

The role of hepatocyte growth factor activator inhibitor-1 (HAI-1) as a prognostic indicator in cervical cancer

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Abstract. Hepatocyte growth factor activator inhibitor-1 (HAI-1) is a Kunitz-type serine protease inhibitor that has a broad inhibitory spectrum against serine proteases. This is the first study to investigate the role of HAI-1 and its clinical importance in cervical cancer. We attempted to investigate the inhibitory effects of HAI-1 using cervical carcinoma cell lines SiHa with integrated human papillomavirus (HPV) 16 and HeLa with integrated HPV 18. HAI-1 expression in cervical cancer (n=91) were assessed by immunohistochemistry. HAI-1 was found to be a potential inhibitory effects mediated by reduction of hepsin, matriptase and prostasin expression. This led to apoptosis through a reduction in the levels of Bcl-2, Bcl-xL, MUPP-1 and MAGI-3 in cervical cancer cell lines. There were significant correlations between HAI-1 expression and stage (p=0.013), tumor size (p=0.002), stromal invasion (p<0.001), vaginal invasion (p=0.031), parametrial invasion (p=0.012), lymph-node metastasis (p=0.019), and LVS involvement (p=0.002). The disease-free and overall survival rates of patients exhibiting high HAI-1 expression were significantly higher than those of patients exhibiting low HAI-1 expression (p=0.022 and p=0.011, respectively). The present study proposes that these findings may be considered HAI-1 as a therapeutic target for

treatment and identify as a favorable prognostic marker for cancer patients of cervical cancer.

Introduction

Cervical cancer is the most important manifestation of human papillomavirus (HPV) infection and is one of the leading causes of cancer mortality in women worldwide. The causal roles of some high-risk HPV types in cervical carcinogenesis has now been clearly established by studies that take into account the many molecular, epidemiological, virological, cytological and histological complexities of the natural history of the disease (1).

Cell surface proteolysis is suggested to play a major role in cancer progression and metastasis through the processing of macromolecules important for regulating the extracellular environment. The cell surface localization, high activity, and exquisite specificity of the type II transmembrane serine protease suggest a role in outside-in signaling and interaction with the microenvironment. Members of the type II transmembrane serine protease family, such as hepsin and matriptase are highly expressed in many cancers, including those of the cervix, prostate, breast and ovarian cancer (2-6). Matriptase and hepsin have been proposed to initiate signaling and proteolytic cascades through its ability to activate pro-urokinase plasminogen activator (uPA) (7-9). Matriptase is known to interact with the glycosylphosphatidylinositol-anchored membrane serine protease of prostasin (10). Matriptase, hepsin and prostasin are potential candidates for the cellular activities of pro-hepatocyte growth factor (pro-HGF) in cancer tissues (11-13). Importantly, the activities of these proteinases are closely controlled in the pericellular microenvironment by HGF activator (HGFA) inhibitor (HAI). HAI is a type I transmembrane protein with two Kunitz-type serine proteinase domains (KDs) and in its extracellular portion. Two types of HAI have been identified so far, HAI-1 and HAI-2, both of which show cell surface expression on epithelial cells. Therefore, overall structures of the characteristic domains are very similar between HAI-1 and HAI-2. In addition, between KDs, HAI-1 has the ligand binding region of low-density lipoprotein (LDL) receptor. HAI-1 is synthesized as a transmembrane protein on the cell surface and appears to be subsequently shed, but membrane-form HAI-2 cannot. The membrane-bound HAI-1 is able to

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Abbreviations: HAI-1, hepatocyte growth factor activator inhibitor-1; HPV, human papillomavirus; uPA, urokinase plasminogen activator; HGF, hepatocyte growth factor; HGFA, HGF activator; KDs, Kunitz-type serine proteinase domains; LDL, low-density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FIGO, International Federation of Gynecology and Obstetrics; PI, propidium iodide

Key words: hepatocyte growth factor activator inhibitor-1, cervical cancer, apoptotic pathway, therapeutic target, favorable prognostic marker

form a complex with active HGFA and may function to temporarily sequester HGFA to the cell surface and serve as a reservoir for HGFA (14). The shed form of HAI-1 is active in inhibiting HGFA, matriptase, hepsin and prostasin, all of which are pro-HGF activators (11,15,16). The functional relevance of HAI-1 inhibition of matriptase was confirmed in a transgenic mouse model in which matriptase-induced skin tumorigenesis was completely prevented by the over-expression of HAI-1 in the same tissue (17). To date, several studies on HAI-1 expression in tumor tissues have been published. In prostate, breast and gastric cancer it has been reported that the reduced expression of HAI-1 is possibly involved in the cancer progression (18-20). However, it is not clear how HAI-1 is involved that the role of HAI-1 in cervical cancer.

In order to shed light on the role of HAI-1 in cervical cancer, we examined whether HAI-1 protein expression correlates with clinicopathological characteristics in patients suffering from cervical cancer. The main aim of this study was to determine whether HAI-1 protein could be considered as a therapeutic target for treatment and used as a favorable prognosis marker for patients of cervical cancer.

