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Full Length Research Paper

Construction of retroviral recombinant containing human tissue inhibitor of metalloproteinase-2 (TIMP-2) gene and spontaneous invasion of gastric carcinoma cell lines *in vitro*

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Recombinant retroviral vector containing human tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) gene was constructed and investigation of the *in vitro* invasion and metastasis of gastric cancer cells transfected with TIMP-2 was carried out. Human TIMP-2 was isolated from recombinant vector Bluescript 1/TIMP-2(+), and then inserted into the retroviral vector pL-MT. Correct orientation was verified by restriction endonuclease digestion. Human full length TIMP-2 gene was ligated into a plasmid, which was then transfected into PA317 cell line. G418-resistant individual clones were selected to transfect human SGC-7901 cell line. Cell proliferation, cell electrophoresis, soft agar colony formation and *in vitro* invasion were detected to analyze the bio-behavioral changes of cancer cells. The results from restriction endonuclease digestion were as theoretically expected. The cell electrophoresis rate, colony number and invasion ability in SGC-7901 cells and MFC cells transfected with TIMP-2 gene were significantly decreased when compared with control group. However, no significant changes were noted in the proliferation of cancer cells. We successfully construct a recombinant retroviral vector containing human TIMP-2. TIMP-2 transfection could markedly alter the membrane charge of cancer cells, resulting in decreased electrophoresis capacity, cell migration and invasion. However, cell growth was not affected by TIMP-2. These results suggested TIMP-2 transfection might exert effects on the malignant phenotype of cancer cells through affecting extracellular environment, which provided a new way to investigate gene regulation of *in vitro* collagen metabolism.

Key words: Tissue inhibitor of matrix metalloproteinase-2, gastric cancer cell, recombinant retroviral vector, invasion, metastasis.

INTRODUCTION

The molecular mechanisms underlying the invasion and metastasis of cancer cells have been a hot topic in cancer research (Declereck et al., 1992). Numerous studies have indicated that tissue inhibitor of matrix metalloproteinases (TIMPs) are involved in the regulation of extracellular matrix through affecting the degradation of metalloproteinases (MMPs) and can reverse the malignant phenotype of some types of cancer cells (Koyama, 2005; Shih et al., 2009). However, few studies have been conducted to directly investigate the effects of TIMPs on cancer cell migration. Recently, more attention has been paid to the transfection of TIMPs into cancer cells leading to increased expression of TIMPs, which may regulate the metabolism of extracellular matrix and affect invasion capability of cancer cells. In the present study, a recombinant retroviral vector containing the human TIMP-2 gene was constructed and transfected into human cancer cell line (SGC-7901). *In vitro* construction of human full-length TIMP-2 gene was performed and, through

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Abbreviations: TIMP-2, Tissue inhibitor of metalloproteinase-2; **MMPs,** metalloproteinases; **MFC,** mouse forestomach carcinoma cell line; **OD,** optical density; **LTR,** long terminal repeats.

lipofectin transfection, TIMP-2 was transfected into human gastric cancer cells (SGC-7901) and mouse forestomach carcinoma (MFC) cell line. Cell proliferation, cell electrophoresis, soft agar colony formation assay and *in vitro* invasion assay were performed to evaluate the effects of TIMP-2 transfection on the malignant phenotype of these cells

MATERIALS AND METHODS

Materials

The recombinant retroviral vector containing NeoR gene was provided by Prof. Beifeng Shen, Institute of Basic Medical Sciences, Academy of Military Medical Science, China. Bluescriptl/TIMP-2(+) plasmid containing human full-length TIMP-2 gene (EcoRl/Xbal) was kindly provided by Dr. Stetler-Stevenson, National Cancer Institute, USA. DNA purification kit was purchased from Promega, USA. Competent Escherichia coli strain DH5a and hb101 were prepared using $CaCl_2$. The restriction endonucleases and other reagents were commercial products. Human SGC-7901 cell line was from Institute of Digestive Diseases, Xijing Hospital, the Fourth Military Medical University, China. MFC cell line was provided by Dr. Jin Gao, Chinese Academy of Medical Sciences, China. PA317 cell line and NIH3T3 cell line were provided by Institute of Basic Medical Sciences, Academy of Military Medical Science, China. Boyden Chamber was modified and provided by Dr. Jin Gao, Chinese Academy of Medical Sciences. Polycarbonate membrane with a pore size of 12 μm and a filter size of 13 mm was purchased from Proctiches, USA. Matrigel was provided by Cell Biology Laboratory, Beijing Medical University, China.

