Expression and clinical significance of cathepsin B and stefin A in laryngeal cancer

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Abstract. The aim of this study was to investigate the roles of the cathepsin B cysteine protease and its endogenous inhibitor stefin A in laryngeal cancer. Immunohistochemistry was employed to detect the expression of cathepsin B and stefin A in 84 patients with laryngeal cancer, respectively. The protein expression of stefin A was negatively associated with lymphatic metastasis, recurrence of laryngeal cancer and the survival rate, which was not observed with cathepsin B protein expression. Both down-regulation of cathepsin B and up-regulation of stefin A *in vitro* significantly inhibited the migration, invasion and proliferation of laryngeal cancer cells, respectively. Our results strongly suggest that stefin A may be a potential predictor of laryngeal cancer and may be used in the molecular diagnosis and gene therapy of laryngeal cancer. Cathepsin B may be used as a promising therapeutic target in the treatment of laryngeal cancer.

Introduction

Laryngeal cancer is a common head and neck malignancy and accounts for 7% of all malignancies, and the incidence of laryngeal cancer has been increasing over the years. Laryngeal cancer is associated with a high prevalence of local invasion and cervical lymph node metastasis, and a majority of patients die of cervical recurrence and metastasis. Investigations concerning the invasion and metastasis of laryngeal cancer may clarify the exact mechanisms underlying these processes and may identify novel therapeutic targets. The invasion and metastasis of cancers are complex, multistep, multifactor interacting, and dynamic biological processes involving cancer cells, host cells and the extracellular matrix (1). Invasion is a prerequisite of metastasis that depends on adherence and degradation of the extracellular matrix by degradative enzymes (2). Liotta *et al* proposed that the invasion and metastasis of cancers involve three steps: i) adherence between cancer cells and the extracellular matrix; ii) the release of proteolytic enzymes causing the degradation of the extracellular matrix; and iii) migration of cancer cells under the action of chemokines. This theory suggests that adherence, degradation and migration act sequentially and repetitively, ultimately resulting in sustained invasion and distal metastasis of cancer cells (3). The impairment of the basement membrane has been considered an important marker of cancer invasion, and the degradation of the extracellular matrix by proteolytic enzymes is a critical step of invasion and metastasis of cancer cells. Based on the substrates and pH values for enzyme catalysis, proteolytic enzymes can be classified into four types: serine proteases, matrix metalloproteinases, cysteine proteases, and aspartic proteases. These proteases have different biological functions related to cancer and play an important role in the occurrence, invasion, angiogenesis, and metastasis of cancers. Cancer invasion depends on the local concentration of proteases and the balance between proteases and their inhibitors. Under normal conditions, proteases and their endogenous inhibitors are in a dynamic balance. The interaction between proteases and their inhibitors contributes to the integrity or degradation of the extracellular matrix.

Cathepsins are intracellular proteases located in the lysosomes. They are activated under acidic conditions, and stefins (cystatins) are the inhibitors of cathepsins. Stefin A is the first reported inhibitor of the cathepsins that is linked with malignant tumors. Stefin A is highly expressed in epithelial cells and lymphoid tissues (4-6) and is closely associated with the occurrence and development of cancers. It has been reported that the expression and activities of cathepsin B and stefin A are altered in patients with malignancies, such as

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breast, lung, renal cell, colorectal, and head and neck cancers. These proteins are related to the progression and prognosis of cancers, and therefore, researchers have proposed that cathepsin B and stefin A may be used as predictors of certain types of cancers (7). The cathepsin B content in breast cancer is significantly higher than in adjacent normal mucosa; furthermore, high expression of cathepsin B predicts recurrence and a low 5-year survival rate (8). In addition, overexpression of stefin A was found to suppress the distant metastasis of breast cancer, which may be associated with the suppression of proteases such as cathepsin B (9). In patients with head and neck squamous cell carcinoma, no relationship has been found between cathepsin B expression and survival rate or prognosis, while stefin A was found to be closely associated with survival rate. Moreover, stefin A can be used as a potential predictor of head and neck carcinoma (10-12). Stefin A is an inhibitor of cysteine proteases and is negatively associated with malignancy of cancers. Therefore, stefin A may be a potential predictor of laryngeal cancer. Suppression of the urokinase plasminogen activator receptor (uPAR) and cathepsin B expression in glioma cells by RNA interference was found to reduce angiogenesis and the invasion of glioma (13). Reduced expression, both *in vivo* and *in vitro*, of cathepsin B and MMP-9 may also compromise the invasion and metastasis of malignant meningioma, resulting in a decreased tumor volume (14).