Materials and methods

Cell culture, media and generation of transfectants. HeLa (ATCC no. CCL-2), Caski [Japanese Collection of Research Bioresources (JCRB) no. IFO50007], SiHa (ATCC no. HTB-35) and Yumoto cancer cell lines were derived from human cervical carcinomas. Yumoto cells were kindly provided by Dr A. Mitsunashi (Chiba University, Chiba, Japan), as described previously (21). HPV 16 type Caski and SiHa cell lines, 18 type HeLa cell lines and non-HPV type Yumoto were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere with 5% CO₂ in air. A cDNA encoding the whole coding region of HAI-1 was constructed by polymerase chain reaction using full-length HAI-1 cDNA as a template. Primers used were, 5'-TTGGAATTCGCGATGGCCCCTGCGAGGAC-3' and 5'-TTAGTCTGACTCAGAGGGGCGGGTGGTGT-3' for HAI-1. The polymerase chain reaction products were sub-cloned into the EcoRI/SalI site of an expression plasmid pCIneo (Promega, Madison, WI). A HAI-1 cDNA expression vector was transfected into all cervical cancer cell lines using TransFast transfection reagent (Promega). Mock transfected cells served as a control.

Western blotting analysis. Cell lysates were collected and estimated using a Protein Assay system (Bio-Rad, Hercules, CA) according to the manufacturer's protocols. Proteins from each cell line were subjected to SDS-PAGE and were transferred onto a nitrocellulose membrane. The polyclonal and monoclonal antibodies used for immunoblotting were as follows: HAI-1, matriptase, prostasin, Bcl-2, Bcl-xL, MUGG-1 and MAGI-3 (Santa Cruz Biotechnology, Santa Cruz, CA), hepsin (Cayman Chemical, Ann Arbor, MI) and β -actin (Sigma Chemical, St. Louis, MO). The working dilution of all primary antibodies was 1:1000. After incubation of membranes with the appropriate secondary antibodies,

antigen-antibody complexes were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Matrigel invasion assay. To investigate differences in the invasive abilities of cells expressing HAI-1, we used BioCoat Matrigel Invasion Chambers (BD Bioscience, Bedford, MA). SiHa and HeLa cervical cancer cells with or without transient transfection of the HAI-1 vector were incubated *in situ* with 10 μ g/ml of Dil (Invitrogen) in DMEM containing 10% FBS for 1 h. The labeled cells (5×10^4) of each genotype were then added to the chambers inserts, and 0.75 ml of medium was added to the bottom of each well. After 48 h of incubation, the membranes were removed from the insert and mounted on slides, and the numbers of invading cells were counted under the microscope. The matrigel assays were performed in triplicate.

MTS assay. The effects of HAI-1 on cell proliferation were evaluated using the MTS assay (Promega). Cells were seeded into 96-well plates and cultured until the cell density reached 5×10^4 cells/well. The cells were then transiently transfected with the HAI-1 vector for 48 h. After incubation with MTS for 1 h, the absorbances were measured at a wavelength of 490 nm using an ELISA plate reader (Bio-Rad Systems).

Cell cycle and FITC-conjugated Annexin V analysis. The cells subjected to transient transfection of the HAI-1 vector were trypsinized, washed twice with phosphate-buffered saline, incubated with trypsin for 10 min at room temperature, incubated with trypsin inhibitor and RNase buffer for 10 min at room temperature, and stained with 200 μ l propidium iodide staining solution for 10 min in the dark, at room temperature (Becton-Dickinson, San Jose, CA). Finally, the cell cycles were analyzed with the FACS cytometer. In addition, apoptosis was measured by staining with FITC-conjugated Annexin V using a MEBCYTO apoptosis kit (MBL, Nagoya, Japan) according to the manufacturer's recommendations and flow cytometry analysis (Calibur™, Becton-Dickinson).

Cell growth in a monolayer and in soft agar. To obtain a monolayer of cell growth, cells were plated at a density of 3×10^4 cells/well in 6-well plates containing DMEM with 10% FBS. The cell numbers were counted in triplicate after 2, 4, 6, 8 and 10 days using a hemocytometer to assess cell proliferation. For the soft agar determination of cell growth, a cell suspension (1×10^4 cells/well) in 1 ml 0.2% Noble Agar DMEM with 10% FBS was overlaid onto a 35 mm dish containing a 0.5% agar base. Colonies >0.2 mm in diameter were counted 21 days after culture. Soft agar assays were performed in triplicate.

Patients. The patient population consisted of 91 patients presenting with the International Federation of Gynecology and Obstetrics (FIGO) stage IB-IIB cervical cancers. Each of these patients underwent radical hysterectomy and pelvic lymphadenectomy at the Department of Obstetrics and Gynecology of Okayama University Hospital. Patients with neoadjuvant chemotherapy were excluded from this study.