Methods

In vitro cell growth curve and doubling time

The cells were seeded in a 74-well plate at a density of 1×10^6/ml and maintained in an incubator. Cell counting was performed every 24 h. Four samples were included in each group and experiment was performed in triplicates. About 2 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole (MTT) was added into each well, followed by incubation for 4 h. Approximately 1 ml of supernatant was removed and 0.1 ml of lysis buffer (20% SDS, 5%DMSDD; pH 7.4) was supplemented followed by incubation overnight at 37℃. The optical density (OD) was detected at 570 nm using a microplate reader (El309, USA). The degree of dilution under which the OD value was half of the maximum OD value was considered as the end point of titration.

Soft-agar colony formation assay

The medium was mixed with 0.7% agarose followed by sterilization. Then, the mixture was cooled to 50℃ and supplemented with Roswell Park Memorial Institute (RPMI) 1640 medium containing 20% fetal calf serum of equal volume. The underlay of plate was added with 2 ml of mixture followed by solidification. Then, 1 ml of 0.3% soft-agar medium containing cells (50, 100, 300 or 500 cells) was added followed by incubation in a 37℃ incubator with 5% CO_2 for 7 days. Experiment was performed in quintuplicates. The number of foci was counted under a microscope followed by averaging.

Cell electrophoresis

After routine staining and counting, the cells were digested with trypsin and then loaded onto Mark II automatic electrophoresis apparatus for electrophoresis. This experiment was performed with the assistant of Beijing Anzhen Hospital.

Cell migration assay

NIH3T3 cells were maintained in serum free medium for 24 h. The supernatant was obtained and placed in the lower chamber. The upper chamber and lower chamber was separated by polycarbonate filters (pore size: 12 μm; filter size: 13 μm), on which Matrigel (artificial basement membrane, type IV collagen, laminin, etc) was put (Tondreau et al., 2009; Takahia et al., 1995). The cells with designed density were placed into the upper chamber. The chamber was incubated at 37℃ and 5% CO_2 for 6 h. The filters were then removed, and the cells on the upper side of the filter were scraped off with a cotton-tipped swab. Cells that migrated to the lower side of the filter were fixed *in situ* in methanol and stained with hematoxylin. Five randomly chosen fields were examined for cell density at 200× magnification with a phase-contrast microscope (Leica). Experiments were performed in duplicate and repeated at least four times.

Statistical analysis

Results were expressed as the means ± standard deviation. Comparison of means was performed using one-way analysis of variance followed by Dumentt test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Synthesis of gene fragment of TIMP-2 signal peptide

Single strand DNAs of 60 or 45 bp were synthesized using an automatic DNA synthesizer. Restriction endonuclease EcoR I/Pst I sites were included at the end of DNA and a complementary region of 15 bp was observed at the center of the single DNAs. After annealing and primer extension with Klenow polymerase, electrophoresis was performed and a signal peptide with 87 bp in length was identified.

Subcloning of TIMP-2 signal peptide into pGEM-4Z vector

The pGEM-4Z vector was digested with cohesive end-producing restriction endonucleases (EcoR I and Pst I) followed by ligation with signal peptides containing restriction endonuclease EcoR I/Pst I sites with T4 DNA ligase. Correct orientation was verified by restriction endo-nuclease digestion.

Subcloning of TIMP-2 into pGEM-4Z vector containing signal peptide

The Bluescript/TIMP-2 plasmid was digested with PSt I, and TIMP-2 cDNA with Bluescript I multiple cloning site and restriction endonuclease Pst I site was obtained. The pGEM-4Z vector containing TIMP-2 signal peptide was

