Overexpression of Stefin A in Human Esophageal Squamous Cell Carcinoma Cells Inhibits Tumor Cell Growth, Angiogenesis, Invasion, and Metastasis

Wendong Li, Fang Ding, Liyong Zhang, Zhongmin Liu, Yu Wu, Aiping Luo, Min Wu, Mingrong Wang, Qimin Zhan, and Zhihua Liu

Abstract
Purpose: Evidence is accumulating that an inverse correlation exists between stefin A and malignant progression. The aim of this study is to investigate the role of stefin A in human esophageal squamous cell carcinoma cells and to evaluate the possibility of stefin A for cancer therapy.

Experimental Design: We stably transfected stefin A cDNA into human EC9706 or KYSE150 esophageal squamous cell carcinoma cells. Subsequently, we evaluated the effect of stefin A overexpression on cell growth, cathepsin B activity, cell motility and invasion, tumor growth, and metastasis. Immunohistochemistry was done to assess the expression of factor VIII and to support the localization of stefin A and cathepsin B. We also evaluated the effect of CA074Me, a selective membrane-permeant cathepsin B inhibitor.

Results: Both transfection of stefin A and treatment with 10 μmol/L CA074Me significantly reduced cathepsin B activity and inhibited the Matrigel invasion. Combination of both further reduced cathepsin B activity and inhibited the Matrigel invasion. Overexpression of stefin A delayed the in vitro and in vivo growth of cells and significantly inhibited lung metastasis compared with 50% of lung metastasis in xenograft mice from EC9706 or empty vector cells. Transfection with stefin A showed a dramatic reduction of factor VIII staining in the tumors of xenograft mice.

Conclusions: Our data strongly indicate that stefin A plays an important role in the growth, angiogenesis, invasion, and metastasis of human esophageal squamous cell carcinoma cells and suggest that stefin A may be useful in cancer therapy.

The most important characteristic of malignant tumors is their ability to invade surrounding tissues and metastasize. Tumor invasion and metastasis are usually the major causes that lead cancer patients to death. Although many advances have been made in cancer therapy, most cancer deaths still result from metastatic disease. Tumor invasion and metastasis are aggressive processes during which tumor cells invade, migrate, and proliferate. Penetration and degradation of components of extracellular matrix and basement membrane are key steps in the metastatic cascade of cancer cells. In the metastatic process, a variety of proteolytic enzymes are triggered to be expressed, secreted, and activated. Production of proteolytic enzymes of different classes is a property strongly associated with the metastatic state of cancer cell (1). These enzymes include matrix metalloproteinases, serine proteases (urokinase-type plasminogen activator, plasmin, etc.), and lysosomal cysteine proteinase cathepsin B.

Cathepsin B has been implicated in the progression of tumors from a premalignant to a malignant state and in various disease states, including tumor growth, angiogenesis, invasion, and metastasis (2–4); rheumatoid arthritis; cholestatic liver injury; and pancreatitis. It is widely held that invasion is facilitated by a membrane or secreted form of cathepsin B that acts outside the cell to degrade extracellular matrix components at or adjacent to the surface of the invading cell (5). Cathepsin B has a broad pH optimum and is able to degrade the components of the extracellular matrix and basement membrane either directly or indirectly by activating other proteases like pro-urokinase-type plasminogen activator (6). Overexpression of cathepsin B mRNA, increased cathepsin B staining, and elevated cathepsin B activity have been found in different human cancers (7). An amplicon at 8p22-23 resulting in cathepsin B gene amplification and overexpression supports an important role for cathepsin B in esophageal adenocarcinoma and possibly in other tumors (8). Many investigators have shown a correlation between increased activity of cathepsin B and increased metastatic capability of animal tumors or the malignancy of human tumors (4, 9–12). These increases in