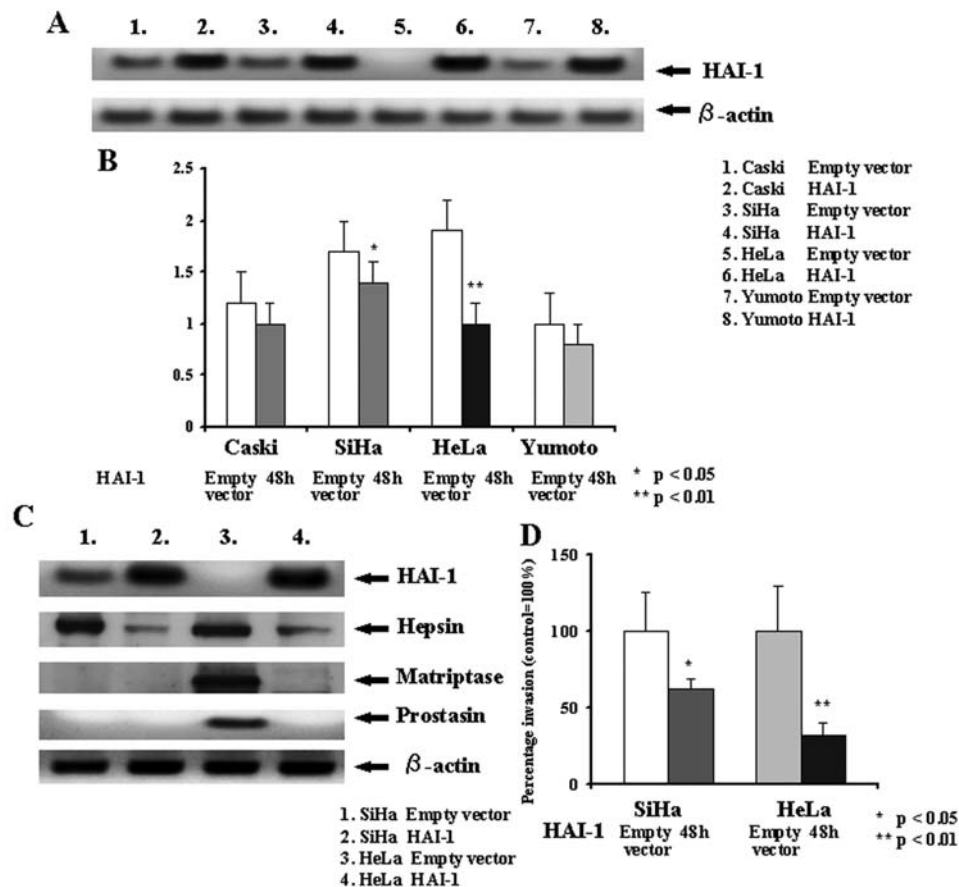


Figure 1. Evaluation of the transient transfection of the hepatocyte growth factor activator inhibitor-1 (HAI-1) vector into cervical cancer cells for 48 h. (A) Western blot analysis of HAI-1 expression after the transient transfection of the HAI-1 vector into Caski, SiHa, HeLa and Yumoto cervical cancer cells for 48 h. β-actin antibody was used as the loading control in the same blot. (B) MTS assays of cell proliferation of Caski, SiHa, HeLa and Yumoto cervical cancer cells after transient transfection of the HAI-1 vector into cervical cancer cells for 48 h. The assays were carried out for quadruplicate transfection experiments. (C) Western blot analysis of HAI-1, hepsin, matrilysin and prostatein expression levels after transient transfection of the HAI-1 vector into SiHa and HeLa cervical cancer cells for 48 h. β-actin antibody was used as the loading control in the same blot. (D) Matrigel invasion assay after transient transfection of the HAI-1 vector into SiHa and HeLa cervical cancer cells. Following incubation, membranes were removed from the insert and mounted on slides. The numbers of invading cells were counted under the microscope. The matrigel assays were performed in triplicate.

Tumor specimens were obtained at the time of surgery, immediately fixed in 10% neutral-buffered formalin and then embedded in paraffin. The histological cell types were conducted according to the WHO classification as follows: 54 were classified as squamous cell carcinomas, 27 as adenocarcinomas and 10 as adenosquamous carcinomas. Clinical staging was assessed based on the FIGO staging system as follows: 45 were allocated to stage IB, 5 to stage IIA and 41 to stage IIB. The median age at the time of surgery was 45 years (range 27-66 years). Patients with lymph node metastasis, parametrial involvement, deep stromal invasion or marked lymph-vascular space involvement were treated with an adjuvant external whole pelvic irradiation, combination chemotherapy or chemoradiation. The disease-free survival (DFS) and overall survival (OS) rates were defined as the interval from the initial surgery to clinically or radiologically proven recurrence and death, respectively. The end date of the follow-up study for conducting the analysis was 30th September 2007, and median duration of the follow-up was 79 months (range 4-146).

Immunohistochemical analysis and staining evaluation. Formalin-fixed paraffin-embedded sections at 4 μm thick

were deparaffinized with xylene and rehydrated in ethanol. Endogenous peroxidase activity was quenched by treatment with methanol containing 0.3% hydrogen peroxidase for 15 min. Then, the sections were incubated with a primary antibody for HAI-1 at room temperature, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were counterstained with hematoxylin. The levels of HAI-1 staining in the tumors were classified into three groups by scoring the percentages of positively stained cell: 2, strong staining, >50% of cells; 1, moderate staining, 10-50% of cells; and weak staining, <10% of cells. Two independent examiners with no prior knowledge of the patient clinical data conducted the microscopic evaluations. Controversial cases were evaluated using a conference microscope.