In the present study, we performed immunohistochemistry analysis of cathepsin B and stefin A expression in tumor tissues and adjacent normal mucosa of 84 patients with laryngeal cancer, and analyzed the correlation between both expression and clinicopathological parameters, respectively. Furthermore, migration, invasion and proliferation of laryngeal cancer cells after cathepsin B down-regulation and stefin A up-regulation were investigated *in vitro,* respectively. Our results strongly suggest that stefin A may be a potential predictor of laryngeal cancer and may be used in the molecular diagnosis and gene therapy of laryngeal cancer. Additionally, cathepsin B may be used as a promising therapeutic target in the treatment of laryngeal cancer.

Table I. Patient and tumor characteristic of the laryngeal carcinoma cases.

Characteristics	No. (%) for TMA
All patients	84 (100)
Male	68 (81.0)
Female	16 (19.0)
Age (years)	
Median	61
Range	39-84
Histological grade	
Well differentiated	44 (52.4)
Moderately differentiated	33 (39.3)
Poorly differentiated	7 (8.3)
Tumor site	
Supraglottic	45 (53.6)
Glottic	36 (42.9)
Subglottic	3 (3.5)
TNM stage	
I	8 (9.5)
II	24 (28.6)
III	23 (27.4)
IV	29 (34.5)
Recurrence	
Yes	24 (28.6)
No	60 (71.4)

Patients and methods

Sample collection. Laryngeal cancer tissues were obtained from 84 patients at the Department of Head and Neck Surgery, Cancer Hospital, Chinese Academy of Medical Sciences, from 1994 to 2004 (Table I). The range of age of the patients was 39-84 years, with a median age of 63 years. Follow-up was carried out for 11-122 months (median, 48 months). Local recurrence was noted in 24 patients, and 31 patients had cervical lymph node metastasis. Repeat cancer was noted in 3 patients. Radiotherapy and chemotherapy were not administered before surgery. The study was approved by the Ethics Committee of the Chinese PLA General Hospital and the patients gave their informed consent before their inclusion into the study.

Tissue microarray. Based on H&E staining, representative cancer tissues and adjacent normal mucosa were selected, and a total of 152 cancer tissues and adjacent normal mucosa was collected. Subsequently, a sampling needle was used to collect tissues (1 mm in diameter) at the designated region, which were then put into the pores of blank paraffin blocks. The paraffin blocks were then incubated at 38˚C for 30 min and pressurized with coverslips. Finally, these blocks were incubated at 50˚C in an oven for 10 h followed by continuous sectioning. In the cathepsin B and stefin A tissue microarray, 1 and 2 cases were dropped, respectively.

Immunohistochemistry. The tissues were embedded in paraffin and sectioned. These sections were heated at 80˚C for 4 h followed by deparaffinization with xylene. Hydration was then performed with an ethanol series followed by antigen retrieval with citrate buffer (pH 6.0). Sections were treated with 3% hydrogen peroxide at room temperature for 15 min to inactivate endogenous peroxidase followed by washing three times with distilled water. Sections were blocked with animal serum at room temperature for 10 min to block non-specific binding sites followed by incubation with the primary antibody at 4˚C overnight. After rinsing with PBS, sections were treated with the secondary antibody at room temperature for 20 min followed by washing with PBS. After incubation with streptavidin-peroxidase solution for 20 min at room temperature, development was performed with DAB solution for 5-10 min, and the reaction was terminated by PBS treatment. Counter staining was performed with hematoxylin, and sections were then air-dried and mounted. The sections were then observed under a microscope, and representative images were captured. In addition, the primary antibody was replaced

with PBS as a negative control. The primary antibodies against cathepsin B and stefin A were ab30443 and ab10442 (Abcam, Cambridge, UK), respectively.