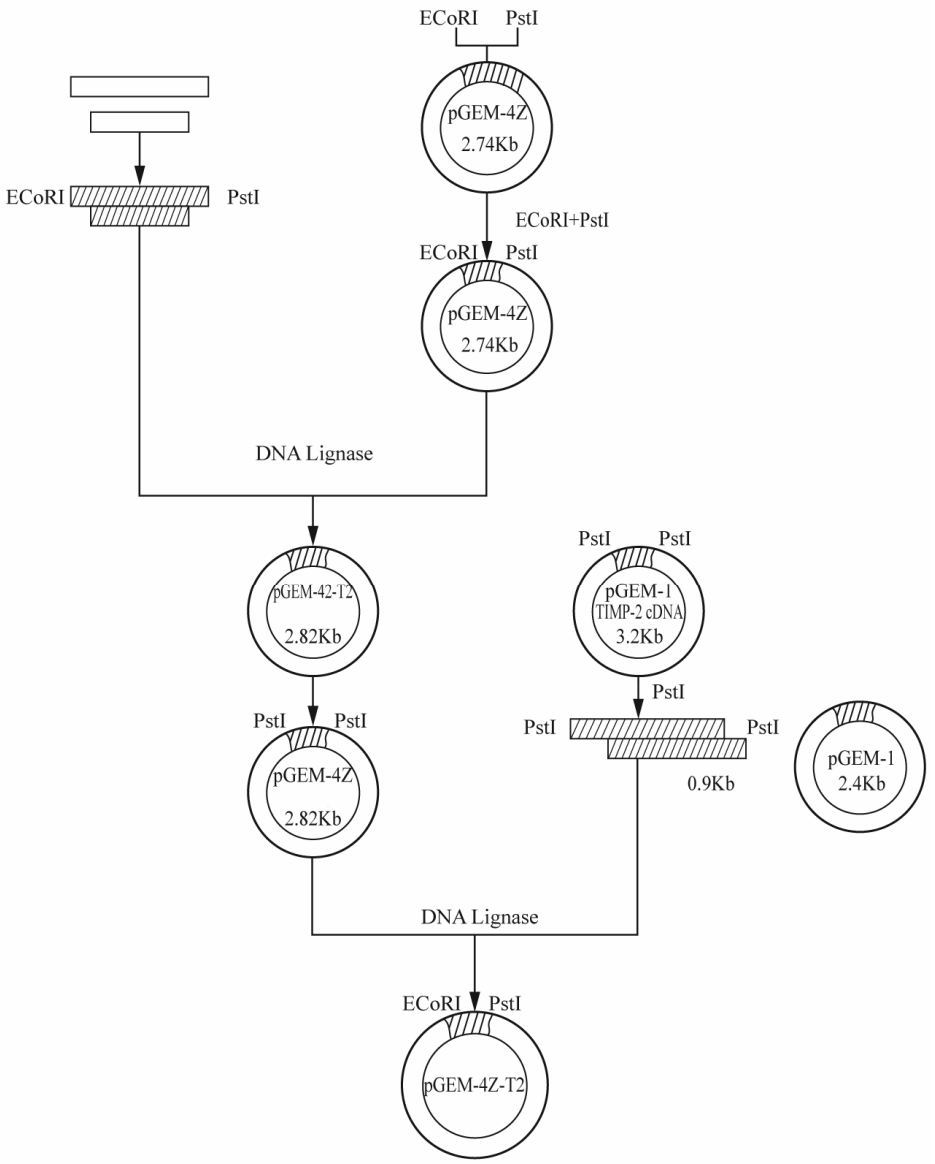

Figure 1. Construction of the plasmid containing human full length TIMP-2.

prepared by digestion with restriction endonuclease Pst I and non-directional ligation was performed between TIMP-2 cDNA and pGEM-4Z vector. Correct orientation was verified by restriction endonuclease digestion. DNA sequencing was performed in pGEM-4Z vector containing human full length TIMP-2 gene to confirm the correct sequences of exogenous fragment and signal peptide (Figure 1).

Subcloning of human full length TIMP-2 and its signal peptide into the pLXSN retroviral vector

After confirmation of correct orientation, plasmid amplification and purification were performed. The MT-1 promoter in pXMT vector was digested with restriction endonuclease Ecor I and BgII followed by recovery. The products were subcloned into endonuclease EcoR I site in pLXSN-TIMP-2 vector. Correct orientation was verified by restriction endonuclease digestion and the TIMP-2 cDNA retroviral vector containing MT-1 promoter was obtained (Figure 2).

Growth curve of cells transfected with TIMP-2 gene

The cell growth of SGC-7901 cells and MFC cells with and without transfection of TIMP-2 was determined. The growth of cells transfected with TIMP-2 was markedly slowed with prolonged time of culture. It could clearly be