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cathepsin B activity correspond in part to increased amount of cathepsin B mRNA transcripts and in part to reduced regulation by endogenous low molecular weight cysteine proteinase inhibitors (3). The major means of regulation of mature cysteine proteinases is by its endogenous inhibitors of the cystatin superfamily (stefins, cystatins, and kininogens) and thyropins. Intracellularly, the most abundant inhibitors are stefin A (cystatin A) and stefin B (cystatin B), whereas extracellularly, cystatin C is the most widely distributed inhibitor. Quantitatively different combinations of cystatins are the major constituents of the inhibitory potential against cathepsin B in squamous cell lung cancer and normal lung tissue (13). Cathepsin B and its endogenous inhibitors may facilitate proteolysis by the hepatoma cells and thereby contribute to the invasive phenotype of this type of cancer (14). Stefin A was the first inhibitor of cysteine proteases reported to be associated with malignant tumors. Stefin A is decreased in many cancer tissues and cells. For example, in breast cancer patients, an inverse correlation of stefin A expression with metastatic potential and relapse-free period was suggested. Lowered stefin A had an effect on total cystatins activity and was associated with cathepsin B activity (3). An inverse correlation between increased cathepsin B expression and decreased stefin A level has been shown in a variety of human tumors (15–18). Evidence is accumulating that an inverse correlation exists between the level of stefin A and malignant progression. Our previous work as well as the work from other group showed obvious down-regulated expression of stefin A in human esophageal squamous cell carcinoma using cDNA microarray technology (18, 19). Our cDNA microarray results showed a 6- to 7-fold decrease in stefin A levels and a 3-fold increase in cathepsin B levels in human esophageal squamous cell carcinoma tissue (18). To investigate the role of stefin A in human esophageal squamous cell carcinoma cells, we stably transfected sense-oriented stefin A cDNA into EC9706 or KYSE150 esophageal squamous cell carcinoma cells. Our study showed that overexpression of stefin A not only obviously reduced the tumor growth, angiogenesis, and invasion but also significantly inhibited the lung metastasis in xenograft mice from EC9706 cells.

Materials and Methods

Cell culture and reagents. Human EC9706 esophageal squamous cell carcinoma cell line was established and maintained in our laboratory (20), two other human esophageal squamous cell carcinoma cell lines KYSE150 and KYSE510 (21) were generous gifts from Dr. Shimada Y (First Department of Surgery, Faculty of Medicine, Kyoto University, Japan).
University, Japan). All the three cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 μg/mL penicillin (pH 7.2-7.4) in a humidified incubator containing 5% CO₂, at 37°C. CA074Me (Sigma, St. Louis, MO), a highly selective cathepsin B inhibitor, which is readily cell permeant, was dissolved in DMSO.

Preparation of constructs and transfection. Human stefin A cDNA was subcloned into a eukaryotic expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA) in the sense orientation. EC9706 and KYSE150 cells were transfected with either the stefin A cDNA construct or the pcDNA3.1(+) empty vector alone by using LipofectAMINE 2000 (Invitrogen). After G418 (400 μg/mL, Life Technologies, Rockville, MD) selection, the stable clones were identified by Western blot for stefin A expression. Two stable clones (A1 and A2) expressing higher stefin A and one empty vector clone (Vector from EC9706 cells or Vector(150) from KYSE150 cells, respectively) were harvested for cathepsin B activity using a fluorescence microscope. For observation of cathepsin B inhibitor, cells were treated with 10 μmol/L CA074Me for 12 hours before cathepsin B activity assay was done. The cell fluorescence intensity was assessed by Leica QWin image analysis and image processing software. For observation of cathepsin B inhibitor, cells were treated with 10 μmol/L CA074Me for 12 hours before cathepsin B activity assay was done. The cell fluorescence intensity was assessed by Leica QWin image analysis and image processing software. The mean cell fluorescence intensity for cathepsin B activity in untreated EC9706 or KYSE150 cells was considered as 100%.

