Suppression of the invasion and migration of cancer cells by *SERPINB* family genes and their derived peptides

RUEY-HWANG CHOU[1-4], HUI-CHIN WEN[1,7], WEI-GUANG LIANG[1,5], SHENG-CHIEH LIN[1,5], HSIAO-WEI YUAN[1], CHENG-WEN WU[1,5,6] and WUN-SHAING WAYNE CHANG[1]

[1]National Institute of Cancer Research, National Health Research Institutes, Miaoli 35053; [2]Center for Molecular Medicine, China Medical University Hospital, Taichung 40402; [3]China Medical University, Taichung 40402; [4]Department of Biotechnology, Asia University, Taichung 41354; [5]College of Life Science, National Tsing Hua University, Hsinchu 30013; [6]Institute of Biochemistry and Molecular Biology, National Yang Ming University, Taipei 11221, Taiwan, R.O.C.

Received June 28, 2011; Accepted August 17, 2011

DOI: 10.3892/or.2011.1497

Abstract. Apart from *SERPINB2* and *SERPINB5*, the roles of the remaining 13 members of the human *SERPINB* family in cancer metastasis are still unknown. In the present study, we demonstrated that most of these genes are differentially expressed in tumor tissues compared to matched normal tissues from lung or breast cancer patients. Overexpression of each *SERPINB* gene effectively suppressed the invasiveness and motility of malignant cancer cells. Among all of the genes, the *SERPINB1*, *SERPINB5* and *SERPINB7* genes were more potent, and the inhibitory effect was further enhanced by co-expression of any two of them. In addition, single treatment of the synthetic peptides corresponding to the P5-P5' sequences of the reactive center loop (RCL) of SERPINB1, SERPINB5 or SERPINB7 markedly suppressed the invasive and migratory properties of the cancer cells in a dose-dependent manner. More significantly, combination treatment of these peptides in cancer cells further improved the suppressive effect by 20-40%. Here, we determined the expression of all *SERPINB* family members in lung and breast cancer patients, and identified those members with potent inhibitory ability toward invasion and migration, and designed RCL-derived peptides to suppress the malignancy of cancer cells. Forced re-expression of these anti-invasive *SERPINB* genes or application of the SERPINB RCL-peptides may provide a reasonable strategy against lethal cancer metastasis.

Correspondence to: Dr W.S.W. Chang, National Institute of Cancer Research, National Health Research Institutes, No. 35, Keyan Road, Zhunan, Miaoli 35053, Taiwan, R.O.C.
E-mail: wayne@nhri.org.tw

Dr R.H. Chou, Center for Molecular Medicine, China Medical University Hospital, No. 6, Hsueh-Shih Road, Taichung 40447, Taiwan, R.O.C.
E-mail: rhchou@gmail.com

Present address: [7]Institute of Cellular and System Medicine, National Health Research Institutes, Taiwan, R.O.C.

Key words: SERPINB family, cancer, migration, invasion

Introduction

Cancer metastasis is the leading cause of morbidity and mortality in cancer patients. It is a highly complex process, including cell detachment, migration, invasion, circulation in blood vessels, adhesion, colonization at other sites and formation of secondary tumors (1). Prior to tumor cell detachment from the primary site, which leads to cell migration and invasion in the metastasis process, the extracellular matrix (ECM) microenvironment must be degraded by proteases, such as urokinase plasminogen activator (uPA), uPA receptor (uPAR) and the plasmin network (2,3) and matrix metalloproteinases (MMPs) (4). On the other hand, protease inhibitors negatively regulate the proteolysis process in cancer metastasis, e.g. plasminogen activator inhibitors (PAIs), PAI-1 (SERPINE1) and PAI-2 (SERPINB2) against uPA/uPAR/plasmin network and the tissue inhibitor of matrix metalloproteinases (TIMPs), TIMP-1 to TIMP-4 against MMPs.

Serine protease inhibitors (serpins) regulate many physiological processes, such as blood coagulation, fibrinolysis, inflammation, complement activation and cell migration (5). Based on their phylogenic relationships, the superfamily is divided into 16 different clades (A-P), in which human serpins are the first 9 clades (A-I) (6). The clade B serpins (*SERPINB* family) is the largest one within the human serpin superfamily. It contains 13 genes located on chromosome 6p25 (*SERPINB1*, *SERPINB6* and *SERPINB9*) and 18q21 (the remaining members of the family). Unlike circulating serpins, the SERPINB family genes lack the N and C terminus extension regions common to other serpins, and lack a secretory signal peptide; thus they primarily reside within cells (7).

Various *SERPINB* genes have been reported to be cancer-related serpins, but few have been shown to be associated with cancer invasion and metastasis. Clinical results reveal that a lower level of *SERPINB2* expression is associated with poor prognosis and outcome in gastric (8,9), breast (10) and lung cancers (11). Overexpression of *SERPINB2* was found

Table I. Primers for RT-PCR of *SERPINB* family genes.

Gene symbol	Forward primers	Reverse primers	PCR products (bp)
SERPINB1	5'-TCAGCTTGCCCAGGTTCAAACTG-3'	5'-GGATGCTACCTGAGGAATTATGC-3'	300
SERPINB2	5'-GCTGGAGATGTTAGCATGTTCTTG-3'	5'-GGCTTGGTGGAACACTTCAGAAAG-3'	300
SERPINB3	5'-GCGGTCTCGTGCTATCTGG-3'	5'-GTAGGTGATGATCCGAATC-3'	109
SERPINB4	5'-TGGAGCCACGGTCTCTCAG-3'	5'-GGAGATGATAATTCGACTA-3'	115
SERPINB5	5'-CATGGAGGCCACGTTCTGTATG-3'	5'-CCTGGCACCTCTATGGAATCCC-3'	417
SERPINB6	5'-CTCCCGCGGTTTAAACTAGAGG-3'	5'-GCAGAAGAGAATCCCGTTGGTC-3'	300
SERPINB7	5'-GGACCAATCCAAGGCGAATGACC-3'	5'-TAAACAGCGTGGACTGAGGGAGTT-3'	285
SERPINB8	5'-AGCTGGAGGAGAGTTATGACTTG-3'	5'-AGAACCTGCCACAGAACAAGATG-3'	300
SERPINB9	5'-CAAGCCAGACTGTATGAAGAGTAC-3'	5'-AAAGGGTGGTCAGCACAGAACCTG-3'	300
SERPINB10	5'-GCAGACATGATGGAGTTGTATGAAG-3'	5'-GAAGAGGAATGGGTGATTTGCATTG-3'	300
SERPINB11	5'-CGTTTCATGAGTGGACAAGCTCTTC-3'	5'CCTTGAACTGAGCTCTCATTGGTAG-3'	300
SERPINB12	5'-AAGGCACAGATCCTGGAAATGAG-3'	5'-ATTGGGACTTGGAGAGATTCCAG-3'	300
SERPINB13	5'-GCTTCTGCCCAACGACATCG-3'	5'-GCAGCCTCGGTGCCTTCCTC-3'	300