Pathological analysis. According to the number of positive cells evaluated under a microscope, scoring was determined as follows: 0, <1%; 1, 1-24%; 2, 25-49%; 3, 50-74%; and 4, 75-100% positively stained cells. In addition, semi-quantitative analysis was carried out based on the staining intensity as follows: 0, no staining; 1, light yellow (weakly positive); 2, brownish yellow (intermediate staining); 3, brown (strong positive). The final scores were the products of both scores (15).

Cell culture, siRNA transfection, plasmid transfection and Western blot analysis. HEP-2 cells were purchased from the Institute of Basic Medical Sciences of Peking Union Medical College, China. HEP-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY), 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified 5% CO_2 incubator. siRNA sequences for cathepsin B (GGGATTCATAGAGGCCACC) and non-silencing siRNA (TTCTCCGAACGTGTCACGT) were used at 50 nmol/l, respectively. pcDNA3.1 (+)-stefin A was a kind gift from Dr Zhihua Liu (Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China). siRNAs and plasmids were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, respectively. Cell lysates were size-fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The antibodies mentioned in 'Immunohistochemistry' were used to detect cathepsin B and stefin A, respectively. β-actin (A5441, Sigma-Aldrich) was used as a loading control.

Cell migration and invasion assay. Matrigel (1 mg/ml; BD Biosciences, Bedford, MA) was used to cover the chambers of the Transwell inserts (Corning, Corning, NY), which were then placed into a 24-well plate. HEP-2 cells (10^5/100 μl) in DMEM were added to the upper chamber and DMEM containing 10% FBS was added to the lower chamber, followed by culture for 48 h. The cells were then fixed in 75% methanol followed by staining with 0.1% crystal violet. The procedures for the detection of migration were similar to those for the detection of invasion except that the chambers were not coated with Matrigel. In addition, $5x10^4$ HEP-2 cells were seeded followed by incubation for 12 h and subsequent cell counting.

Cell proliferation assay by MTT. HEP-2 cells were harvested 48 h after siRNA and pcDNA3.1 (+)-stefin A transfection and then seeded in a 96-well plate at a density of 10^3/well. Cells were grown in DMEM containing 10% FBS, and 20 μl of MTT solution (5 mg/ml; Sigma-Aldrich, St. Louis, MO) was added to each well after 1, 2, 3, 4, 5 and 6 days of culture followed by incubation for another 4 h. The supernatant was then removed, and 150 μl DMSO was added followed by gentle vortexing for 10 min. When the crystals were dissolved, the absorbance was detected at 490 nm with a microplate reader.

Statistical analysis. Statistical analysis was performed using SPSS version 12 software (SPSS Inc., Chicago, IL). Independent t-tests or ANOVAs (for more than one group) were used to determine the significance of the difference between means. P-values <0.05 were considered significant. Survival curves were established according to the Kaplan-Meier method, and comparisons between survival curves were performed using the log-rank test. Overall survival was calculated from the day of tumor challenge to death determined by tumor size and ethical guidelines.

Table II. The analysis of cathepsin B/stefin A expression in laryngeal cancer patients.

	n	χ^2	P-value
Cathepsin B	83	7.301	0.008
Stefin A	82	3.979	0.046

Results

Expression levels of cathepsin B and stefin A in laryngeal squamous cell carcinoma and their association with clinicopathological characteristics. The patient population consisted of 70 males and 14 females, with a median age of 63 years (range, 39-84 years). Follow-up was performed for 11-122 months (median, 48 months). Local recurrence was noted in 24 patients, and 31 patients had cervical lymph node metastasis. Repeat cancer was found in 3 patients. In the cathepsin B and stefin A tissue microarray, 1 and 2 cases were dropped respectively.