Motility and invasion assay. AP48 invasion chamber (Neuro Probe, Inc., Gaithersburg, MD) was used for in vitro motility and invasion assays according to the manufacturer’s instructions. For treatment with cathepsin B inhibitor, cells were treated with 10 μmol/L CA074Me for 12 hours. Cells (6 × 10⁴) in serum-free media were plated in the upper chamber and incubated in a humidified CO₂ incubator at 37°C. 10% fetal bovine serum was added to RPMI 1640 in the lower chamber as a chemoattractant. For invasion assay, the upper side of the filter was covered with 300 μg/mL of Matrigel (BD Biosciences, Bedford, MA). After 8 hours for motility assay or 24 hours for invasion assay, cells on
the upper side of the filter were mechanically removed. Cells migrated to the lower side were fixed with 100% methanol and stained with 0.25% crystal violet. The number of cells on the lower side was counted under microscope. The counting was done for five different fields in each sample. Experiments were carried out in triplicate, and the results were shown as mean ± SD of three independent experiments. The mean cell counting in untreated EC9706 or KYSE150 cells was considered as 100%.

**Experiments in nude mice.** Single-cell suspensions of each of the transfectants and parental cells were trypsinized and collected. The cell viability was >95% as determined by trypan blue staining. Cells (2 × 10⁶) in a 0.1 mL volume of PBS were inoculated s.c. into the right flank of 4- to 5-week-old female BALB/c nude mice (eight for each group).

Once palpable tumors were established, tumor volume measurements were taken once a week using calipers along two major axes. Tumor volume was calculated as follows: \( V = \frac{4}{3} \pi R_1 R_2 \), where \( R_1 \) is radius 1 and \( R_2 \) is radius 2 and \( R_1 < R_2 \). At the end of 3 months, all mice were sacrificed, and the tumor volume and weight were measured. All the tumors were fixed in 4% polyformaldehyde and cut into 6-μm sections. For observation of metastasis, all the lungs, livers, and brains were excised, fixed in 4% polyformaldehyde, and cut into consecutive 6-μm sections. All these sections above were stained with H&E and observed under a microscope. The presence of lung metastasis was determined and confirmed by the pathologists in a cancer hospital (Chinese Academy of Medical Sciences). The number of lung metastases for each group was counted.

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**Fig. 3.** Effect of transfection with stefin A or treatment with 10 μmol/L CA074Me on cathepsin B activity and invasion in EC9706 (A-C) or KYSE150 (D and E) cells. **A,** cathepsin B activity assay: photographic results from a fluorescence microscope. Inhibition of cathepsin B activity expressed as % untreated EC9706 (B) or KYSE150 (D) cells according to Materials and Methods. Columns, mean of three independent experiments; bars, SD. *, \( P < 0.05 \); **, \( P < 0.001 \). n.s.d., no significant difference.

Inhibition of invasion expressed as % untreated EC9706 (C) or KYSE150 (E) cells according to Materials and Methods. Columns, mean of three independent experiments; bars, SD. *, \( P < 0.05 \); **, \( P < 0.001 \). n.s.d., no significant difference.
Factor VIII staining and microvessel quantitation. Tumor tissue sections were analyzed by immunohistochemical staining using PV-9000 detection kit (Golden Bridge International, Inc., Suite G Lynnwood, WA) according to manufacturer’s instructions with some modifications. Briefly, sections were deparaffinized, quenched with 3% hydrogen peroxide in deionized water for 20 minutes, microwaved 10 minutes in 0.01 mol/L sodium citric acid buffer (pH 6.0), and blocked for 30 minutes with PBS containing 3% goat serum, 0.1% Triton X-100, and 0.05% Tween 20. Slides were incubated with rabbit antihuman factor VIII IgGs (1:80; Chemicon International, Inc., Temecula, CA) overnight at 4°C. The poly-horseradish peroxidase/anti-mouse/rabbit IgG was used as the secondary antibody. Sections were exposed to diaminobenzidine peroxidase substrate (Sigma) for 2 to 3 minutes and counterstained with hematoxylin. Corresponding tissue sections without primary antibody served as negative controls. Microvessel density was quantified by examining areas of vascular hotspots as previously described by Weidner et al. (23) with some modifications. Sections were scanned at low magnification (×200 and ×100) for the localization of vascular hotspots. The three most vascular areas of the tumor, not containing necrosis, were determined and then counted in the high power field (×200). The values of the three sections were averaged, and the results were analyzed. Branching structures were counted as a single vessel as previously shown (24).

