

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

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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.

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 phylogenetic 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

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Key words: *SERPINB* family, cancer, migration, invasion

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

Gene symbol	Forward primers	Reverse primers	PCR products (bp)
<i>SERPINB1</i>	5'-TCAGCTTGCCAGGTTCAAAGT-3'	5'-GGATGCTACCTGAGGAATTATGC-3'	300
<i>SERPINB2</i>	5'-GCTGGAGATGTTAGCATGTTCTTG-3'	5'-GGCTTGGTGGAAACACTTCAGAAAG-3'	300
<i>SERPINB3</i>	5'-GCGGTCTCGTGCTATCTGG-3'	5'-GTAGGTGATGATCCGAATC-3'	109
<i>SERPINB4</i>	5'-TGGAGCCACGGTCTCTCAG-3'	5'-GGAGATGATAATTCGACTA-3'	115
<i>SERPINB5</i>	5'-CATGGAGGCCACGTTCTGTATG-3'	5'-CCTGGCACCTCTATGGAATCCC-3'	417
<i>SERPINB6</i>	5'-CTCCCGCGGTTTAAACTAGAGG-3'	5'-GCAGAAGAGAATCCCGTTGGTC-3'	300
<i>SERPINB7</i>	5'-GGACCAATCCAAGGCGAATGACC-3'	5'-TAAACAGCGTGGACTGAGGGAGTT-3'	285
<i>SERPINB8</i>	5'-AGCTGGAGGAGAGTTATGACTTG-3'	5'-AGAACCTGCCACAGACAAGATG-3'	300
<i>SERPINB9</i>	5'-CAAGCCAGACTGTATGAAGAGTAC-3'	5'-AAAGGGTGGTCAGCACAGAACCTG-3'	300
<i>SERPINB10</i>	5'-GCAGACATGATGGAGTTGTATGAAG-3'	5'-GAAGAGGAATGGGTGATTTGCATTG-3'	300
<i>SERPINB11</i>	5'-CGTTTCATGAGTGGACAAGCTCTTC-3'	5'-CCTTGAAGTCTGAGCTCTCATTGGTAG-3'	300
<i>SERPINB12</i>	5'-AAGGCACAGATCCTGGAAATGAG-3'	5'-ATTGGGACTTGGAGAGATTCCAG-3'	300
<i>SERPINB13</i>	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% CO₂ 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- μ l reaction volume containing 0.2 μ M of each primer, 200 μ M dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 1 U Taq DNA polymerase (Takara, Kyoto, Japan) and 1 μ l 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 *EcoRV* 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)
<i>SERPINB1</i>	EB	5'-AGAATTCACCATGGAGCAGCTGAG-3'	5'-AGGGATCCACAGGGGAAGAAAATCT-3'	1137
<i>SERPINB2</i>	EB	5'-CGCGAATTCACCATGGAGGATCTTTGT-3'	5'-TCAGGATCCACGGGTGAGGAAAATCT-3'	1245
<i>SERPINB3</i>	XB	5'-CTCGAGCCACCATGAATTCAGTCAAGC-3'	5'-GGATCCTTCGGGGATGAGAATCTGCCAT-3'	1170
<i>SERPINB4</i>	XB	5'-CTCGAGCCACCATGAATTCAGTCAAGC-3'	5'-GGATCCTTCGGGGATGAGAATCTGCCAT-3'	1170
<i>SERPINB5</i>	EE	5'-GAATTCACCATGGATGCCCTGCAACTAGC-3'	5'-GATATCCACAGGAGAACAGAATTTGCCAA-3'	1125
<i>SERPINB6</i>	EE	5'-GAATTCACCATGGATGTTCTCGCAGAAGC-3'	5'-GATATCCACCGGAGAGGAAAAGCGGCCGC-3'	1128
<i>SERPINB7</i>	EB	5'-TTTGAATTCACCATGGCCTCCCTTGCT-3'	5'-GCAGGATCCACAGGGCAAGAACTTT-3'	1140
<i>SERPINB8</i>	XB	5'-CTCGAGCCACCATGGATGACCTCTGTGAAGC-3'	5'-GGATCCTTCGGAGAAGAGAACCTGCCAC-3'	1122
<i>SERPINB9</i>	EB	5'-CGAATTCGCCCATGGAACTCTTTTC-3'	5'-ATGGATCCTTTGGCGATGAGAACCT-3'	1128
<i>SERPINB10</i>	XB	5'-CTCGAGCCACCATGGACTCTCTAGCAACATC-3'	5'-GGATCCTTGGGGGAGCATAATCTTCCAT-3'	1191
<i>SERPINB11</i>	XB	5'-CTCGAGCCACCATGGGTTCTCTCAGCACAGC-3'	5'-GGATCCTTGGGAGAGGCAAGCTTGCCAC-3'	1176
<i>SERPINB12</i>	XB	5'-CTCGAGCCACCATGGACTCTCTTGTGTACAGC-3'	5'-GGATCCTTAGGAGAGCAGACCCTGCCAT-3'	1215
<i>SERPINB13</i>	EB	5'-CGAATTCGCCCATGGATTCACTTGG-3'	5'-GACCGGTGGATCCCGTGAAGGAGAAGAAAATC-3'	1173