Immunohistochemical examination showed that cathepsin B and stefin A were mainly located in the membrane and cytoplasm of cells in the cancer nests. Cathepsin B expression was markedly higher in laryngeal cancer than in the adjacent normal mucosa (P=0.008, <0.05). However, cathepsin B expression was not related to the site, stage, or differentiation of the cancer. In addition, cathepsin B expression was not statistically different between the patients with and without lymph node metastasis (P=0.593) or between those with and without recurrence (P=0.872).

Stefin A expression was significantly higher in laryngeal cancer than in the normal mucosa (P=0.046). Stefin A expression was also not associated with the site, stage, or differentiation of the cancers. However, stefin A expression was markedly lower in the patients with lymph node metastasis than in those without lymph node metastasis (P=0.004), and a significant difference was also observed between patients with and without recurrence (P=0.001). These findings suggest that patients with low stefin A expression are at risk for lymph node metastasis and cancer recurrence (Fig. 1, Tables II and III).

Survival analysis. Statistical analysis revealed that cathepsin B protein expression was not related to prognosis (P=0.889). Kaplan-Meier survival curves were delineated and log-rank tests showed that there was no significant difference in the survival rate between patients with high and low cathepsin B protein expression.

As an endogenous inhibitor, stefin A protein expression was closely related to the prognosis of laryngeal cancer

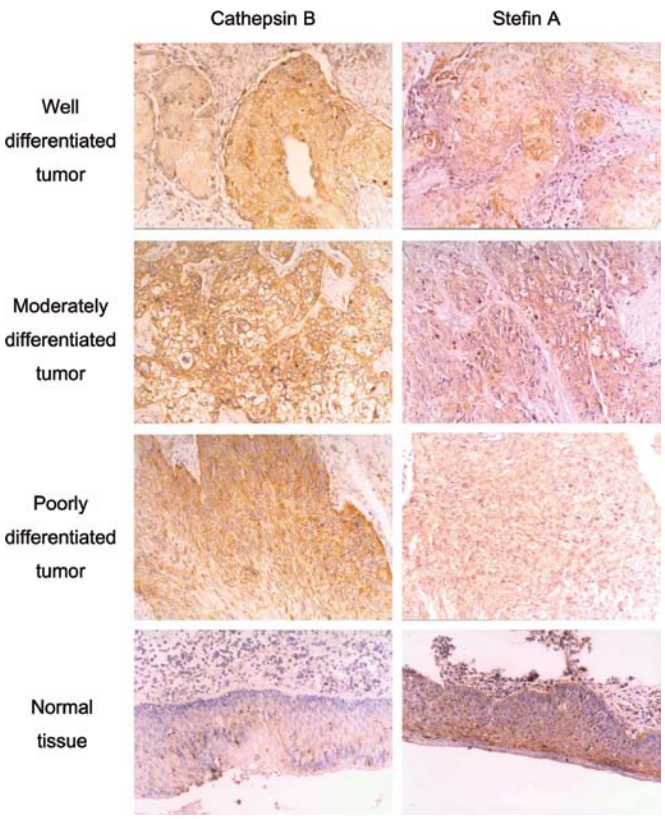

Figure 1. Immunohistochemical staining of cathepsin B and stefin A in the laryngeal cancer tissue microarray, respectively (x200).