to completely inhibit soluble and cell-surface bound plasminogen activator activity, resulting in the suppression of the metastasis of melanoma cells in SCID mice (12). *SERPINB5* (*MASPIN*) has been well studied and was confirmed to be a tumor-suppressor gene, which suppresses tumor growth, invasion and metastasis in different types of cancers, and has potential therapeutic perspectives (13-15). The squamous-cell carcinoma antigens (SCCAs), SCCA1 (SERPINB3) and SCCA2 (SERPINB4), have been used as diagnostic markers for advanced squamous-cell carcinoma. The relative mRNA ratio (higher than 0.2) of SERPINB4 over SERPINB3 has been correlated to primary tumor recurrence in head and neck squamous-cell carcinoma (16). *SERPINB13* was found to be down-regulated in squamous-cell carcinoma of the oral cavity and in squamous-cell carcinoma cell lines of head and neck cancer (17), but up-regulated in skin carcinoma (18,19). Recently, decreased expression of *SERPIN* genes including *SERPINB2*, *SERPINB3*, *SERPINB4*, *SERPINB7*, *SERPINB11*, *SERPINB12* and *SERPINB13* on chromosome 18q21 has been demonstrated in oral squamous-cell carcinomas, while the T/N (tumor vs. matched normal tissues) expression ratio of these *SERPIN* genes was found to have no significant association with clinicodemographic characteristics (20).

Apart from the previously mentioned members of the human *SERPINB* family, however, whether the remaining members, such as *SERPINB2* and *SERPINB5*, are associated with cancer and whether they are functionally important in cancer metastasis, are still unclear. In the present study, we determined the expression patterns of each *SERPINB* gene in matched normal and tumor tissues from lung and breast cancer patients, constructed individual *SERPINB* genes, and screened their effects on the invasive and migratory properties of malignant cancer cells. Based on the results, we also designed the SERPINB RCL-peptides, which effectively suppressed invasion and migration of the cancer cells.

Materials and methods

Cell culture. Human lung adenocarcinoma cell line, CL1-5, and breast carcinoma cell line, MDA-MB-231, were cultured, respectively, in RPMI-1640 and MEM α media supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, Logan, UT, USA) at 37˚C in a humidified 5% CO_2 atmosphere.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using TRIzol reagent, and cDNA was generated by M-MLV reverse transcriptase according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). The PCR was carried out in a thermocycler (Biometra, Göttingen, Germany) and conducted in a 25-μl reaction volume containing 0.2 μM of each primer, 200 μM dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Takara, Kyoto, Japan) and 1 μl cDNA under the following conditions: 1 cycle at 94˚C for 2 min; 30 cycles at 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec; and 1 cycle at 72˚C for 10 min. The primers are listed in Table I. For determining the expression patterns of each *SERPINB* gene in normal and tumor tissues, the human lung matched cDNA pair panel and the human breast matched pair total RNA panel (BD Clontech, Palo Alto, CA, USA) were used.

Cloning of the full-length SERPINB genes and stable transfection. The full-length *SERPINB* genes were amplified by PCR from human keratinocyte Matchmaker cDNA Library (BD Clontech), apart from *SERPINB4*, *SERPINB9*, *SERPINB11* and *SERPINB12*, which were amplified from the MGC: 27150 cDNA clone (Invitrogen Life Technologies), HEK-293 cell line cDNA, normal lung cDNA (Ambion, Austin, TX, USA), and normal brain cDNA (BD Clontech), respectively. The PCR program was as followings: 1 cycle at 94˚C for 2 min; 35 cycles at 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 2 min and 1 cycle at 72˚C for 10 min. The cloning sites and primer sequences used in the PCR of each gene are listed in Table II. Subsequently, each amplified *SERPINB* gene was cloned into the modified pEGFP-N1 vector (BD Clontech) with replacement of the *Eco*RV cloning site at nt673-nt678, and the sequence of each construct was verified by DNA auto-sequencing (DNA Sequencing Core Laboratory, National Health Research Institutes, Miaoli, Taiwan). Each construct was transfected into the desired cell lines with Lipofectamine 2000 (Invitrogen

Table II. Primers for the cloning of *SERPINB* family genes.