^aEB, *EcoRI* and *BamHI*; EE, *EcoRI* and *EcoRV*; XB, *XhoI* and *BamHI*. ^bThe restriction enzyme sites are underlined, and the gene-specific regions are in bold. ^cORF (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 μ l 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 2.5×10^4 cells/well instead.

In vitro wound healing assay. Approximate 2×10^5 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 μ g/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)					
<i>SERPINB1</i>	4	1	0	0	↓
<i>SERPINB2</i>	2	3	0	0	
<i>SERPINB3</i>	1	4	0	0	↑
<i>SERPINB4</i>	3	2	0	0	
<i>SERPINB5</i>	0	4	1	0	↑
<i>SERPINB6</i>	5	0	0	0	↓
<i>SERPINB7</i>	0	4	0	1	↑
<i>SERPINB8</i>	4	0	1	0	↓
<i>SERPINB9</i>	4	0	1	0	↓
<i>SERPINB10</i>	1	4	0	0	↑
<i>SERPINB11</i>	1	1	0	3	
<i>SERPINB12</i>	1	2	0	2	
<i>SERPINB13</i>	1	4	0	0	↑
In breast cancer (n=5)					
<i>SERPINB1</i>	2	1	2	0	
<i>SERPINB2</i>	3	1	1	0	↓
<i>SERPINB3</i>	3	1	0	1	↓
<i>SERPINB4</i>	3	2	0	0	
<i>SERPINB5</i>	3	1	1	0	↓
<i>SERPINB6</i>	3	0	2	0	↓
<i>SERPINB7</i>	0	4	1	0	↑
<i>SERPINB8</i>	3	2	0	0	
<i>SERPINB9</i>	3	2	0	0	
<i>SERPINB10</i>	3	0	0	2	↓
<i>SERPINB11</i>	0	0	0	5	
<i>SERPINB12</i>	4	1	0	0	↓
<i>SERPINB13</i>	3	0	1	1	↓

^aThe gene expression levels were rather consistent between the tumor and matched normal tissues. ^bThe 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