Confocal imaging. Tissue sections of the tumor from stefin A–transfected EC9706 cells were mounted on poly-1-lysine-coated slides. After simultaneous overnight incubation at 4°C with mouse anti-human stefin A IgGs (1:400; Axxora, LLC) and rabbit anti-human cathepsin B IgGs (1:200; Serotec Ltd., Oxford, United Kingdom) as primary antibodies, the slides were washed in PBS and incubated with the secondary antibodies: FITC-conjugated goat anti-mouse IgGs (1:50; Molecular Probes, Eugene, OR) and TRITC-conjugated goat anti-rabbit IgG (1:50; Molecular Probes). Nuclei were counterstained with 1 μg/mL 4’,6-diamidino-2-phenylindole (Sigma). Slides were examined with an ultra-spectral confocal microscopy system (Leica TCSSP2-AOBS-UV Leica-Microsystems, Wetzlar, Germany). Series of images were processed and analyzed with the accompanying software package.

Statistical analysis. Results were showed as mean ± SD. Student’s t test was used for comparison unless particular test was notified. *P < 0.05 was considered statistically significant.
Results

High invasive EC9706 cells show low stefin A protein level. We grew EC9706, KYSE150, and KYSE510 cells to 90% to 95% confluence and assessed the stefin A protein levels by Western blot (Fig. 1A and B) as well as the invasiveness using invasion assay (Fig. 1C and D). In the three cancer cell lines, the most invasive EC9706 cells showed the lowest stefin A protein expression; KYSE510 cells with the highest protein level were found to be least invasive; KYSE150 cells were less invasive with less protein. Densitometric quantification verified that the amount of stefin A protein was significantly four to five times higher in KYSE510 cells, and 17 to 18 times higher in KYSE150 cells, than that in EC9706 cells (Fig. 1B; \( P < 0.01 \)). In Fig. 1A, cathepsin B was detected in two forms (about 31 and 26 kDa), which was consistent with the previous study (25). To investigate the role of stefin A in human esophageal squamous cell carcinoma cells, we chose EC9706 cells for transfection and further study.

Transfection with sense-stefin A construct inhibits cell growth in vitro. EC9706 cells were transfected with sense stefin A construct or empty vector for stable clones. As shown in Fig. 2A, two stable cell clones (A1 and A2) expressed higher stefin A protein. Densitometric quantification indicated that the amount of stefin A protein was significantly four to five times higher in KYSE150 cells, and 17 to 18 times higher in KYSE510 cells, than that in EC9706 cells (Fig. 1B; \( P < 0.01 \)). In Fig. 1A, cathepsin B was detected in two forms (about 31 and 26 kDa), which was consistent with the previous study (25). To investigate the role of stefin A in human esophageal squamous cell carcinoma cells, we chose EC9706 cells for transfection and further study.

Stefin A–transfected esophageal squamous cell carcinoma cells show reduced cathepsin B activity and reduced invasiveness in vitro. Previous studies confirmed that stefin A was one of cathepsin B inhibitors. Using MR–cathepsin B detection kit, we evaluated the effect of overexpression of stefin A on cathepsin B activity in EC9706 cells. As shown in Fig. 3A and B, cathepsin B activities were reduced either by transfection with stefin A or by treatment of CA074Me. Transfection of stefin A reduced cathepsin B activity in EC9706 cells by 87% to 92%. CA074Me at a concentration of 10 \( \mu \)mol/L reduced cathepsin B activity by 80% to 83%. Combination of both further reduced cathepsin B activity by 95% to 96%.

Many studies confirmed that cathepsin B was involved in invasion and metastasis of tumor (4, 12, 13). As stefin A was endogenously a cathepsin B inhibitor, we examined whether transfection with stefin A inhibited cell invasion. Using AP48 invasion chamber coated with or without Matrigel, we investigated the effects of transfection with stefin A combined with or without CA074Me on cell motility and invasion. There was no significant difference between EC9706 or empty vector cells and A1 or A2 cells in cell motility. Treatment with cathepsin B inhibitor did not affect cell motility either (data not shown). However, as shown in Fig. 3C, transfection with stefin A reduced cell invasion by 78% to 83%, whereas treatment with 10 \( \mu \)mol/L CA074Me reduced invasion by 65% to 68%. The most significant reduction in invasiveness was observed in combination of both above, and cell invasion was reduced