Statistical analysis. Statistical analyses were performed using the Mann-Whitney U test for comparison with the control and one-factor ANOVA followed by Fisher's protected least significance difference test for all-pair wise comparisons. Analyses were performed with the software package Stat View version 5.0 (Abacus Concepts, Berkeley, CA). Differences were considered significant at p<0.05.

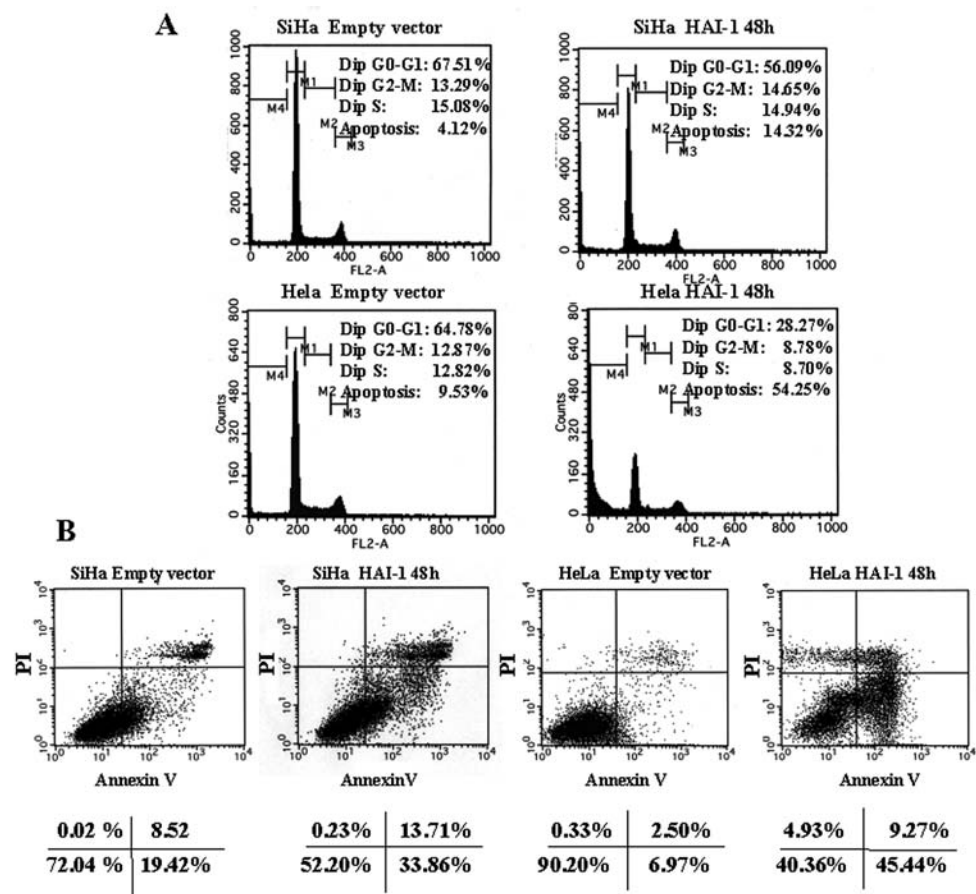


Figure 2. (A) Effects on the cell cycle by the transient transfectoin of the hepatocyte growth factor activator inhibitor-1 (HAI-1) vector into SiHa and HeLa cervical cancer cells for 48 h. Representative cell cycle profiles, measured by propidium iodide (PI) staining with the percentages of cells in G₀-G₁ phase, S phase, G₂-M phase and apoptosis phase are shown. (B) Effects on apoptosis by the transient transfection of the HAI-1 vector into SiHa and HeLa cervical cancer cells. Representative flow cytometric data of apoptosis in transient transfectoin of the HAI-1 vector into SiHa and HeLa cervical cancer cells for 48 h.

Results

Expression of HAI-1 in cervical cancer cell line. We first examined HAI-1 expression in various cervical cancer cell lines. As shown in Fig. 1A, HAI-1 protein level was almost absent in the HeLa cell lines and abundantly expressed in Caski, SiHa and Yumoto cell lines. To examine functional role of HAI-1, transient transfection of the HAI-1 vector into cervical cancer cell lines which overexpress HAI-1 were generated as described in Materials and methods. The transient transfection of the HAI-1 vector caused significant increase HAI-1 protein levels in all fourth cervical cancer cell lines.

MTS assay by HAI-1. We used MTS assays to examine cell proliferation into Caski, SiHa, HeLa and Yumoto cervical cancer cell lines caused by HAI-1. The transient transfection of the HAI-1 vector into these fourth cervical cancer cell line caused reduced cell growth by MTS assay. And, the inhibitory effect was most prominent in HeLa cervical cancer cell lines (Fig. 1B).

Effect of the target enzyme by HAI-1. Serine protease has been implicated in degradation of the extracellular matrix, and in modulation of cell-substratum adhesions in tumor cells associated with metastasis. The functional roles of HAI-1 were

examined by transient transfection of the HAI-1 vector into SiHa and HeLa cell lines followed by Western blotting. The transient transfection of the HAI-1 vector into SiHa and HeLa cell lines caused a significant increase in expression of HAI-1. The levels of hepsin expression were decreased following transient transfection of the HAI-1 vector into SiHa and HeLa cells. The level of matriptase and prostatic expressions was significantly decreased by transient transfection of the HAI-1 vector into HeLa cells. Matriptase and prostatic expression was not detected in SiHa cells without or without transient transfection of the HAI-1 vector (Fig. 1C).