Gene symbol	Cloning sites[a]	Forward primers[b]	Reverse primers[b]	ORF[c] (bp)
SERPINB1	EB	5'-AGAATTCCACC**ATGGAGCAGCTGAG**-3'	5'-AGGGATCCAC**AGGGGAAGAAAATCT**-3'	1137
SERPINB2	EB	5'-CGCGAATTCCACC**ATGGAGGATCTTTGT**-3'	5'-TCAGGATCCAC**GGGTGAGGAAAATCT**-3'	1245
SERPINB3	XB	5'-CTCGAGCCACC**ATGAATTCACTCAGTGAAGC**-3'	5'-GGATCCTT**CGGGGATGAGAATCTGCCAT**-3'	1170
SERPINB4	XB	5'-CTCGAGCCACC**ATGAATTCACTCAGTGAAGC**-3'	5'-GGATCCTT**TGGGGATGAGAATCTGCCAT**-3'	1170
SERPINB5	EE	5'-GAATTCCACC**ATGGATGCCCTGCAACTAGC**-3'	5'-GATATCCAC**AGGAGAACAGAATTTGCCAA**-3'	1125
SERPINB6	EE	5'-GAATTCCACC**ATGGATGTTCTCGCAGAAGC**-3'	5'-GATATCCAC**CGGAGAGGAAAAGCGGCCGC**-3'	1128
SERPINB7	EB	5'-TTTGAATTCCACC**ATGGCCTCCCTTGCT**-3'	5'-GCAGGATCCAC**AGGGCAAGAAACTTT**-3'	1140
SERPINB8	XB	5'-CTCGAGCCACC**ATGGATGACCTCTGTGAAGC**-3'	5'-GGATCCTT**CGGAGAAGAGAACCTGCCAC**-3'	1122
SERPINB9	EB	5'-CGAATTCCGCC**ATGGAAACTCTTTC**-3'	5'-ATGGATCCTT**TGGCGATGAGAACCT**-3'	1128
SERPINB10	XB	5'-CTCGAGCCACC**ATGGACTCTCTAGCAACATC**-3'	5'-GGATCCTT**GGGGGAGCATAATCTTCCAT**-3'	1191
SERPINB11	XB	5'-CTCGAGCCACC**ATGGGTTCTCTCAGCACAGC**-3'	5'-GGATCCTT**GGGAGAGGCAAGCTTGCCAC**-3'	1176
SERPINB12	XB	5'-CTCGAGCCACC**ATGGACTCTCTTGTTACAGC**-3'	5'-GGATCCTT**AGGAGAGCAGACCCTGCCAT**-3'	1215
SERPINB13	EB	5'-CGAATTCCGCC**ATGGATTCACTTGG**-3'	5'-GACCGGTGGATCCCG**TGAAGGAGAAGAAAATC**-3'	1173

[a]EB, *Eco*RI and *Bam*HI; EE, *Eco*RI and *Eco*RV; XB, *Xho*I and *Bam*HI. [b]The restriction enzyme sites are underlined, and the gene-specific regions are in bold. [c]ORF (open reading frame) does not include stop codon here.

Life Technologies) and, after 24 h of transfection, cells were sub-cultured at a 1:10 dilution in growth medium. Culture medium containing 0.5 mg/ml G418 was then used to select stable transfectants.

Cell viability. Cells were seeded onto a 96-well plate at a density of 2000 cells/well and cultured overnight. Four hours before harvest, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) solution was added to a final concentration of 0.5 mg/ml with incubation at 37˚C. At the end of the incubation, the cultured medium was replaced with 100 μl dimethyl sulfoxide (DMSO) (J.T. Baker, Phillipsburg, NJ, USA), and the plate was shaken at room temperature for 10 min followed by measurement of the absorbance at 550 nm by a microplate reader, Molecular Devices Spectra Max 250 (GMI, Inc., Ramsey, MN, USA).

In vitro invasion/migration assay. The *in vitro* invasion assay was performed using a modified Boyden chamber as described previously (21). The migration assay was similar to that of the invasion assay, except the inserted polycarbonate membrane was not coated with any extracellular matrix proteins and that test cells were seeded at a density of $2.5x10^4$ cells/well instead.

In vitro wound healing assay. Approximate $2x10^5$ cells were placed onto a 6-well culture dish and grown to confluence. Confluent cells were wounded with a P-200 pipette tip (0 h), washed several times with phosphate-buffered saline to remove floating cells, and cultured in medium containing 1% FBS and 0.5 μg/ml mitomycin C (Sigma) to limit proliferation for 24 h. Scraped cells were photographed at 0, 12 and 24 h.

Peptide synthesis. A variety of peptides corresponding to the P5-P5' sequences of the reactive center loop (RCL) of SERPINB1, SERPINB5 or SERPINB7 were synthesized by the Institute of Biological Chemistry, Academia Sinica (Taipei, Taiwan). The purity and the composition were confirmed by high performance liquid chromatography and mass spectroscopy. The synthetic SERPINB RCL-peptides were dissolved in DMSO.

Results

SERPINB family genes are differentially expressed in the tumor tissues compared to that in the matched normal tissues. In order to evaluate the significance of the expression of all *SERPINB* family genes in tumorigenesis, we determined the expression of each *SERPINB* gene in 5 matched normal and tumor tissue pairs from lung and breast cancer patients. As summarized in Table III, while *SERPINB1*, *SERPINB6*, *SERPINB8* and *SERPINB9* were shown to be down-regulated, *SERPINB3*, *SERPINB5*, *SERPINB7*, *SERPINB10* and *SERPINB13* were up-regulated in lung cancer patients. Notably, 7 out of 13 *SERPINB* genes (*SERPINB2*, *SERPINB3*, *SERPINB5*, *SERPINB6*, *SERPINB10*, *SERPINB12* and *SERPINB13*) were down-regulated while *SERPINB7* was up-regulated in breast cancer patients. The gene expression patterns of *SERPINB3*, *SERPINB5*, *SERPINB10* and *SERPINB13* were rather different between lung and breast cancer patients. Similarly, the differential expression of several *SERPIN* genes has been observed, e.g., *SERPINB5* is up-regulated in lung cancer (22) but down-regulated in breast cancer (23); *SERPINB13* is up-regulated in skin carcinoma (18,19) but down-regulated in oral squamous cell carcinoma (17).

The invasiveness and mobility of malignant cancer cells are inhibited by members of the SERPINB family. Apart from *SERPINB2* and *SERPINB5*, whether the remaining *SERPINB* family genes are also involved in cancer invasion and migration is still unclear. To address this, we cloned individual *SERPINB* genes, and screened the effects on these properties of malignant cancer cells. The results revealed that different *SERPINB* genes inhibited the invasiveness by 30-60% and the migratory

Table III. Expression patterns of *SERPINB* family genes in human lung and breast tumor tissues relative to matched normal tissues.