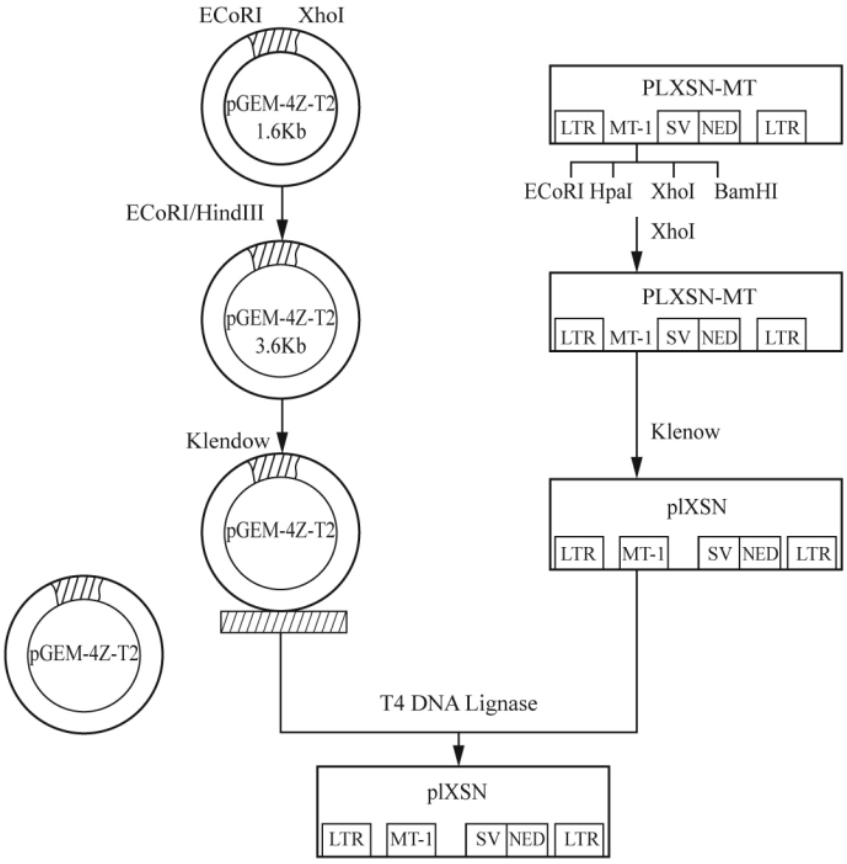

Figure 2. Construction of the TIMP-2 retroviral vector containing MT-1 promoter.

seen that, for both MFC and SGC-7901, the growth of cells with and without TIMP-2 were significantly different. These results implied that the TIMP-2 transfection might affect cancer cell growth (Figure 3).

Cell electrophoresis rate

The cell migration velocity was determined by the amount of surface changes. Therefore, the cell electrophoresis rate could represent the cell migration velocity to a certain extent. The cell line with high invasive potential and high migration had a relatively high electrophoresis rate. The cancer cells transfected with TIMP-2 had a significantly lowered electrophoresis rate compared to those without transfection of TIMP-2. These results suggest that surface charges of cancer cells transfected with TIMP-2 gene were altered. Significant difference in the cell electrophoresis rate was observed between cells with and without TIMP-2 transfection (Table 1).

Soft-agar colony formation

The number of foci in the SGC-7901B was markedly higher than the other two groups. The SGC-7901B clone had a vigorous growth and clustered colonies could be observed after 5 - 7 days of culture. The number and size of colonies in SGC-7901B clone were dramatically larger than those in the other two groups (Table 2).

Cell migration

In vitro reconstituted basement membrane invasion assay was employed to determine if TIMP-2 expression affected cancer cell invasion. As shown in Table 3, after 6 h of incubation, cancer cells with different numbers could be observed on the back of polycarbonate filter. As for MFC, its invasion was fairly strong, so the MFC transfected with TIMP-2 tended to be readily inhibited. On the contrary, the invasion of SGC-7901 was moderate, so the number of invasive cells with and without TIMP-2 was slightly different.

DISCUSSION

In recent years, numerous studies have found that cancer metastasis is closely related with proteases (Wu et al.,