Matrigel invasion assay by HAI-1. We assessed the motility and invasiveness of SiHa and HeLa cells overexpressing HAI-1 by using a BioCoat Matrigel invasion chamber. Cells were plated on the upper surface of the membrane, and transient transfection of HAI-1 after 48 h. Next, the cells invading to the bottom side of the membrane were stained and counted. The percentage of cells reaching the bottom of the filter were decreased to 58.32 and 38.81% at 48 h after transient transfection of the HAI-1 vector into SiHa and HeLa cells, respectively (Fig. 1D).

Cell cycle assay by HAI-1. Cells usually respond to genotoxic stress by activating cell cycle checkpoints and by blocking further progression into the cell cycle, allowing the cells to

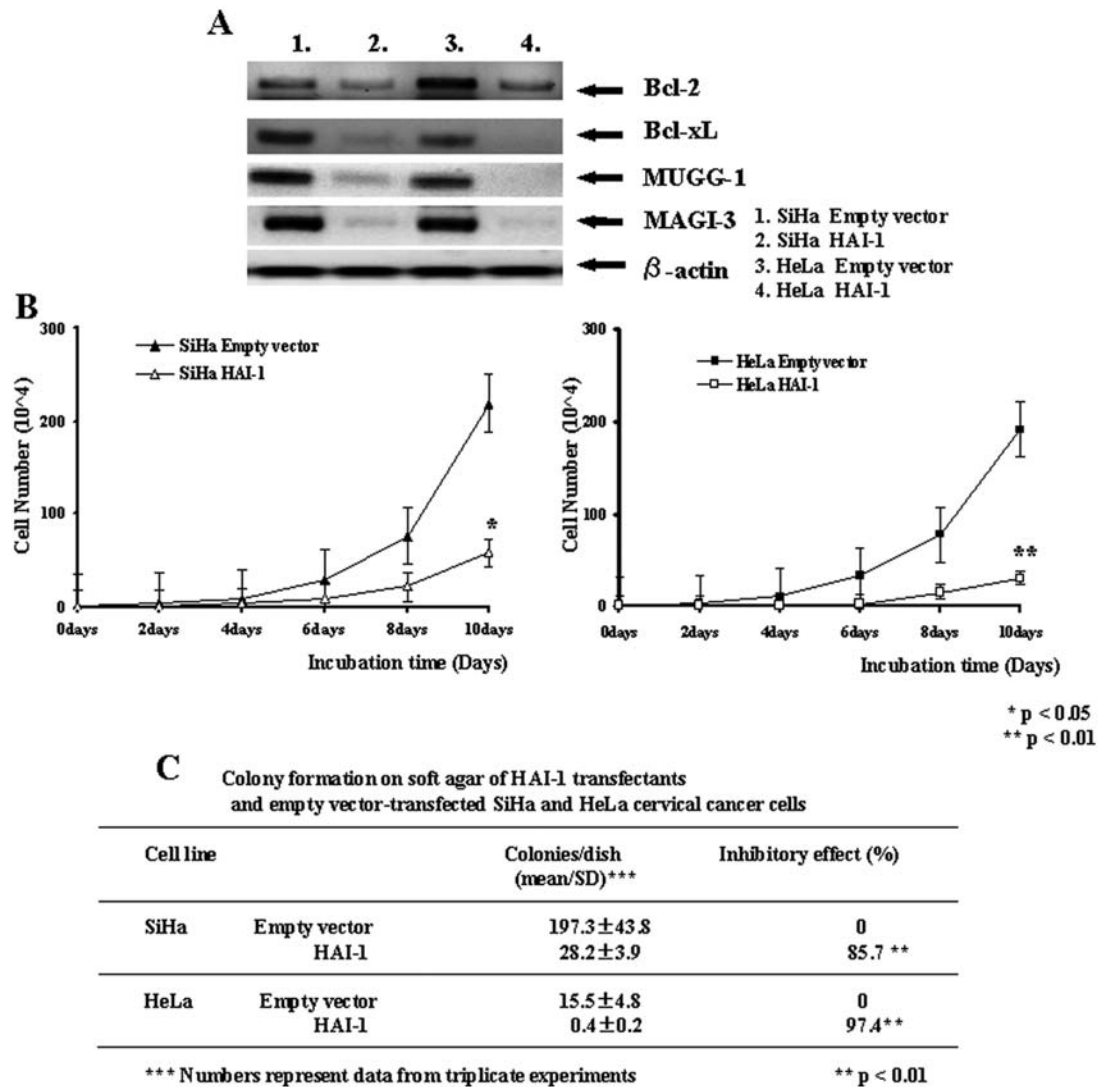


Figure 3. (A) Effects on the apoptotic and PDZ domain-containing protein pathway by the transient transfection of the hepatocyte growth factor activator inhibitor-1 (HAI-1) vector into SiHa and HeLa cervical cancer cells. The expression levels of Bcl-2, Bcl-xL, MUPP-1 and MAGI-3 were detected by Western blotting. β-actin antibody was used as the loading control in the same blot. Effects on cell growth by the transient transfection of the HAI-1 vector into SiHa and HeLa cervical cancer cells for 48 h. (B) Monolayer growth of the transient transfection of the HAI-1 vector into SiHa and HeLa cervical cancer cells incubated for 2, 4, 6, 8 and 10 days in DMEM supplemented with 10% FBS. Numbers represent data from triplicate experiments. (C) Colony formation on soft agar of the transient transfection of the HAI-1 vector into SiHa and HeLa cervical cancer cells. Numbers represent data from triplicate experiments.