	Down-regulated	Up-regulated	Undistinguishable[a]	Undetectable[b]	Expression patterns
In lung cancer (n=5)					
SERPINB1	4	1	0	0	↓
SERPINB2	2	3	0	0	
SERPINB3	1	4	0	0	↑
SERPINB4	3	2	0	0	
SERPINB5	0	4	1	0	↑
SERPINB6	5	0	0	0	↓
SERPINB7	0	4	0	1	↑
SERPINB8	4	0	1	0	↓
SERPINB9	4	0	1	0	↓
SERPINB10	1	4	0	0	↑
SERPINB11	1	1	0	3	
SERPINB12	1	2	0	2	
SERPINB13	1	4	0	0	↑
In breast cancer (n=5)					
SERPINB1	2	1	2	0	
SERPINB2	3	1	1	0	↓
SERPINB3	3	1	0	1	↓
SERPINB4	3	2	0	0	
SERPINB5	3	1	1	0	↓
SERPINB6	3	0	2	0	↓
SERPINB7	0	4	1	0	↑
SERPINB8	3	2	0	0	
SERPINB9	3	2	0	0	
SERPINB10	3	0	0	2	↓
SERPINB11	0	0	0	5	
SERPINB12	4	1	0	0	↓
SERPINB13	3	0	1	1	↓

[a]The gene expression levels were rather consistent between the tumor and matched normal tissues. [b]The gene transcript was not detectable in any of the tumor or normal tissues in 3 individual experiments.

ability by 20-50% in the CL1-5 lung adenocarcinoma cells; in particular, *SERPINB1*, *SERPINB5* and *SERPINB7* were more potent (Fig. 1A). The anti-migratory ability of these genes was further validated by a wound healing assay. Overexpression of these *SERPINB* genes significantly reduced the migratory distance of CL1-5 cells in comparison with that of the control cells (Fig. 1B). Similar inhibitory effects of the *SERPINB* genes on the invasiveness and motility were observed in MDA-MB231 breast cancer cells as well (Fig. 1C). These results indicate that *SERPINB* family genes are invasion/migration-suppressing serpins to different degrees.

Co-transfection of SERPINB genes facilitates the inhibitory effect on the invasive and migratory properties of cancer cells. In order to further decrease the invasive and migratory abilities of cancer cells, any two of the more effective invasion-suppressing genes, *SERPINB1*, *SERPINB5* and *SERPINB7*, were stably transfected into CL1-5 cells. To confirm the expression of each recombinant *SERPINB* gene, RT-PCR with the forward primer of the *SERPINB* gene (Table I) and the reverse primer of the EGFP gene (5'-CCGTCCAGCTCGACCAGGAT-3') was performed to amplify the fragment of each ectogenic gene. As shown in Fig. 2, the indicated *SERPINB* genes were indeed expressed in the stable transfectants (top panel). Co-transfection of any two of the indicated *SERPINB* genes further decreased the invasiveness (middle panel) and motility (bottom panel) of the CL1-5 cells by 10-20% compared to the individual transfection of single *SERPINB* gene. The results indicate that forced overexpression of more than one *SERPINB* gene synergistically suppresses the invasiveness and motility of malignant cancer cells.

Peptides referring to the RCL sequences of SERPINB suppress invasion and migration of cancer cells. Serpins are known to

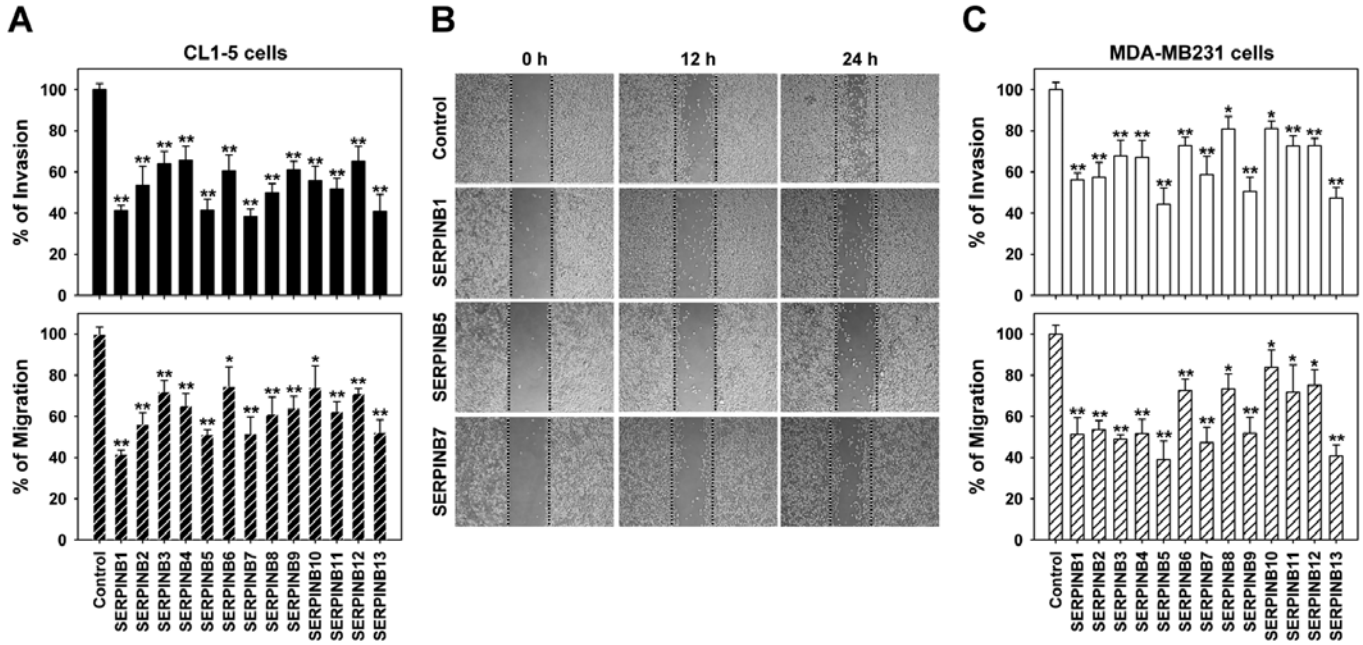

Figure 1. *SERPINB* family genes inhibit the invasiveness and motility of cancer cells. (A and B) CL1-5 and (C) MDA-MB231 cells were stably transfected with each *SERPINB* gene. Each stable transfectant was applied to the *in vitro* invasion or migration assay for 24 h. The relative invasive (upper panel) or migratory ability (lower panel) was normalized with that of the control transfectant as shown in A and C. (B) Images of the wound healing assay for the CL1-5 stable transfectants at the indicated time intervals. (Error bars, ±SE; $^{*}p<0.05$ and $^{**}p<0.01$ relative to the control by the t-test).