<table>
<thead>
<tr>
<th>Nude mouse</th>
<th>No. mice with lung metastasis</th>
<th>Total no. lung tumor nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC9706 ( (n = 8) )</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>Vector ( (n = 8) )</td>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>A1 ( (n = 8) )</td>
<td>0*</td>
<td>0</td>
</tr>
<tr>
<td>A2 ( (n = 8) )</td>
<td>0*</td>
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* \( P < 0.05 \) (Fisher’s exact test), clone A1 or A2 group versus EC9706 or vector group.
by 88% to 95%. Reduced cathepsin B activity and reduced invasiveness were also observed in stefin A–transfected KYSE150 cells (Fig. 3D and E).

**Stefin A–transfected cells show reduced tumor growth in vivo.**

Our data showed that the tumors from stefin A–transfected cells in nude mice grew more slowly than that from EC9706 or empty vector cells (Fig. 4A-C), which was consistent with the cell growth curve above. At the end of 3 months, the weights of tumors from stefin A–transfected cells were five times less than that of tumors from EC9706 or empty vector cells. As shown in Fig. 4D and E, stefin A transfection in KYSE150 cells also inhibited tumor growth and reduced tumor weight.

**Stefin A–transfected EC9706 cells show a significant inhibition of lung metastasis in nude mice.**

At the end of 3 months, all the mice were sacrificed. All the lungs, livers, and brains were cut to consecutive 6-μm sections. The metastases were carefully observed on serial microscopic sections of whole specimens in all mice. Lung metastases were seen in two of eight mice from EC9706 cells without the help of a microscope. By a microscope, parental or empty vector EC9706 cells metastasized to the lungs in half of nude mice, resulting in a total number of 27 or 26 lung tumor colonies, respectively. In contrast, no lung metastasis was seen in nude mice from stefin A–transfected EC9706 cell clones (A1 and A2) even with the help of microscope (Fig. 5A and B; Table 1). Overexpression of stefin A inhibited the lung metastasis in nude mice ($P = 0.038$, Fisher’s exact test). Neither liver metastases nor brain metastases were found in all mice. Nevertheless, no lung metastasis was observed in nude mice from parental KYSE150 cells or transfected KYSE150 cells.

**Stefin A–transfection shows the colocalization of stefin A with cathepsin B.**

As shown in Fig. 6, a merged image of stefin A and cathepsin B proteins in the tumor tissue section from A2 cells showed the interaction and colocalization of stefin A and cathepsin B, which supported the inhibition of cathepsin B by stefin A. In tumor and transformed cells, cathepsin B is translocated to the peripheral cytoplasmic and plasma membrane region or secreted into the surrounding space along with enhanced invasive potential, whereas in normal cells, it is localized predominantly in the lysosomes of the perinuclear region (26). As shown in Fig. 6, both stefin A and cathepsin B protein are mainly localized in the peripheral cytoplasmic and plasma membrane region. However, no significant change was observed with immunohistochemical staining for cathepsin B (data not shown).

**Stefin A transfection inhibits tumor angiogenesis.**

As shown in the tumor sections stained with H&E, the tumors from stefin A–transfected EC9706 cells showed obvious necrosis compared with the tumors from parental or empty vector cells (Fig. 7A). In this study, we assumed that tissue necrosis was due to inhibition of tumor angiogenesis. Immunohistochemical staining with factor VIII antibody was employed. Transfection with stefin A showed a dramatic reduction of factor VIII staining (Fig. 7B). As shown in Fig. 7C, microvessel density was significantly decreased in the tumors from EC9706 cells or empty vector cells (54 ± 7.3/high power field or 57 ± 5.5/high power field, respectively) compared with the tumors from A1 or A2 cells (17 ± 3.8/high power field or 12 ± 3.6/high power field, respectively; $P < 0.001$), which supported the assumption. Similar inhibition of tumor angiogenesis was obtained in the tumor sections from stefin A–transfected KYSE150 cells (Fig. 7D).