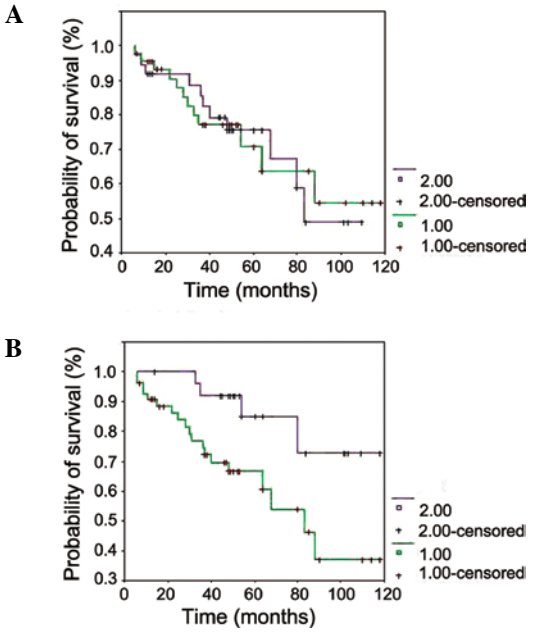

Figure 2. The prognostic significance of cathepsin B and stefin A expression in the laryngeal cancer patient population. Kaplan-Meier survival analysis demonstrates the effect of cathepsin B and stefin A expression on overall survival. (A) Cathepsin B expression level shows no relationship with prognosis in laryngeal cancer (P=0.889). (B) Stefin A expression level shows a significant relationship with prognosis (P=0.0179); high expression of stefin A indicates a high survival rate. 1.00, low expression or no expression; 2.00, high expression.

Table III. Correlation between cathepsin B/stefin A expression and clinicopathological characteristics of the human laryngeal cancer cases.

	Cathepsin B		Stefin A	
	n	P-value	n	P-value
Clinical stage		0.928		0.953
I-II	31		30	
III-IV	52		52	
Tumor type		0.433		0.086
Supraglottic	45		45	
Glottic	35		34	
Subglottic	3		3	
Histological grade		0.227		0.228
Well differentiated	43		43	
Moderately differentiated	33		32	
Poorly differentiated	7		7	
Lymph node metastasis		0.593		0.004
pN0	52		51	
pN1-3	31		31	
Recurrence		0.872		0.001
Yes	24		24	
No	59		58	

(P=0.0179). Low stefin A expression predicted a low survival rate. The results revealed that the 3- and 5-year survival rates were 74.6 and 66.7%, respectively, in patients with low stefin A protein expression and were 92 and 84.9%, respectively, in patients with high stefin A protein expression. Log-rank tests showed a marked difference in the survival rate (Fig. 2).

Effects of cathepsin B down-regulation on the biological functions of HEP-2 cells. The siRNA targeting cathepsin B was applied to down-regulate cathepsin B expression in HEP-2 cells. Cell migration and invasion were determined by transwell assays, and cell proliferation was detected by MTT assays. The results showed that down-regulation of cathepsin B expression significantly inhibited invasion (P<0.05) and migration (P<0.05) of HEP-2 cells, accompanied by decreased cell proliferation (Fig. 3).

Effects of stefin A up-regulation on the biological functions of HEP-2 cells. After transfected with pcDNA3.1 (+)-stefin A, migration and invasion of HEP-2 cells were significantly decreased (P<0.05), compared with the cells transfected with the empty vector. In addition, cell growth was also inhibited (Fig. 4).

Discussion

Cathepsins are involved in multiple biological processes including tissue remodeling during embryonic development, wound healing, antigen-presentation, bone resorption, and apoptosis. High cathepsin B expression has been noted in