repair DNA damage. The following cell cycle profiles were obtained from transient transfection of the HAI-1 vector into SiHa and HeLa cells. The cell cycle arrest was mainly observed at the apoptosis phases in the transient transfection of the HAI-1 vector into SiHa and HeLa cells, supporting the idea that HAI-1 acts as an inhibitor of cell growth (Fig. 2A).

Apoptotic assay by HAI-1. We used FITC-conjugated Annexin V assays to examine apoptosis expression after transient transfection of the HAI-1 vector into SiHa and HeLa cells. Representative flow cytometric data revealed that transient transfection of the HAI-1 vector for 48 h showed an increase in Annexin V-FITC- and propidium iodide (PI)-positive signals. HAI-1 induced apoptosis in these cancer cells, which was assessed by Annexin V-FITC assay as shown in Fig. 2B. HAI-1 increased the total apoptotic and necrotic cells to 47.80 and 59.64% as compared with the control of 27.96 and 9.80% for SiHa and HeLa cells, respectively.

Furthermore, PI-negative and Annexin V-FITC-positive signals were significantly correlated more than PI-positive and Annexin V-FITC-negative signals, and PI-positive and Annexin V-FITC-positive signals. Therefore, HAI-1 was likely to regulate the early apoptosis process rather than late apoptosis and necrosis.

We also investigated the effect of HAI-1 on the protein levels of some apoptotic molecules such as anti-apoptotic Bcl-2 and Bcl-xL, and PDZ domain-containing MUPP-1 and MAGI-3 protein. As shown in Fig. 3A, the expression of Bcl-2, Bcl-xL, MUPP-1 and MAGI-3 were down-regulated in the transient transfection of the HAI-1 vector into SiHa and HeLa cells.

Cell growth in monolayers and anchorage-independent cell growth in soft agar by HAI-1. Effects of HAI-1 expression on cell proliferation were analyzed using the transient transfection of the HAI-1 vector into SiHa and HeLa cervical cancer cell

