running on a Macintosh personal computer was used for the statistical analysis of the data.

Results

Figure 1 shows the gross morphology of the two urinary bladder tumor subpopulations, Hi-T24 cells (Fig. 1a) and Lo-T24 cells (Fig. 1b), grown on plastic tissue culture plates. The cells were similar in gross morphology, and were also similar to the cell shape of the parental T24 cell line (data not shown). In Fig. 2, the growth curves of the Hi-T24 and Lo-T24 cells are shown. It is apparent that the two cell subpopulations grew at similar rates, with a doubling time of 21 h.

The adhesion activities of the Hi-T24 and Lo-T24 cells to various concentrations of matrigel were concentration dependent and are demonstrated in Fig. 3. The difference in the adhesion activities between the two subpopulations of T24 cells was not significant, but the Hi-T24 cells attached to the matrix at a slightly higher rate than the Lo-T24 cells ($P > 0.05$).

The Hi-T24 cells and Lo-T24 cells infiltrated and penetrated the ECM layer at different rates, and Table 2 shows the number of cells that penetrated the ECM layer during the 10-day test period: 214.3 ± 9 cells/well of the Hi-T24 cells passed through the matrigel layer, and 56.3 ± 9.5 cells/well of the Lo-T24 cells passed through the matrigel layer ($P < 0.05$).

When the MMP-2 specific antibody was added to the incubation medium of Hi-T24 cells, the number of Hi-T24 cells that passed through the matrix layer was

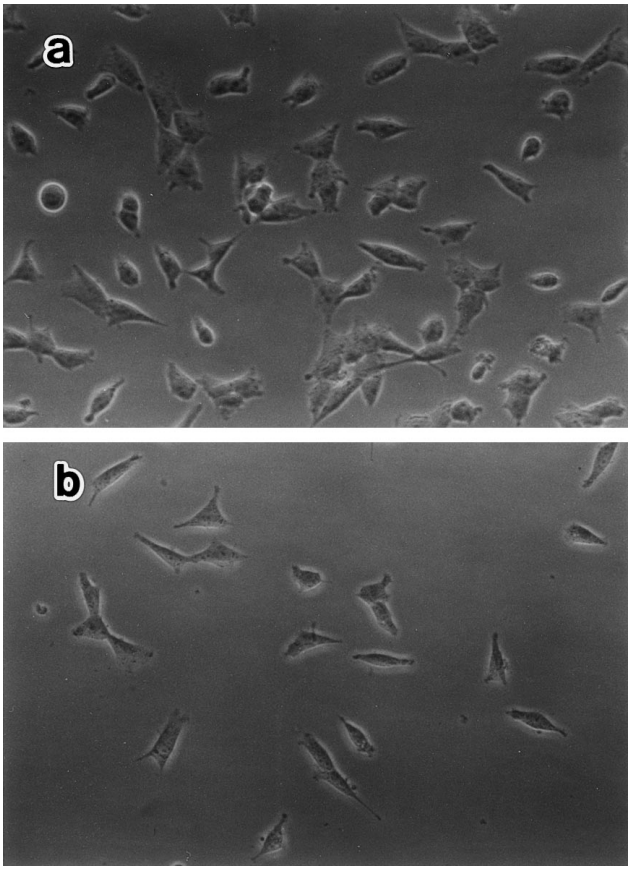

Fig. 1 Gross morphology of urinary bladder carcinoma cells. The Hi-T24 (**a**) and Lo-T24 (**b**) subpopulations of a human urinary bladder carcinoma cell line were grown on plastic culture plates for 10 days

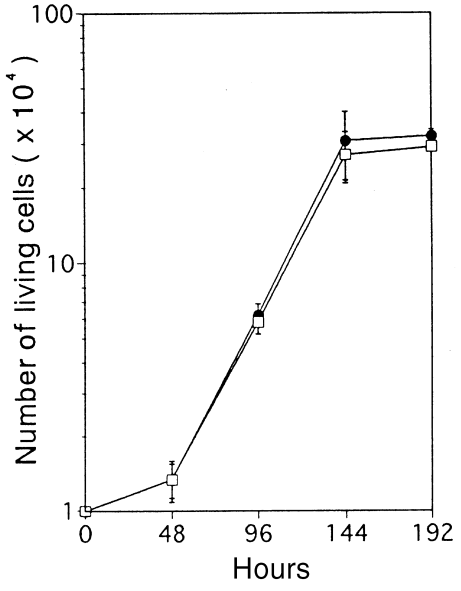

Fig. 2 Growth curves for the Hi-T24 and Lo-T24 cells. *Open squares* Hi-T24 cells, *filled circles* Lo-T24 cells. Bars represent SD