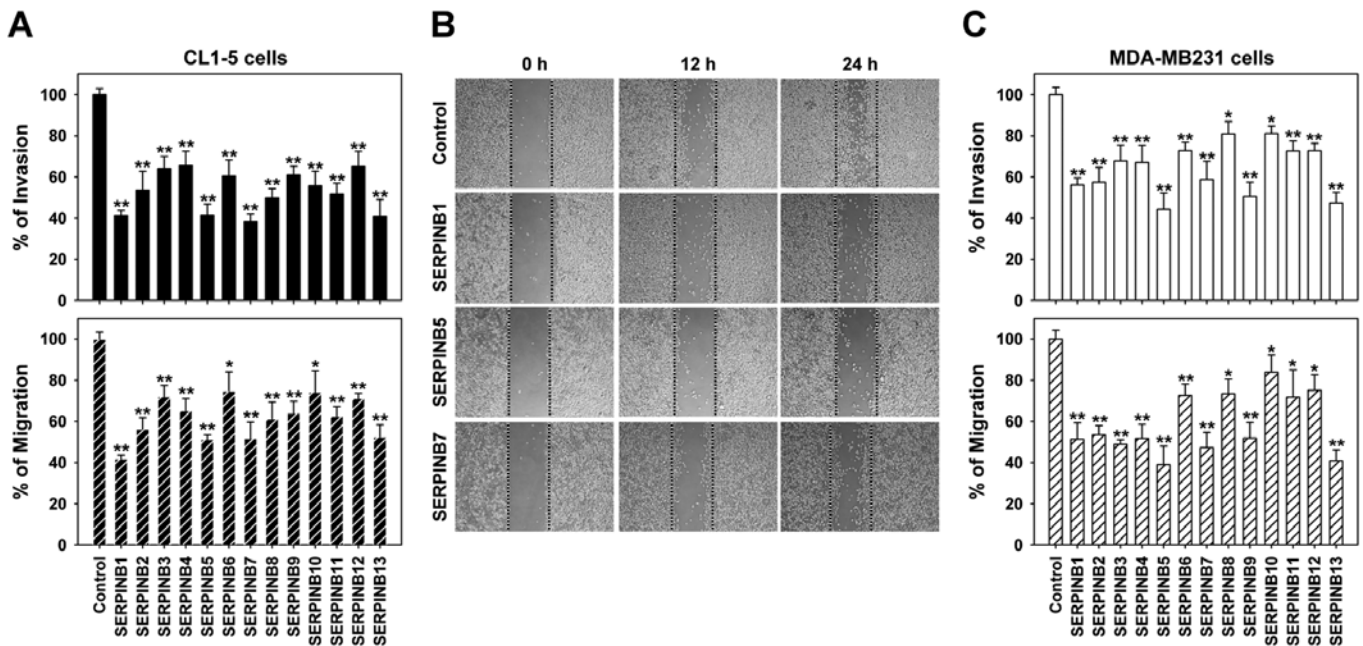


Figure 1. *SERPINB* family genes inhibit the invasiveness and motility of cancer cells. (A and B) CL1-5 and (C) MDA-MB231 cells were stably transfected with each *SERPINB* gene. Each stable transfectant was applied to the *in vitro* invasion or migration assay for 24 h. The relative invasive (upper panel) 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, \pm SE; * p <0.05 and ** p <0.01 relative to the control by the t-test).

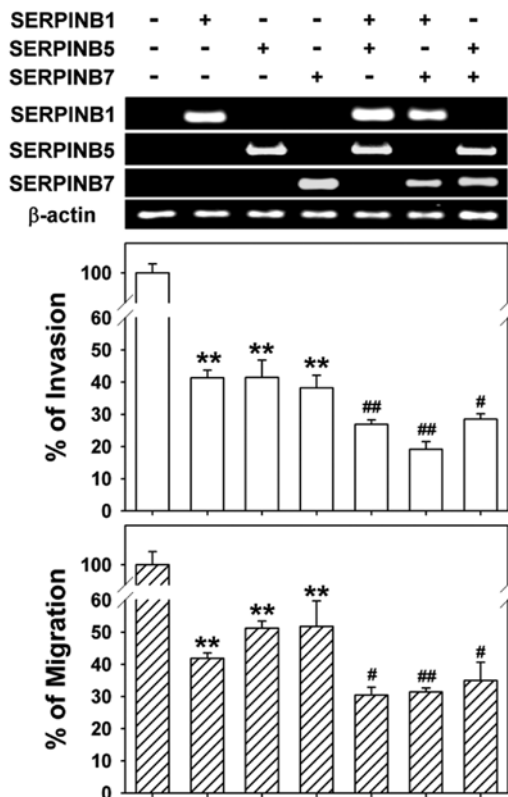


Figure 2. Co-transfection of the *SERPINB* genes strengthens the inhibitory effect on cancer cell invasion and migration. CL1-5 cells were stably transfected with *SERPINB1*, *SERPINB5* or *SERPINB7*, or with any two. The expression of each recombinant *SERPINB* gene was examined by RT-PCR (top panel). Subsequently, each transfectant was applied to the *in vitro* invasion (middle panel) or migration (bottom panel) assay for 24 h. (Error bars, \pm 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.