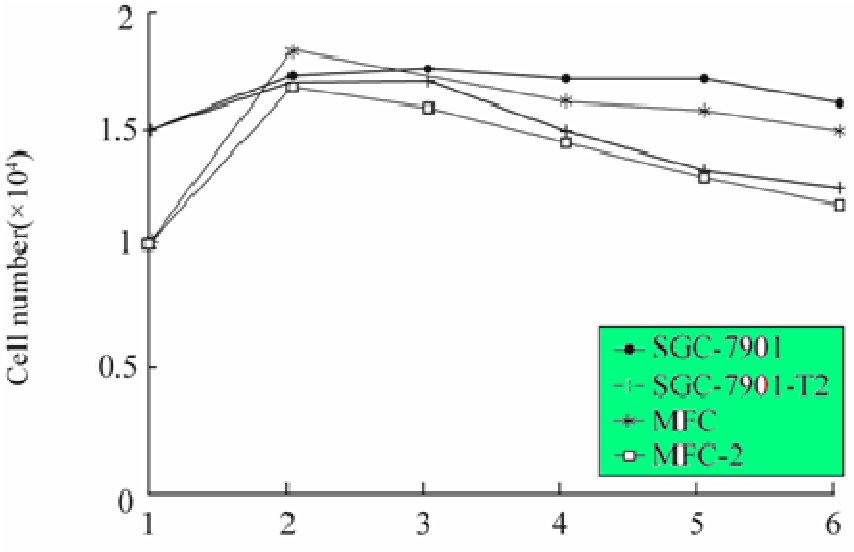

Figure 3. Growth curve of cells with the time of culture (days).

Table 1. Cell electrophoresis rate in difference cell lines.

Group	Number of cells (0.1×10^6)	Cell electrophoresis rate	P value
MFC	1.0	1.87	< 0.01
MFC-T2	1.0	0.81*	-
SGC-7901	1.0	1.33	-
SGC-7901-T2	1.0	1.03**	< 0.05

*P < 0.01, MFC-T2 *vs.* MFC group; **P < 0.05, SGC-7901-T2 *vs.* SGC-7901 group.

Table 2. Number of foci in different cell lines transfected with TIMP-2.

Cell line	Number of cells	Number of foci/ plate					mean	P value
		1	2	3	4	5		
MFC	0.1×10^6	77	79	65	66	56	69.2	-
MFC-T2	0.1×10^6	77	38	29	40	31	35.0*	< 0.05
SGC-7901	0.1×10^6	48	32	43	37	42	41.8	-
SGC-7901-T2	0.1×10^6	15	18	19	18	6	18.8**	< 0.01

*P < 0.05, MFC-T2 *vs.* MFC group **P < 0.01, SGC-7901-T2 *vs.* SGC-7901 group.

2007; Sadatmansoori et al., 2001), in which the MMPs secreted by cancer cells play an important role in cancer metastasis through destroying the integrity of basement membrane (Shiomi and Okada, 2003). Several types of TIMPs have been found in human being. TIMPs can bind some MMPs including collagenase resulting in the inactivation of these MMPs, which protects the integrity of basement membrane. Since the discovery of TIMP-2 in 1989, transfection with TIMP-2 has been performed in several types of cells. Results showed TIMP-2 transfection could, at least partly, suppress the malignant phenotype of some cancers (Grignon et al., 1996; Imai et al., 1996; Tsuchiya et al., 1993). However, no study has been con- ducted to explore the effects of TIMP-2 on cancer metastasis and the mechanisms.

In the present study, the pL-MT-2 retroviral vector was constructed. In this vector, the virus structure was removed and long terminal repeats (LTR) were preserved in which gene transcription was driven by 5'-LTR promoter. In addition, this vector also included the SV40 promoter which controlled the transcription of NeoR gene. In the construction of the viral vector, restriction endonuclease EcoR I site and EcoR I/BgL-1 sites in MT-1 promoter were modified, which were then ligated with human MT-1 promoter. Because MT-1 is sensitive to heavy metal ions, the protein could be express in an

Table 3. Cell invasion of different cell lines.

Group	Time to invasion (h)	Number of cells (1×10^6)	Cell density (cells)
MFC	6	2	73.09 ± 11.66
MFC-T2	6	2	52.80 ± 12.54*
SGC-7901	6	2	33.50 ± 10.67
SGC-7901-T2	6	2	31.47 ± 11.15

*P<0.05, MFC-T2 *vs.* MFC group

environment with heavy metal ions including zinc (Zn) and chromium (Cr) (Unger et al., 1999). The human full length TIMP-2 gene was correctly inserted into restriction endonuclease EcoR I/BamH-I sites in pGEM-4Z vector. Because the multiple cloning site EcoR I in the pL-MT was occupied by MT-1 promoter, the Pst I/BamH I sites in the pGEM-4Z-TIMP-2 vector and XhoL I/BamH I multiple cloning sites in the pL-MT were initially modified followed by ligation. The recombinant vector was verified by restriction endonuclease digestion (Nawrocki-Raby et al., 2003).