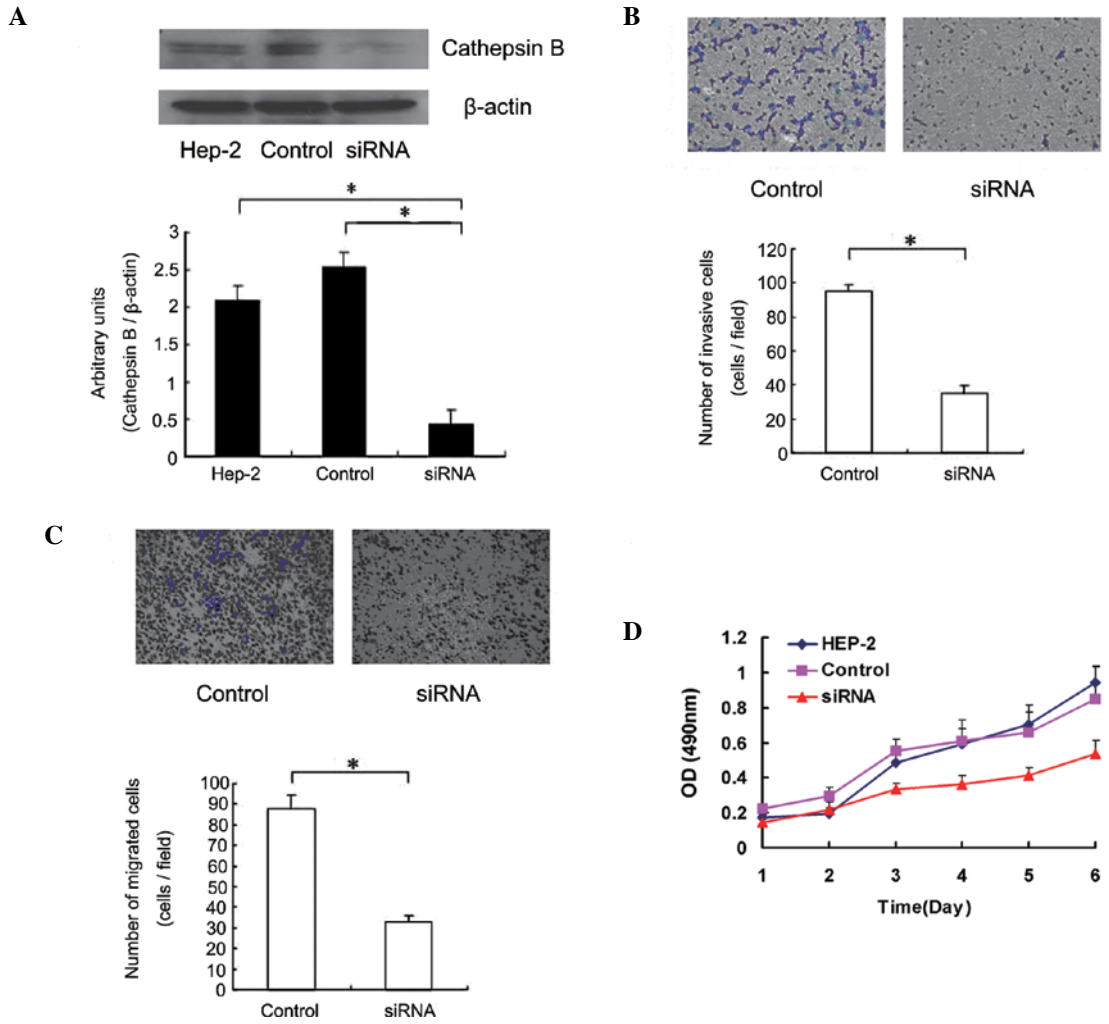

Figure 3. Cathepsin B RNAi inhibits the invasion, migration and growth of HEP-2 cells. (A) Western blot analysis of cathepsin B RNAi. β-actin was used as a loading control. Ratios of cathepsin B/corresponding β-actin quantification were calculated and represent the relative expression level of cathepsin B protein. Columns in the histogram represent the mean of three independent experiments; bars, SD; *P<0.01. (B) Matrigel invasion of the HEP-2 cells treated by non-silencing siRNA and specific cathepsin B siRNA, respectively. Columns in the histogram represent the mean of three independent experiments; bars, SD; *P<0.01. (C) Migration of the HEP-2 cells treated by the non-silencing siRNA and the specific cathepsin B siRNA, respectively. Columns in the histogram represent the mean of three independent experiments; bars, SD; *P<0.01. (D) Growth curves of HEP-2, non-silencing-treated HEP-2 and specific cathepsin B siRNA-treated HEP-2 cells. Data points represent the mean of three independent experiments; bars, SD.