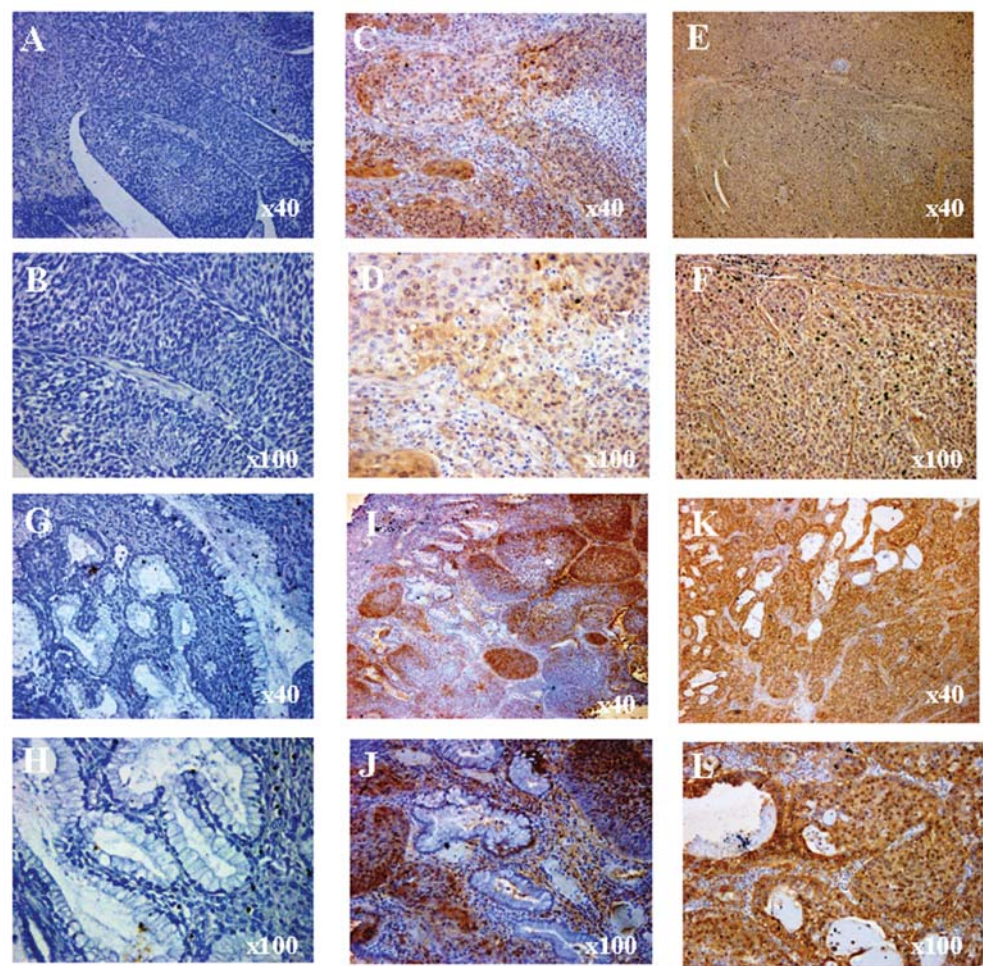


Figure 4. Representative immunostaining patterns of hepatocyte growth factor activator inhibitor-1 (HAI-1). (A) and (B) weak HAI-1 staining in squamous cell carcinoma. (C) and (D) moderate HAI-1 staining in squamous cell carcinoma. (E) and (F) strong HAI-1 staining in squamous cell carcinoma. (G) and (H) weak HAI-1 staining in adenocarcinoma. (I) and (J) moderate HAI-1 staining in adenosquamous cell carcinoma. (K) and (L) strong HAI-1 staining in adenosquamous cell carcinoma (original magnification x40 and x100). The relative strengths of the HAI-1 immunohistochemical staining was assessed qualitatively.

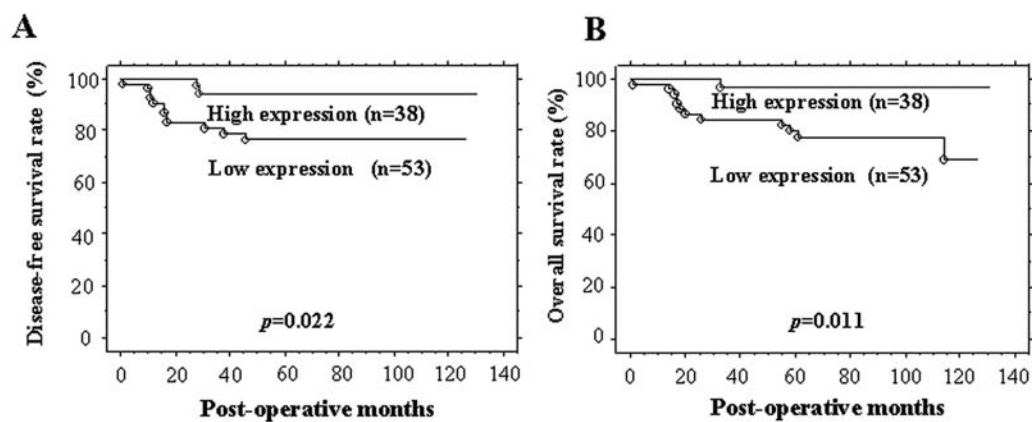


Figure 5. Kaplan-Meier plots for the disease-free and (A) overall survival (B) of the 91 patients with cervical cancer according to their hepatocyte growth factor activator inhibitor-1 (HAI-1) expression status. Low HAI-1 expression, score 0-1; high HAI-1 expression, score 2.

lines. We found a significant inhibitory effect of HAI-1 on cell growth transient transfection of the HAI-1 vector into both the SiHa and HeLa cell compared to control cells ($p<0.05$) (Fig. 3B). Furthermore, the colony forming assay

showed that the transient transfection of the HAI-1 vector into SiHa and HeLa cell resulted in a significantly reduced number of colonies compared with control cells ($p<0.01$) (Fig. 3C).

Table I. Association between HAI-1 and clinicopathological factors in cervical cancer.

Variable	Cases	Staining			(Mean \pm SE)	p-value ^a
		0	1	2		
Age (years)						0.716
<50	61	11	23	27	1.26 \pm 0.75	
\geq 50	30	5	14	11	1.20 \pm 0.71	
FIGO stage						0.013 ^a
I	45	5	16	24	1.42 \pm 0.69	
II	46	11	21	14	1.04 \pm 0.73	
Histological type						0.280
Non-SCC	37	8	16	13	1.14 \pm 0.75	
SCC	54	8	21	25	1.31 \pm 0.72	
Tumor size (cm)						0.002 ^a
\leq 4	74	10	28	36	1.35 \pm 0.71	
>4	17	6	9	2	0.76 \pm 0.66	
Stromal invasion						<0.001 ^a
\leq 2/3	45	4	11	30	1.58 \pm 0.66	
>2/3	46	12	26	8	0.91 \pm 0.66	
Vaginal invasion						0.031 ^a
Negative	77	12	29	36	1.31 \pm 0.73	
Positive	14	4	8	2	0.85 \pm 0.66	
Parametrial invasion						0.012 ^a
Negative	68	10	24	34	1.35 \pm 0.73	
Positive	23	6	13	4	0.91 \pm 0.66	
Lymph node metastasis						0.019 ^a
Negative	72	10	28	34	1.33 \pm 0.71	
Positive	19	6	9	4	0.89 \pm 0.73	
LVS involvement						0.002 ^a
Negative	44	4	15	25	1.48 \pm 0.66	
Positive	47	12	22	13	1.02 \pm 0.74	
Ovarian metastasis						0.170
Negative	88	15	35	38	1.26 \pm 0.73	
Positive	3	1	2	0	0.67 \pm 0.58	

^aMann-Whitney U test; FIGO, International Federation of Gynecology; LVS, lymph-vascular space.