**Discussion**

Cathepsin B is believed to participate in tumor progression from a premalignant to a malignant state. Regulation of cathepsin B expression levels by molecular genetic techniques led to changes of malignant phenotype of tumor cell. Stable transfection (2) or transient transfection (4) of human cathepsin B cDNA into tumor cells resulted in an increase in cathepsin B activity and an increase in invasiveness. Conversely, down-regulation of cathepsin B expression reduces cathepsin B activity and inhibits Matrigel invasiveness of tumor cells by stable transfection with an antisense cathepsin B cDNA (4, 27–30) or by RNA interfering (31, 32). Because invasiveness through Matrigel is often correlated with metastatic potential in vivo (33–36), these data provide further evidence for the relationship between increased levels of cathepsin B and malignancy.

In normal cells or tissues, there is a balance between cathepsin B and its endogenous inhibitors. Alterations in the balance have been postulated to contribute to malignant progression. An inverse correlation between increased cathepsin B expression and decreased levels of its endogenous inhibitors has been shown in a variety of malignant tumors (15–18). Up-regulated expression of the endogenous inhibitors in tumor cells improved the malignant phenotype. Stable transfection of cystatin C into B16F10 melanoma cells with low cystatin C levels inhibited motility and in vitro invasiveness by 50% in both stimulated (autocrine motility factor, laminin) and unstimulated cells (37). Sense cystatin C–transfected SNB19 cells were markedly less invasive than control cells in a Matrigel invasion assay and in a coculture assay of SNB19 spheroids and fetal rat brain aggregates, and sense cystatin C–transfected SNB19 cells did not form tumors in nude mice upon intracerebral injection (25). Larger changes in invasiveness were observed in murine SCC-VII squamous cell carcinoma cells by heterologous expression of cathepsin B and cystatin C.
than were seen in the previous cathepsin B overexpression study (2). Recently, overexpression of cystatin M, a potent endogenous protein inhibitor of lysosomal cysteine proteases, in human MDA-MB-435S breast carcinoma cells significantly suppressed in vitro cell proliferation, migration, and Matrigel invasion and showed significantly delayed primary tumor growth and lower metastatic burden in the lungs and liver in mice when compared with mock controls (38, 39).

In our study, we observed that the relative amounts of stefin A decreased as the invasion ability increased. The human EC9706 esophageal squamous cell carcinoma cell line is normally highly invasive and produces very little stefin A protein. Introducing a sense stefin A construct into EC9706 cells inhibited the in vitro invasiveness by 78% to 83% in a Matrigel invasion assay and lung metastasis in nude mice significantly. The reason why the metastasis was significantly inhibited might be that the invasiveness of EC9706 cells was reduced to some extent, which prevented metastasis. The fact that metastasis was only found in lungs but not in livers and brains indicate lung is the most important target for metastasis of human esophageal squamous cell carcinoma cells in nude mice, which is in agreement with our assumption. This is the

![Image](https://example.com/image.png)

**Fig. 7.** Stefin A transfection inhibited angiogenesis of the tumors from EC9706 (A-C) or KYSE150 (D) cells. A, tumor section stained with H&E from EC9706, empty vector, and A1 and A2 cells. Sections from A1 and A2 cells show obvious necrosis. N, necrosis. Magnification, ×100. B, immunohistochemical analysis of tumor tissue sections stained with anti-human factor VIII antibody. Obviously decreased staining of factor VIII was observed in stefin A–transfected tumor sections compared with high intense staining in the tumor sections from EC9706 cells and empty vector transfectants. Magnification, ×200. MicrovesSEL density analysis in the tumor sections from EC9706 (C) or KYSE150 (D) cells was done according to Materials and Methods. Columns, mean; bars, SD. *, P < 0.01; **, P < 0.001. n.s.d., no significant difference.
first observation for overexpression of endogenous cathepsin B inhibitor in experimental metastasis of esophageal carcinoma cells. Previous study showed that treatment of a liver-homing Lewis lung carcinoma subline H-59 cells with E-64, an exogenous cathepsin inhibitor, inhibited experimental liver metastases formation by up to 90% (40).