several tumor typess and is mainly found in the extracellular space and tumor invasion margins. Therefore, researchers have proposed that cathepsin B plays critical roles in the invasion and metastasis of cancers (16-18). Research demonstrated that the expression and activity of these enzymes are related to cancer stage and metastasis and that these enzymes may be used as predictors (7). Increased cathepsin B activity is partially attributed to its increased expression and partially to decreased expression of stefins, which are inhibitors of cathepsins (19). The balance between cathepsins and their inhibitors is related to metastasis and the phenotype of cancer cells. Cathepsin B is involved in all stages of malignant transformation, including precancerous lesions, and also in growth, angiogenesis, invasion and migration of cancers. Inhibitors of cathepsins include stefin A (cystatin A), cystatin C and kininogens. In prostate, breast, lung, colorectal, head and neck, uterine and metastatic ovarian cancers, it has been reported that the imbalance between cathepsins and their inhibitors may result in metastasis and invasion. As such, the expression and/or activity of cathepsins and their inhibitors may be used as indicators for evaluating survival rates and recurrence risks (10,20-24). Recent studies have shown that small molecule inhibitors of recombinant proteases (stefin A/B, AC-LVK-CHO) and a specific cathepsin B inhibitor (CA-074) can suppress the metastasis of ovarian and breast cancers (25-27). In malignancies including glioma, meningioma, prostate cancer and oral cancer, RNA interference was used to down-regulate tumor-related genes including cathepsin B, and the results showed significantly inhibited invasion and metastasis (13,14,21,28).

Our results found higher cathepsin B expression and lower stefin A expression in laryngeal cancer than in adjacent normal mucosa, respectively, which were consistent with the findings of Strojan *et al* (10), while the difference was not as significant as in the previous study, which may be related to the patients' race and geographical locations. It has been shown that cathepsin B expression is usually not proportional to stefin A expression, which suggests that stefin A is not a unique