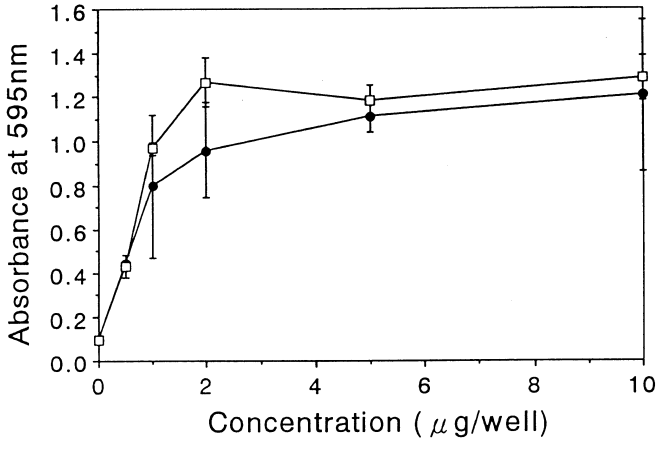

Fig. 3 Adhesion activities of the Hi-T24 and Lo-T24 cells on matrigel. *Open squares* Hi-T24 cells, *filled circles* Lo-T24 cells. Bars represent SD

significantly reduced; 0.5 μg/ml of the anti-MMP-2 antibody added to the medium reduced the number of Hi-T24 cells that passed through the matrix by 15%, compared with the number in the control medium. When 5 μg/ml or 50 μg/ml of the anti-MMP-2 antibody was added to the medium, the number of Hi-T24 cells that passed through the matrix decreased by 44% and 73%, respectively (Table 2).

As the anti-MMP-2 antibody suppressed the infiltrating activity of the cells, the amount of MMP-2 protein secreted by the cells into the medium was measured using an EIA. We also measured TIMP-2 protein. The results are shown in Table 3. The amount of MMP-2 protein secreted by the Hi-T24 cells was significantly higher than that secreted by the Lo-T24 cells ($P < 0.05$), whereas the amount of TIMP-2 protein secreted by the Hi-T24 cells was significantly lower than the amount secreted by the Lo-T24 cells ($P < 0.05$).

To investigate whether the amount of the proteolytic enzyme and the inhibitor protein secreted into the medium reflected the actual gene expression of the cells, the quantity of mRNA of MMP-2, MT-MMP, and TIMP-2 in the cells was measured by RT-PCR. Fig. 4 shows the bands after electrophoresis of the DNA produced by RT-PCR of cellular mRNA. MMP-2 and MT-MMP gene expression of the Hi-T24 cells was apparently higher than that of the Lo-T24 cells (Fig. 4a,b, lanes 1 and 2). TIMP-2 gene expression was slightly greater in the Lo-T24 cells than in the Hi-T24 cells (Fig. 4c, lanes 1 and 2).

Table 2 Infiltrative activities of the two subpopulations of the urinary bladder cancer cell line T24 and effects of anti-MMP-2 antibody

	MMP-2 antibody (μg/ml)	Number of infiltrated cells (cells/well)
Lo-T24 cells	0	56.3 ± 19.5
Hi-T24 cells	0	214.3 ± 9.0
	0.5	169.0 ± 2.0
	5	101.3 ± 6.5
	50	66.3 ± 9.7

Values represent the mean ± SD
Comparison of Lo-T24 cells vs. Hi-T24 cells with no anti-MMP-2 antibody in the medium ($P < 0.05$)

Table 3 Amounts of MMP-2 and TIMP-2 secreted into the spent medium by the Hi-T24 and Lo-T24 cells

	MMP-2 (ng/ml)	TIMP-2 (ng/ml)
Lo-T24 cells	3.8 ± 0.3	168.7 ± 5.6
Hi-T24 cells	7.8 ± 0.2	133.2 ± 4.3

Values represent the mean ± SD
Comparison of Lo-T24 cells vs. Hi-T24 cells ($P < 0.05$ and $P < 0.05$ for MMP-2 and TIMP-2, respectively)

Discussion

A relationship between the proteolytic activity of MMP-2 and the degree of tumor invasion has been reported by several investigators [2]. It has also been suggested that MMP-2 is activated by the transmembrane domain of MT-MMP, and that the expression of MT-MMP correlates with tumor progression [17]. TIMP-2 is another regulatory factor, which inhibits the proteolytic activity of MMP-2 [10]. MMP-9 has been reported to function in a similar way to MMP-2 [12], but we did not detect MMP-9 protein in the two T24 subclones in our preliminary experiments. Although the interaction between MMP proteins has been recognized as one of the critical factors for tumor progression, especially for invasion and metastasis of tumors, the simultaneous expression of MMP genes in cancer cell lines selected from a single cell line has not been reported previously. We focused our investigation on the expression of MMP-2 and its related proteins, TIMP-2 and MT-MMP, because the suppressive effects of anti-MMP-2 antibody on the infiltration of Hi-T24 cells through matrigel suggested that MMP-2 is a key factor in tissue invasion by Hi-T24 cells.

We examined, using RT-PCR, subpopulations of the T24 cell line, which we specifically selected according to their infiltrating activity through matrigel, to compare the expression of MMP-2 between the two subpopulations at the molecular level. The Hi-T24 cells, which were shown to be a high-invasive subpopulation of a urinary bladder carcinoma cell line, expressed higher levels of MMP-2 and MT-MMP mRNA than the low-invasive cells. The quantity of the MMP-2 protein in the spent medium, which was measured using an EIA, was higher in the medium of the Hi-T24 cells than in the medium of the Lo-T24 cells. By contrast, the amount of TIMP-2 protein, and MMP-2 inhibitor, in the spent medium of the Hi-T24 cells was lower than that of the Lo-T24 cells; however, the difference in the amount of secreted inhibitor protein seemed to be insufficient to explain the higher invasiveness of the Hi-T24 cells. These results indicate that the high invasiveness of Hi-T24 cells is mainly due to increased transcription and translation of the MMP-2 gene.