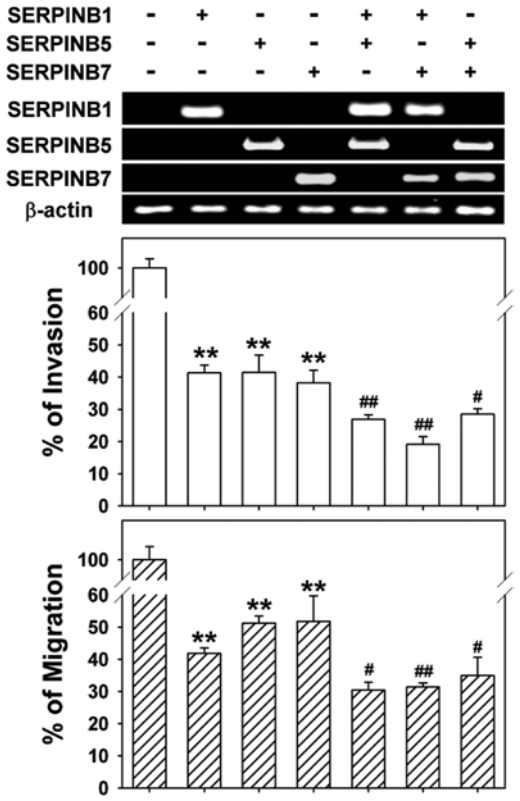

Figure 2. Co-transfection of the *SERPINB* genes strengthens the inhibitory effect on cancer cell invasion and migration. CL1-5 cells were stably transfected with *SERPINB1*, *SERPINB5* or *SERPINB7*, or with any two. The expression of each recombinant *SERPINB* gene was examined by RT-PCR (top panel). Subsequently, each transfectant was applied to the *in vitro* invasion (middle panel) or migration (bottom panel) assay for 24 h. (Error bars, ±SE; statistic analysis, t-test; $^{*}p<0.05$ and $^{**}p<0.01$ relative to the control; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ relative to the single transfection).

bind to their target proteases via a distinct 20-residue RCL (5). The alignment results of the RCL sequence of all SERPINB members showed that the P15-P7 region is conserved; however, the P6-P5' region is more diverse (Fig. 3A). Thus, for better specificity, the peptides corresponding to the diverse P5-P5' regions of RCL of SERPINB1, SERPINB5 or SERPINB7 were synthesized. To demonstrate whether the synthetic peptides could be absorbed, the FITC-labeled RCL-peptides were used to treat the cells. It has been reported that the macromolecular uptake is a spontaneous event during mitosis, and trypsinization of adherent cells could mimic cell retraction and macromolecular uptake during mitosis (24). Thus, the CL1-5 cells were trypsinized to mimic the rounding up of cells during mitosis, and then were treated with the FITC-labeled RCL-peptides at 37˚C for 1 h and observed under a fluorescence microscope. As expected, the FITC-labeled RCL-peptide was spontaneously absorbed into the cells (upper panel, Fig. 3B). These RCL-peptides were further used to examine their inhibitory activity toward invasion and migration of cancer cells. As shown in Fig. 3C, the SERPINB1-, SERPINB5- or SERPINB7- RCL-peptides significantly decreased the invasiveness (top panel) and motility (middle panel) of the CL1-5 cells in a dose-dependent manner, while the synthetic peptides themselves exhibited no toxicity (bottom panel). Moreover, combination treatment of non-toxic SERPINB RCL-peptides further decreased the invasive and migratory abilities of the CL1-5 cells by 20-40% in comparison with single treatment (Fig. 3D). Here, we provided potential peptides with anti-invasive and anti-migratory abilities against malignant cancer cells, based on the results from the functional screening of all the members of the *SERPINB* family in regards to the invasion and migration of cancer cells.