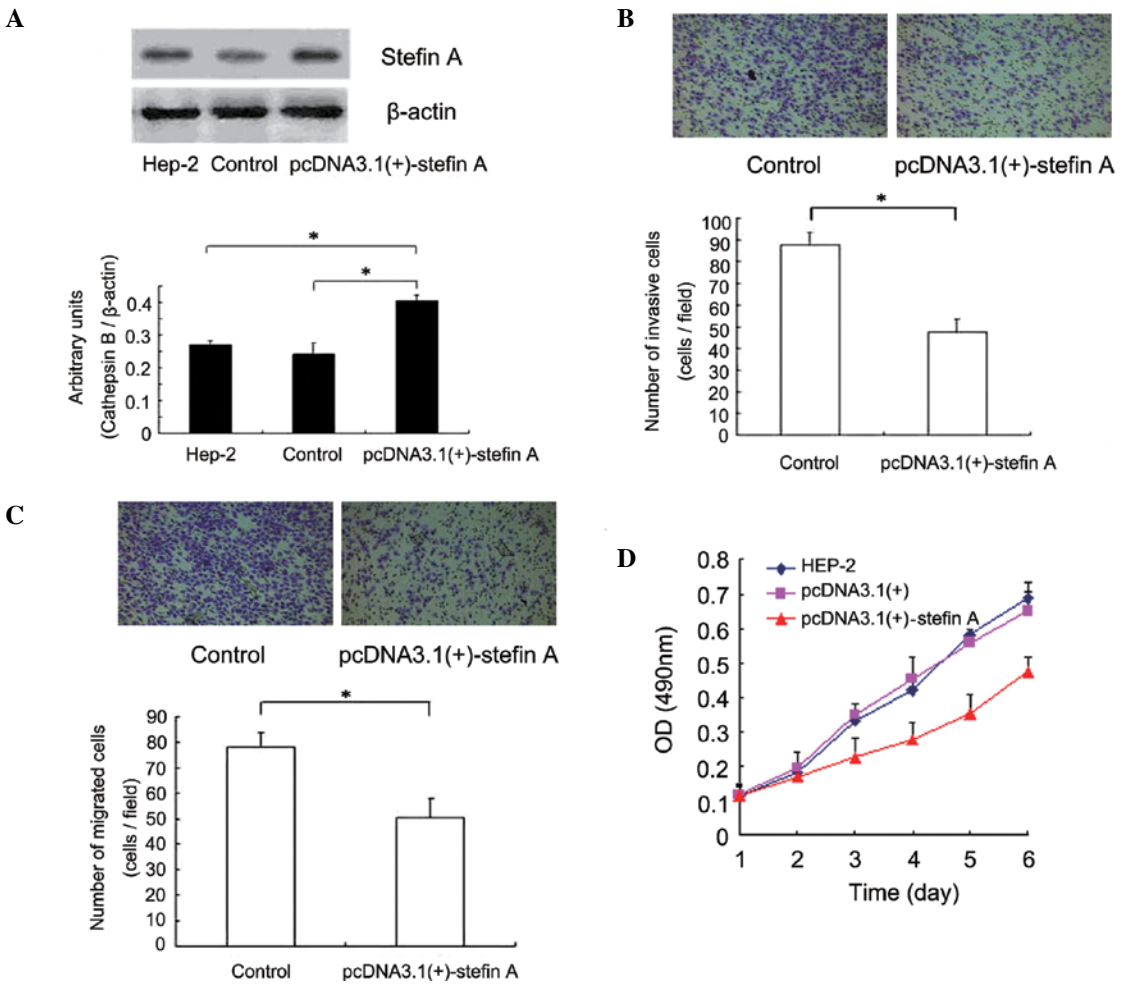

Figure 4. Overexpression of stefin A inhibits the invasion, migration and growth of HEP-2 cells. (A) Western blot analysis of stefin A overexpression. β-actin was used as a loading control. Ratios of stefin A/corresponding β-actin quantification were calculated and represent the relative expression level of stefin A protein. Columns in the histogram represent the mean of three independent experiments; bars, SD; *P<0.01. (B) Matrigel invasion of the HEP-2 cells transfected with pcDNA3.1 (+)-stefin A and the empty vector, respectively. Columns in the histogram represent the mean of three independent experiments; bars, SD; *P<0.01. (C) Migration of the HEP-2 cells transfected with pcDNA3.1 (+)-stefin A and the empty vector, respectively. Columns in the histogram represent the mean of three independent experiments; bars, SD; *P<0.01. (D) Growth curves of the HEP-2 cells, cells transfected with pcDNA3.1 (+)-stefin A and the empty vector, respectively. Data points represent the mean of three independent experiments; bars, SD.

endogenous inhibitor of cathepsin B. Under certain conditions, stefin A and other inhibitors may play important roles in regulating cathepsin B. Stefin A can inhibit cathepsin B through i) a direct but weak interaction between stefin A and cathepsin B or ii) a conformational change resulting in strong inhibition (29). In the present study, the level of cathepsin B was elevated, but no association between cathepsin B activity and metastasis or recurrence was observed. Stefin A regulates not only cathepsin B but also cathepsin H, S, and L. Therefore, stefin A may play a more important role in the metastasis and invasion of cancers than cathepsin B alone. Our results showed that the protein expression of cathepsin B was not associated with lymph node metastasis, cancer recurrence, or prognosis. However, stefin A protein expression was associated with lymph node metastasis. In 31 patients with confirmed lymph node metastasis, stefin A expression was associated with cancer recurrence (P=0.001). In the present study, 24 patients had recurrence. In addition, low stefin A expression predicted a high risk for lymph node metastasis and recurrence. In our study, follow-up was carried out in these patients, and Kaplan-Meier survival curves were delineated. The results showed that stefin A expression was strongly related to prognosis (P=0.0179), and low stefin A expression predicted a low survival rate (discrete trend in survival curves). Based on the above findings, stefin A may be a potential predictor of laryngeal cancer. Therapies based on levels of stefin A may be a promising strategy for improving the survival rate in these patients.

To further confirm the roles of cathepsin B and stefin A in laryngeal cancer, RNA interference of cathepsin B and overexpression of stefin A were carried out in HEP-2 cells *in vitro*, respectively, both of which significantly compromised the invasion, migration, and growth of cancer cells. These results were consistent with previous studies involving prostate cancer, glioma, meningioma, and oral cancer. Our findings provide evidence for the roles of cathepsin B and its inhibitor stefin A in laryngeal cancer and highlighted the possibility of individualized treatment for laryngeal cancer. Suppression of cathepsin B expression may be a potential and promising method by which to prevent invasion and metastasis of laryngeal cancer. Stefin A may be a potential predictor of laryngeal

cancer and may be used in the molecular diagnosis and gene therapy of laryngeal cancer.

Acknowledgements

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