As shown in Figure 1, one Hind site and two Hindl sites were found in pL-MT vector. Among these sites, one Hindl site was 300 bp away from the multiple cloning sites. The two Hindl sites and the exogenous fragment with different length were used to verify the insert orientation. MT-1 promoter was inserted into the upstream of TIMP-2 gene and theoretically, the nearest promoter may exert stronger regulatory effects when multiple promoters have been inserted (Miyagi et al., 2007). In the present study, we attempted to make the MT-1 promoter in the pL-MT vector exert regulatory effects on the expression of TIMP-2. In the construction of signal peptide, the long fragment (87 bp) made the synthesis difficult at one time accompanied by non-specific errors. Based on the aptamer theory, two single stranded DNAs were generated with 60 and 45 bp in length, and these two DNAs had a complementary end with 15 bp in length. After annealing and extension with Klenow polymerase, a signal peptide of 87 bp was obtained as expected.

In this study, the correct orientation in the plasmid was verified and the construction of recombinant vector was done with quadruplicate subcloning. The first was to screen the recombinant containing pGEM-4Z-TIMP-2 signal peptide. In the subcloning, restriction endonuclease EcoR I/Pst I sites were used and fragments with about 90 and 270 bp in length were observed which verify the correct orientation. The second was to screen pGEM-4E-T2 recombinant. Since the restriction endonuclease EcoR I/Pst I sites in the pGEM-4E vector were inserted with signal peptide, the TIMP-2 gene was inserted at Pst I site and ligated with signal peptide. Restriction endonuclease digestion disclosed the Pst I site at the 5' end of TIMP-2 gene was correctly ligated to the Pst I (Ali et al., 1994). The third was to screen PL-MT-T2 recombinant. Since the MT-1 promoter must be inserted at the upstream of TIMP-2, the EcoR I site should be occupied by multiple cloning site. The vector underwent non-specific digestion and end screening followed by ligation with TIMP-2 gene. Correct orientation was confirmed by restriction endonuclease digestion. Therefore, a retroviral vector containing MT-1 promoter and TIMP-2 gene was successfully constructed. To investigate the *in vitro* and *in vivo* expression of TIMP-2 gene in retroviral vector, lipofectin transfection was performed to infect PA 317 cells. After screening with G418, NIH 3T3 cells with positive expression of TIMP-2 were selected to obtain the infectionable recombinant. Then, the TIMP-2 was transfected into gastric cancer cells followed by detection of malignant phenotype of cancer cells. The results showed the cancer cells had altered membrane charge after TIMP-2 transfection accompanied by decreased cell electrophoresis rate. In addition, soft-agar colony formation assay indicated reduced number of foci after TIMP-2 transfection. However, the cell growth was not significantly affected. These findings suggested that cell proliferation was not influenced by TIMP-2, and TIMP-2 transfection might alter the membrane charge as well as the interactions between cancer cells and matrix (Xia et al., 2005; Nie et al., 1999).

The cell migration assay confirmed the results above (Chang et al., 2006). Currently, Boyden chamber method is the best way to detect the cancer cell invasion. In this model, Matrigel contains some main components of human basement membrane including type IV collagen and laminin. The migration of cancer cells on the Matrigel involves adhesion, degradation and movement. To penetrate through the basement membrane, morphological changes of cancer cells and secretion of proteinases are necessary (Ebert et al., 2005; Xia et al., 2007). The secreted proteinases can degrade extracellular matrix promoting cell migration. Previous studies indicated that normal cells seldom penetrated the Matrigel containing type IV collagen and other matrix, but increased penetrability was observed in cancer cells with high metastasis (Li et al., 2002; Caudroy et al., 2002). Our results indicated significant difference in the penetrability which was found between the cell lines (GC-7901 and MFC) with and without TIMP-2 transfection. Previous study postulated that soft agar colony formation assay was the only way to detect the biological activity of cancer cells. According to our findings, the proliferation of cancer cells transfected with TIMP-2 was not markedly affected, which might be difficult to be detected by soft agar colony

formation assay. Sometimes, soft agar colony formation assay was also found to have unacceptable repeatability. The difficulties underlying the soft agar colony formation assay are resolved by Boyden Chamber assay which has been found to have high accuracy and acceptable repeatability. Therefore, we recommend Boyden Chamber assay in the detection of metastasis and/or invasion of cancer cells.

In the present study, the retroviral vector containing human full length TIMP-2 gene was successfully generated. Our results showed caner cells transfected with TIMP-2 had altered membrane charge resulting in decreased cell electrophoresis rate, reduced migration and alleviated penetrability. However, cell proliferation was not affected by TIMP-2 transfection. These findings suggested that through affecting extracellular environment, TIMP-2 might exert effects on the malignant phenotype of cancer cells, which provided a new way to investigate gene regulation of *in vitro* collagen metabolism.

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