Although MT-MMP and TIMP-2 gene expression was detected in both subpopulations, the expression of the MMP-2 gene in the Lo-T24 cells was very low, whereas expression of the MMP-2 gene in the Hi-T24 cells was significant. MT-MMP gene expression was higher, and TIMP-2 mRNA expression was lower, in the Hi-T24 cells compared with the Lo-T24 cells. These results imply that expression of the MMP and TIMP genes by the tumor cells is regulated independently. It is possible that human urinary bladder carcinomas at the tumor progression stage consist of mixed populations of cells with various patterns of expression of the MMP-2, MT-MMP, and TIMP-2 genes.

The interaction of cancer cells with the ECM has been known to cause distinct changes in cell behavior,

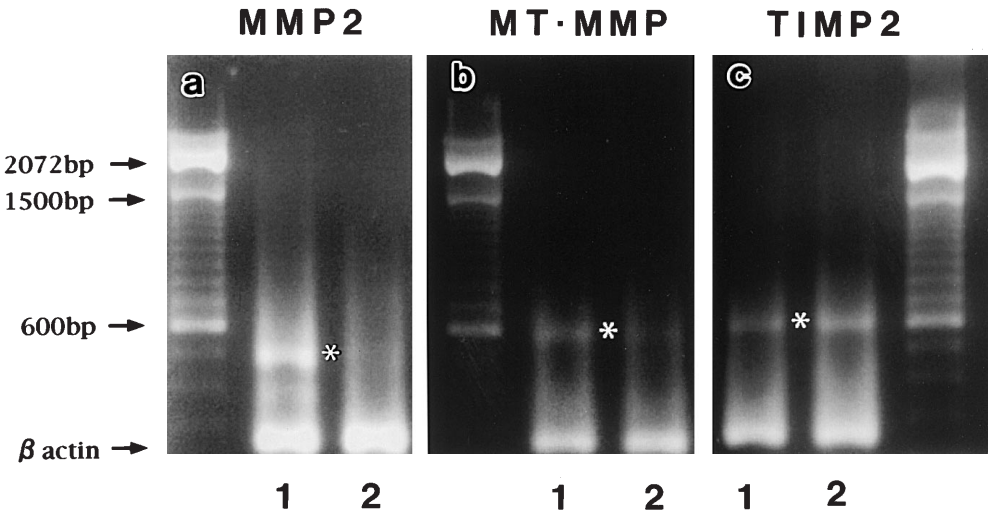

Fig. 4 Electrophoresis of the DNA produced by reverse transcription polymerase chain reaction (RT-PCR) of matrix metalloproteinase-2 (MMP-2), transmembrane metalloproteinase (MT-MMP), and tissue inhibitor of metalloproteinse-2 (TIMP-2) mRNA isolated from the subpopulations of a human urinary bladder cell line. **a** MMP-2, **b** MT-MMP, **c** TIMP-2. Lane 1, Hi-T24 cells, lane 2, Lo-T24 cells. Asterisks indicate the particular bands of DNA

such as reorientation of the cytoskeletal elements, followed by changes in cell morphology, functional differentiation, mode of cell growth, and apoptosis [5, 9, 16]. The adhesion of cancer cells to the ECM has been also reported to stimulate the secretion of MMPs by various kinds of cells [7]. Although the infiltrative activity through the matrigel by the two subpopulations of cells examined in the present report was significantly different, the cells showed similar attachment activities to matrigel. The data shows that, in the case of the human urinary carcinoma cell line, the control mechanism of the expression of the MMP-2 gene was not responsive to signals generated by cell adhesion to matrigel.

Tumor progression is the result of the accumulation of gene mutations [6, 18]. The two subpopulations of cells examined in the present experiment were from a tumor cell line with one genetic trait [4]. Our present data indicate that an imbalance of the control of the three proteins examined is implicated in progression of the urinary bladder cancer cells, and that this imbalance resulted from distinct differences in the expression of the genes. These data suggest that the particular gene mutations that result in an imbalance in the control of the synthesis of matrix-degrading enzymes and their modifiers are involved in the progression of the tumor cells.

One of the major clinical problems in superficial bladder cancer is the lack of biological markers used to estimate accurately the prognosis of the patient [1]. Our present data show that fundamental biochemical differences in the pattern of expression of MMP-2, MT-MMP and TIMP-2 exist between low- and high-invasive bladder cancers, so a rapid assay of gene expression of these proteins is a possible tool for the prognostic diagnosis of bladder cancer. Further investigation of the regulatory mechanism of MMP its inhibitor genes is a prerequisite for developing therapeutic preventive procedures of bladder tumor invasion.

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