Conflicting results about the contributions of cysteine proteinases to cell motility were reported in different studies. Some reports showed that E-64 (41, 42), steffins (42), and transfection with cystatin C (37), cystatin M (38), or antisense cathepsin B (27) reduced the cell motility. Some studies showed no effect of cysteine proteinases on cell motility (2, 4, 43). In our study, both transfection of stefin A CDNA and treatment with CA074Me did not affect the motility of human EC9706 esophageal squamous carcinoma cells but significantly reduced Matrigel invasion. CA074Me, a membrane-permeable proinhibitor for intracellular cathepsin B, which becomes active following internalization and conversion to CA074 (44), is currently the reagent of choice to inactivate cathepsin B within living cells. As stefin A is an endogenous inhibitor of cathepsins B, H, L, and S, here, we employed CA074Me to evaluate the inhibition of cathepsin B by stefin A.

In PC3M cells, CA074Me at a concentration of 10 μmol/L completely abolished intracellular cathepsin B activity and produced a 45% to 75% inhibition of Matrigel invasion, suggesting an important intracellular function for cathepsin B in matrix degradation (4). In our study, CA074Me at a concentration of 10 μmol/L inhibited cathepsin B activity by 80% to 83% and Matrigel invasion by 65% to 68%. Stefin A transfection inhibited cathepsin B activity by 87% to 92% and Matrigel invasion by 78% to 83%. Combined applications of both resulted in the largest inhibition of both cathepsin B activity by 95% to 96% and Matrigel invasion by 88% to 95%. Taken together, transfection with stefin A and treatment with cathepsin B inhibitor contributed synergistically to inhibit cancer invasion by inhibiting cathepsin B activity. In addition, we speculate that cathepsin B serves as a very important role in cancer invasion by inhibiting cathepsin B activity. Confocal results supported the interaction of cathepsin B and stefin A proteins in cells. Stefin A was reported to inhibit cathepsin B by a two-step mechanism, involving an initial weak interaction followed by a conformational change (45). The reason why the invasion inhibition of CA074Me was not strong as other study (4) may be due to the variation of the cell lines.

It has been reported that cathepsin B could promote cell proliferation by activating growth factors (46) or liberating them from the extracellular matrix where they are sequestered (47). Cathepsin B inhibitor was reported to inhibit cell proliferation (48). In our study, transfection with stefin A also inhibited both in vitro cell proliferation and in vivo tumor growth. In addition, we observed a noteworthy phenomenon that the tumors from stefin A–transfected EC9706 cells were prone to necrosis compared with the tumors from parental or empty vector cells. Cathepsin B is believed to participate in tumor growth and angiogenesis. Inhibition of cathepsin B and MMP-9/uPAR gene expression in glioblastoma cell line via RNA interference reduced tumor growth and angiogenesis (31, 32). Adenovirus-mediated expression of antisense urokinase plasminogen activator receptor and antisense cathepsin B also inhibited tumor growth, invasion, and angiogenesis in gliomas (30). Therefore, we assume that transfection with stefin A might inhibit tumor angiogenesis and consequently cause tumor necrosis. Immunohistochemical analysis with factor VIII antibody, an indicator, which is widely used for measure of angiogenesis, supported our hypothesis. Moreover, inhibition of tumor angiogenesis induced by transfection with stefin A must contribute in part to inhibit tumor growth and metastasis. Therefore, we have come to two conclusions: one is that both inhibition of cell proliferation and tumor angiogenesis induced by stefin A transfection may contribute to inhibition of tumor growth; the other is that both reduced invasiveness and inhibition of tumor angiogenesis caused by stefin A transfection may contribute to inhibiting tumor metastasis.

In summary, transfection of stefin A CDNA into human EC9706 esophageal squamous cell carcinoma cells inhibits tumor growth, angiogenesis, invasion, and metastasis, and this is mainly through the inhibiting of cathepsin B activity. Similar results were observed in stefin A–transfected KYSE150 cells, another human esophageal squamous cancer cell line (not all data shown). Therefore, our study provides evidence of the role of stefin A expression in human esophageal squamous cell carcinoma cells and may shed a light of a novel strategy for cancer therapy.

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