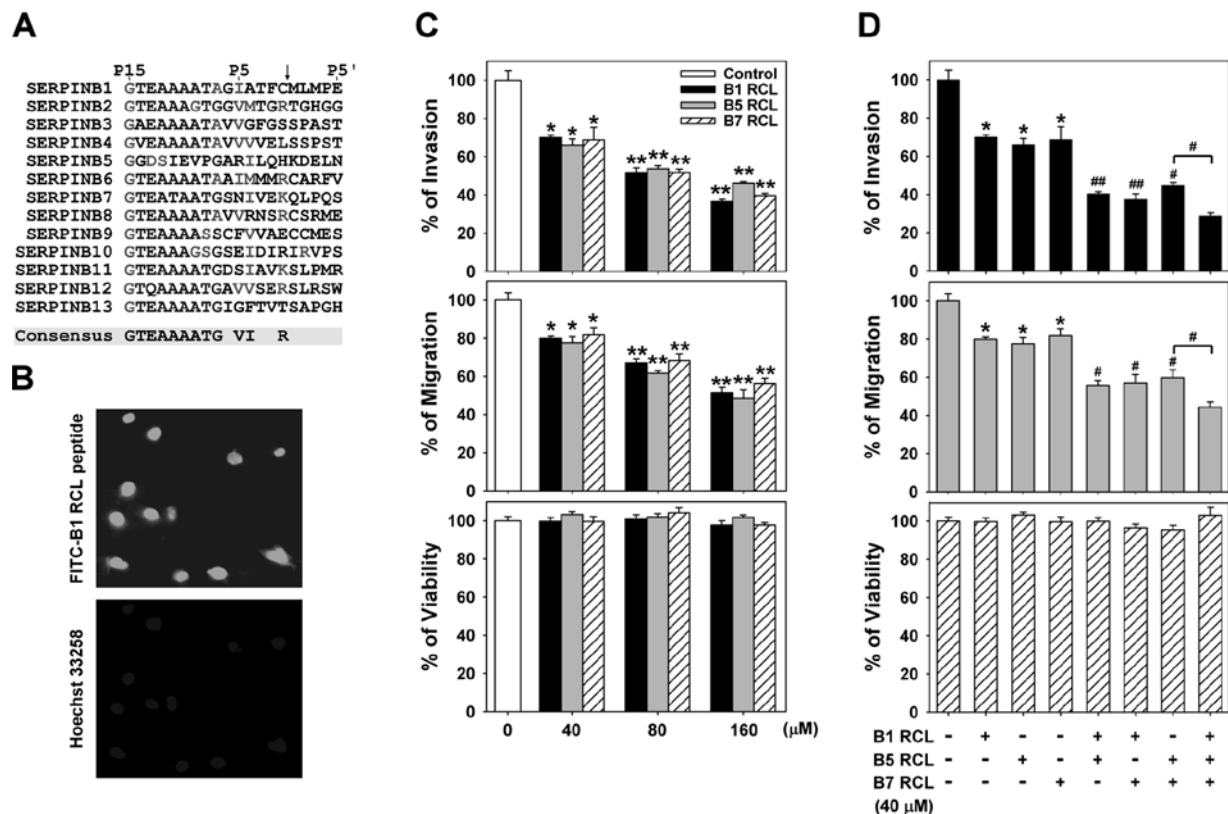


Figure 3. SERPINB RCL-peptides suppress the invasive and migratory properties of cancer cells. (A) The amino acid sequences of the RCL regions of all 13 SERPINB proteins were aligned using the Vector NTI program (Invitrogen Life Technologies). (B) CL1-5 cells were treated with 0.25% trypsin, and then 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, \pm 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).

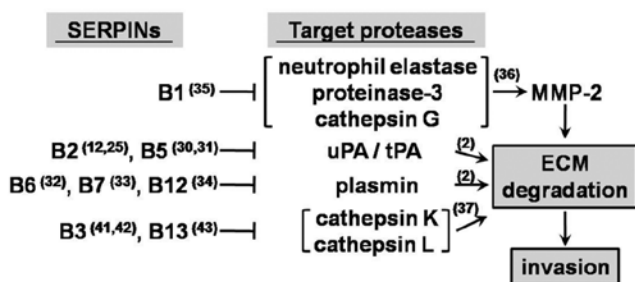


Figure 4. Possible roles of the SERPINB genes in cancer cell invasion are illustrated. The scheme summarizes the proposed roles of the indicated SERPINB genes in the suppression of cancer cell invasion according to previous reports (superscripted numbers in parentheses). uPA, urokinase plasminogen activator; 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.

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