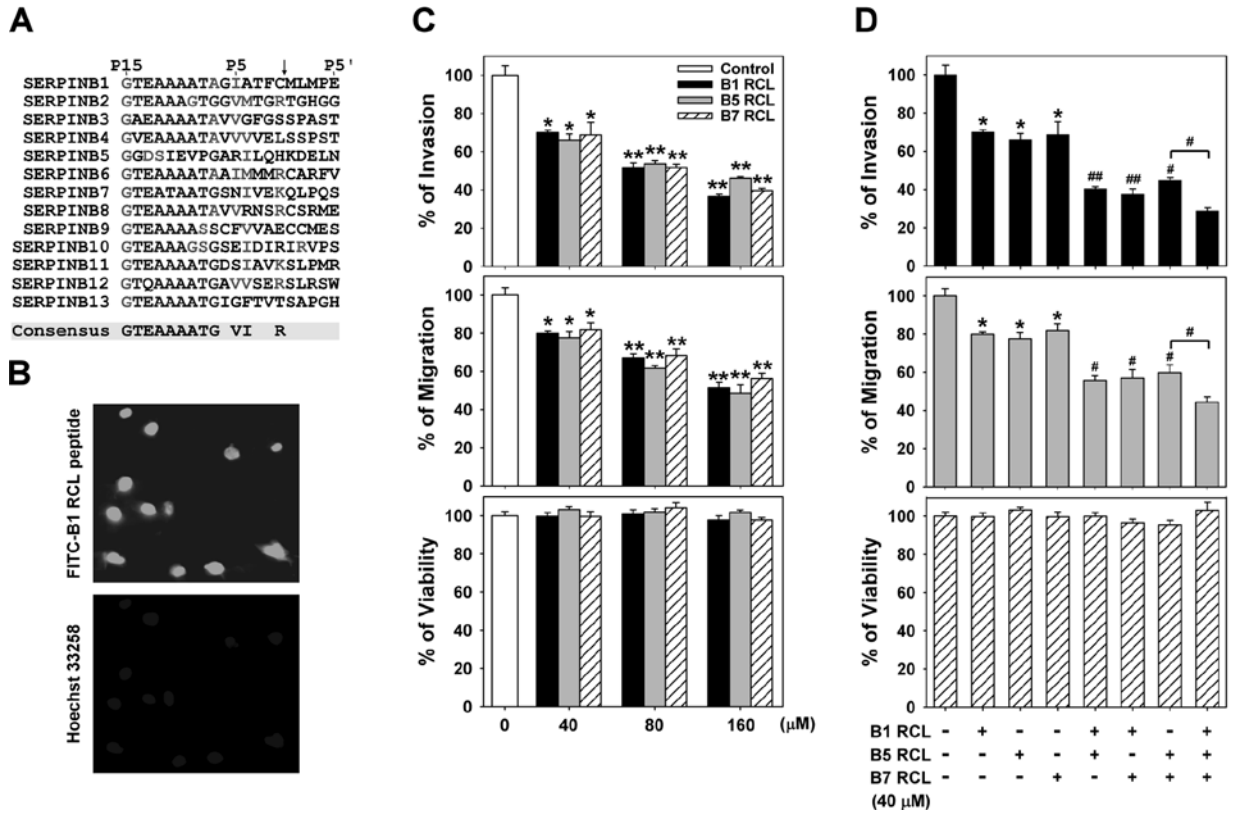

Figure 3. SERPINB RCL-peptides suppress the invasive and migratory properties of cancer cells. (A) The amino acid sequences of the RCL regions of all 13 SERPINB proteins were aligned using the Vector NTI program (Invitrogen Life Technologies). (B) CL1-5 cells were treated with 0.25% trypsin, and then FITC-labeled SERPINB1 RCL-peptides (upper panel) and incubated at 37˚C for 1 h. After fixation, cell nuclei were counterstained with Hoechst 33258 (lower panel) and observed using fluorescence microscopy. (C) CL1-5 cells were treated with or without the SERPINB1, SERPINB5 or SERPINB7 RCL-peptide for 24 h and the numbers of invading (top panel) or migrating (middle panel) cells were counted. The viability of CL1-5 cells after treatment is shown in the bottom panel. (D) The effects of the combination treatment of indicated SERPINB RCL-peptides on invasion, migration and viability of CL1-5 cells. (Error bars, ±SE; statistic analysis, t-test; $^{*}p<0.05$ and $^{**}p<0.01$ relative to the control; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ relative to the single treatment).

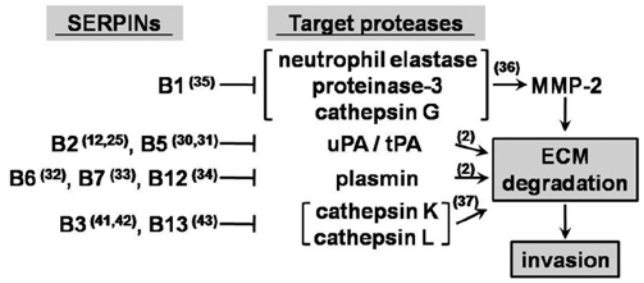

Figure 4. Possible roles of the *SERPINB* genes in cancer cell invasion are illustrated. The scheme summarizes the proposed roles of the indicated *SERPINB* genes in the suppression of cancer cell invasion according to previous reports (superscripted numbers in parentheses). uPA, urokinase plasminogen activator; tPA, tissue-type plasminogen activator; MMP-2, matrix metalloproteinase-2; ECM, extracellular matrix.

Discussion

Cancer cells must degrade the ECM barrier to initiate the process of metastasis. The serine proteases, such as uPA and tissue-type plasminogen activator (tPA), are crucial in this step (2). Here, we provide initial evidence showing that all *SERPINB* genes are invasion- and migration-suppression serpins to a different extent by systematical functional screening (Fig. 1). It has been demonstrated that recombinant SERPINB2 (PAI-2) irreversibly inhibits both cell-surface and secreted uPA and subsequently reduces ECM degradation (25). Conjugation of SERPINB2 with a radioactive bismuth-213 (213Bi) was found to effectively kill different types of cancers, such as prostate (26,27), pancreatic (28) and ovarian (29) cancers modeled in cell lines or preclinical xenograft animal. Recombinant SERPINB5 (rMASPIN) inhibits the activity of single chain tPA *in vitro* (30) and endogenous SERPINB5 inhibits the activity of pericellular uPA (31). Plasmin, another member of the plasminogen activation system, has been reported to be suppressed by SERPINB6 (32), SERPINB7 (33) and SERPINB12 (34). In the present study, overexpression of *SERPINB2*, *SERPINB5*, *SERPINB6*, *SERPINB7* or *SERPINB12* markedly suppressed the malignancy of both CL1-5 and MDA-MB231 cells. This may have been, at least in part, due to the inhibition of the plasminogen activation system.

SERPINB1 has been reported to inhibit the serine protease activities of neutrophil elastase, proteinase-3 and cathepsin G (35), which may activate matrix metalloproteinase, MMP-2, to increase tumor invasion (36). Thus, it is likely that overexpression of *SERPINB1* may have disrupted this neutrophil elastase/proteinase-3/cathepsin G-mediated activation of MMP-2 to suppress the invasion of cancer cells in the present

study. Moreover, cathepsin K and L possess strong proteolytic activity against the ECM (37). The inhibitors of cathepsin K (38) and L (39) effectively prevent tumor bone metastasis. Expression of a single-chain antibody against cathepsin L was found to inhibit the tumorigenicity and metastasis of human melanoma cells (40). A kinetic study showed that SERPINB3 (41,42) and SERPINB13 (43) cross-class inhibit cysteine proteases, cathepsin K and L. Therefore, it is possible that inhibition of cathepsin K or L may have been the cause of the inhibitory effect of *SERPINB3* and *SERPINB13* on cancer cell invasion in our study. The proposed possible roles of the above-mentioned SERPINB genes in the suppression of cancer cell invasion are illustrated in Fig. 4. The detailed mechanisms of the other *SERPINB* genes regarding the inhibition of the malignancy of cancer cells need to be further investigated.

The general inhibitory mechanism of the serpins consist of inhibitory and substrate pathways. Apart from the RCL, serpins require additional regions for the inhibitory pathway (7). Thus, in the present study, the effective inhibition of cancer cell invasion and migration by the SERPINB RCL-peptides (Fig. 3) was probably through substrate binding to their target proteases, which may be quite different from that by the entire family of SERPINB proteins (Figs. 1 and 2). Likewise, it has been reported that both the RCL-peptide (P10-P5' of SERPINB5) and the chimeric ovalbumin with the same RCL of SERPINB5 effectively suppressed carcinoma cell invasion (44). Our SERPINB5 RCL-peptide (P5-P5') was shorter than the previously reported RCL-peptide (P10-P5') with a similar invasion-suppressing effect. Furthermore, we identified the other two effective invasion-suppressing RCL-peptides referred to as SERPINB1 and SERPINB7. Combination treatment of these SERPINB RCL-peptides improved the inhibitory effect on invasion and migration (Fig. 3D), suggesting that more proteases compete in substrate binding by these RCL-peptides.

In summary, we demonstrated that most of the *SERPINB* family genes were differentially expressed in the tumor tissues comparing to that in the matched normal tissues from lung or breast cancer patients. We systematically determined the function of each *SERPINB* gene in regards to the suppression of the invasiveness and motility of cancer cells. Among them, *SERPINB1*, *SERPINB5* and *SERPINB7* were more potent; thus we design their RCL-derived peptides, which were effective for inhibition of the invasive and migratory abilities of the cancer cells. Therefore, forced re-expression of these *SERPINB* genes or treatment of cancer cells with SERPINB RCL-peptides offers a promising approach as a therapeutic option in cancer metastasis.

Acknowledgements

This study was supported by the National Health Research Institutes (CA-097-PP-21 and CA-098-PP-16) and the Department of Health (DOH98-TD-G-111-009 and DOH100-TD-C-111-004), Taiwan.

References

1. Chaffer CL and Weinberg RA: A perspective on cancer cell metastasis. Science 331: 1559-1564, 2011.
2. Andreasen PA, Egelund R and Petersen HH: The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol Life Sci 57: 25-40, 2000.
3. Hildenbrand R, Allgayer H, Marx A and Stroebel P: Modulators of the urokinase-type plasminogen activation system for cancer. Expert Opin Investig Drugs 19: 641-652, 2010.
4. Deryugina EI and Quigley JP: Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 25: 9-34, 2006.
5. Gettins PG: Serpin structure, mechanism, and function. Chem Rev 102: 4751-4804, 2002.
6. Silverman GA, Bird PI, Carrell RW, *et al*: The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem 276: 33293-33296, 2001.
7. Izuhara K, Ohta S, Kanaji S, Shiraishi H and Arima K: Recent progress in understanding the diversity of the human ov-serpin/clade B serpin family. Cell Mol Life Sci 65: 2541-2553, 2008.
8. Nakamura M, Konno H, Tanaka T, *et al*: Possible role of plasminogen activator inhibitor 2 in the prevention of the metastasis of gastric cancer tissues. Thromb Res 65: 709-719, 1992.
9. Ito H, Yonemura Y, Fujita H, *et al*: Prognostic relevance of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitors PAI-1 and PAI-2 in gastric cancer. Virchows Arch 427: 487-496, 1996.
10. Ishikawa N, Endo Y and Sasaki T: Inverse correlation between mRNA expression of plasminogen activator inhibitor-2 and lymph node metastasis in human breast cancer. Jpn J Cancer Res 87: 480-487, 1996.
11. Yoshino H, Endo Y, Watanabe Y and Sasaki T: Significance of plasminogen activator inhibitor 2 as a prognostic marker in primary lung cancer: association of decreased plasminogen activator inhibitor 2 with lymph node metastasis. Br J Cancer 78: 833-839, 1998.
12. Mueller BM, Yu YB and Laug WE: Overexpression of plasminogen activator inhibitor 2 in human melanoma cells inhibits spontaneous metastasis in scid/scid mice. Proc Natl Acad Sci USA 92: 205-209, 1995.
13. Chen EI and Yates JR: Maspin and tumor metastasis. IUBMB Life 58: 25-29, 2006.
14. Marioni G, Staffieri C, Staffieri A, De Filippis C and Blandamura S: MASPIN tumour-suppressing activity in head and neck squamous cell carcinoma: emerging evidence and therapeutic perspectives. Acta Otolaryngol 129: 476-480, 2009.
15. Lonardo F, Li X, Kaplun A, *et al*: The natural tumor suppressor protein maspin and potential application in non-small cell lung cancer. Curr Pharm Des 16: 1877-1881, 2010.
16. Stenman J, Hedstrom J, Grenman R, *et al*: Relative levels of SCCA2 and SCCA1 mRNA in primary tumors predicts recurrent disease in squamous cell cancer of the head and neck. Int J Cancer 95: 39-43, 2001.
17. Nakashima T, Pak SC, Silverman GA, Spring PM, Frederick MJ and Clayman GL: Genomic cloning, mapping, structure and promoter analysis of HEADPIN, a serpin which is down-regulated in head and neck cancer cells. Biochim Biophys Acta 1492: 441-446, 2000.
18. Moussali H, Bylaite M, Welss T, Abts HF, Ruzicka T and Walz M: Expression of hurpin, a serine proteinase inhibitor, in normal and pathological skin: overexpression and redistribution in psoriasis and cutaneous carcinomas. Exp Dermatol 14: 420-428, 2005.
19. Walz M, Kellermann S, Bylaite M, *et al*: Expression of the human Cathepsin L inhibitor hurpin in mice: skin alterations and increased carcinogenesis. Exp Dermatol 16: 715-723, 2007.
20. Shiiba M, Nomura H, Shinozuka K, *et al*: Down-regulated expression of SERPIN genes located on chromosome 18q21 in oral squamous cell carcinomas. Oncol Rep 24: 241-249, 2010.
21. Chou RH, Lin KC, Lin SC, Cheng JY, Wu CW and Chang WS: Cost-effective trapezoidal modified Boyden chamber with comparable accuracy to a commercial apparatus. Biotechniques 37: 724-726, 2004.
22. Smith SL, Watson SG, Ratschiller D, Gugger M, Betticher DC and Heighway J: Maspin - the most commonly-expressed gene of the 18q21.3 serpin cluster in lung cancer - is strongly expressed in preneoplastic bronchial lesions. Oncogene 22: 8677-8687, 2003.
23. Maass N, Hojo T, Rosel F, Ikeda T, Jonat W and Nagasaki K: Down-regulation of the tumor suppressor gene maspin in breast carcinoma is associated with a higher risk of distant metastasis. Clin Biochem 34: 303-307, 2001.

24. Pellegrin P, Fernandez A, Lamb NJ and Bennes R: Macromolecular uptake is a spontaneous event during mitosis in cultured fibroblasts: implications for vector-dependent plasmid transfection. Mol Biol Cell 13: 570-578, 2002.
25. Baker MS, Bleakley P, Woodrow GC and Doe WF: Inhibition of cancer cell urokinase plasminogen activator by its specific inhibitor PAI-2 and subsequent effects on extracellular matrix degradation. Cancer Res 50: 4676-4684, 1990.
26. Li Y, Rizvi SM, Ranson M and Allen BJ: 213Bi-PAI2 conjugate selectively induces apoptosis in PC3 metastatic prostate cancer cell line and shows anti-cancer activity in a xenograft animal model. Br J Cancer 86: 1197-1203, 2002.
27. Abbas Rizvi SM, Li Y, Song EY, *et al*: Preclinical studies of bismuth-213 labeled plasminogen activator inhibitor type 2 (PAI2) in a prostate cancer nude mouse xenograft model. Cancer Biol Ther 5: 386-393, 2006.
28. Qu CF, Song EY, Li Y, *et al*: Pre-clinical study of 213Bi labeled PAI2 for the control of micrometastatic pancreatic cancer. Clin Exp Metastasis 22: 575-586, 2005.
29. Song YJ, Qu CF, Rizvi SM, *et al*: Cytotoxicity of PAI2, C595 and Herceptin vectors labeled with the alpha-emitting radioisotope Bismuth-213 for ovarian cancer cell monolayers and clusters. Cancer Lett 234: 176-183, 2006.
30. Sheng S, Truong B, Fredrickson D, Wu R, Pardee AB and Sager R: Tissue-type plasminogen activator is a target of the tumor suppressor gene maspin. Proc Natl Acad Sci USA 95: 499-504, 1998.
31. Biliran H Jr and Sheng S: Pleiotrophic inhibition of pericellular urokinase-type plasminogen activator system by endogenous tumor suppressive maspin. Cancer Res 61: 8676-8682, 2001.
32. Sun J, Coughlin P, Salem HH and Bird P: Production and characterization of recombinant human proteinase inhibitor 6 expressed in *Pichia pastoris*. Biochim Biophys Acta 1252: 28-34, 1995.
33. Miyata T, Inagi R, Nangaku M, *et al*: Overexpression of the serpin megsin induces progressive mesangial cell proliferation and expansion. J Clin Invest 109: 585-593, 2002.
34. Askew YS, Pak SC, Luke CJ, *et al*: SERPINB12 is a novel member of the human ov-serpin family that is widely expressed and inhibits trypsin-like serine proteinases. J Biol Chem 276: 49320-49330, 2001.
35. Cooley J, Takayama TK, Shapiro SD, Schechter NM and Remold-O'Donnell E: The serpin MNEI inhibits elastase-like and chymotrypsin-like serine proteases through efficient reactions at two active sites. Biochemistry 40: 15762-15770, 2001.
36. Shamamian P, Schwartz JD, Pocock BJ, *et al*: Activation of progelatinase A (MMP-2) by neutrophil elastase, cathepsin G, and proteinase-3: a role for inflammatory cells in tumor invasion and angiogenesis. J Cell Physiol 189: 197-206, 2001.
37. Obermajer N, Jevnikar Z, Doljak B and Kos J: Role of cysteine cathepsins in matrix degradation and cell signalling. Connect Tissue Res 49: 193-196, 2008.
38. Le Gall C, Bonnelye E and Clezardin P: Cathepsin K inhibitors as treatment of bone metastasis. Curr Opin Support Palliat Care 2: 218-222, 2008.
39. Katunuma N, Tsuge H, Nukatsuka M and Fukushima M: Structure-based development of cathepsin L inhibitors and therapeutic applications for prevention of cancer metastasis and cancer-induced osteoporosis. Adv Enzyme Regul 42: 159-172, 2002.
40. Rousselet N, Mills L, Jean D, Tellez C, Bar-Eli M and Frade R: Inhibition of tumorigenicity and metastasis of human melanoma cells by anti-cathepsin L single chain variable fragment. Cancer Res 64: 146-151, 2004.
41. Schick C, Pemberton PA, Shi GP, *et al*: Cross-class inhibition of the cysteine proteinases cathepsins K, L, and S by the serpin squamous cell carcinoma antigen 1: a kinetic analysis. Biochemistry 37: 5258-5266, 1998.
42. Schick C, Bromme D, Bartuski AJ, Uemura Y, Schechter NM and Silverman GA: The reactive site loop of the serpin SCCA1 is essential for cysteine proteinase inhibition. Proc Natl Acad Sci USA 95: 13465-13470, 1998.
43. Jayakumar A, Kang Y, Frederick MJ, *et al*: Inhibition of the cysteine proteinases cathepsins K and L by the serpin headpin (SERPINB13): a kinetic analysis. Arch Biochem Biophys 409: 367-374, 2003.
44. Ngamkitidechakul C, Warejcka DJ, Burke JM, O'Brien WJ and Twining SS: Sufficiency of the reactive site loop of maspin for induction of cell-matrix adhesion and inhibition of cell invasion. Conversion of ovalbumin to a maspin-like molecule. J Biol Chem 278: 31796-31806, 2003.