Immunoassays by HAI-1. HAI-1 expression in human cervical cancer specimens was examined by immunoassays. The representative immunoassaying patterns of HAI-1 are shown in Fig. 4. Weak HAI-1 staining was observed in 16 cases (17.6%), moderate HAI-1 staining in 37 cases (40.6%) and strong HAI-1 staining in 38 cases (41.8%).

Table I shows the distribution of cases scored as positive for each of the biological parameters examined, according to the clinicopathological characteristics in the overall population. As expected, the results showed a statistically significant association with clinicopathological parameters such as stage (p=0.013), tumor size (p=0.002), stromal

invasion (p<0.001), vaginal invasion (p=0.031), parametrial invasion (p=0.012), lymph-node metastasis (p=0.019) and LVS involvement (p=0.002), but age, histological type and ovarian metastasis were not statistically significant (Mann-Whitney U test, p<0.05).

Univariate survival analysis. The results of the univariate survival analyses of other variables are shown in Table II. Overall, HAI-1 was most significantly exhibited on DFS and OS analysis of the prognostic factor for cervical cancer using the log-rank test. The DFS and OS curves of 91 cervical cancer patients according to their HAI-1 expression status are

Table II. DFS and OS analysis of prognostic factor using the log-rank test in cervical cancer.

Variable	Cases	Estimated 5-year DFS (%)	p-value ^a	Estimated 5-year OS (%)	p-value ^a
Age (years)			0.392		0.649
<50	61	86.9		86.9	
≥50	30	80.0		83.3	
FIGO stage			0.263		0.392
I	45	88.9		88.9	
II	46	80.4		82.6	
Histological type			0.172		0.090
Non-SCC	37	78.4		78.4	
SCC	54	88.9		90.7	
Tumor size (cm)			0.075		0.048 ^a
≤4	74	87.8		89.2	
>4	17	70.6		70.6	
Stromal invasion			0.004 ^a		0.008 ^a
≤2/3	45	95.6		95.6	
>2/3	46	73.9		76.1	
Vaginal invasion			0.901		1
Negative	77	84.4		85.7	
Positive	14	85.7		85.7	
Parametrial invasion			0.021 ^a		0.010 ^a
Negative	68	89.7		91.2	
Positive	23	69.6		69.6	
Lymph node metastasis			<0.001 ^a		<0.001 ^a
Negative	72	93.1		93.1	
Positive	19	52.6		57.9	
LVS involvement			<0.001 ^a		0.002 ^a
Negative	44	97.7		97.7	
Positive	47	72.3		74.5	
Ovarian metastasis			0.380		0.423
Negative	88	84.2		85.2	
Positive	3	66.7		66.7	
HAI-1			0.023 ^a		0.007 ^a
Low (0-1)	53	77.4		77.4	
High (2)	38	94.7		97.4	

^aMann-Whitney U-test; FIGO, International Federation of Gynecology and Obstetrics; LVS, lymph-vascular space.

shown in Fig. 5A and B, respectively. The DFS and OS rates of patients exhibiting high HAI-1 expression (score 2) were significantly higher than those of patients exhibiting low HAI-1 expression (score 0-1) ($p=0.022$ and $p=0.011$, respectively) (Mann-Whitney U test, $p<0.05$).

Discussion

Cervical cancer is the most important manifestation of genital HPV infection. HPV viruses encode the E6 and E7 oncogenes,

which are essential for malignant transformation as well as maintenance of the malignant phenotype of cervical cancer. The E6 protein forms a tertiary complex with ubiquitin-mediated degradation, including p53 and PDZ domain-containing protein (MUGG-1 and MAGI-3) (22-24). The E7 protein binds directly to inactivate a number of cell cycle regulatory proteins, including family of retinoblastoma protein (25). Several studies have demonstrated that these oncogenes make ideal targets for gene-specific therapy for cervical cancer. However, to date, their effectiveness has

been limited. It is now widely accepted that new approaches for the treatment of cervical cancer are pivotal in further improving the prognosis of this disease.

This is the first study to investigate the role of HAI-1 and evaluate its clinical importance in cervical cancer. HAI-1 is a Kunitz-type serine protease inhibitor that has a broad inhibitory spectrum against serine proteases. HAI-1 is an endogenous inhibitor of hepsin, matriptase and prostatic. Hepsin, matriptase and prostatic are a serine proteinase domain, and show enhanced expression in a variety of tumor tissues (26). Hepsin and matriptase has been proposed to initiate signaling and proteolytic cascades through its ability to activate pro-uPA, and matriptase is known to interact with prostatic (7-10). In this study, hepsin, matriptase and prostatic were significantly inhibited by HAI-1 in cervical cancer cell lines (Fig. 1C).

Parr and colleagues have reported that HAI-1 expression significantly enhanced the migratory, proliferative and invasive nature of breast cancer cell lines (27). However, the impact on apoptosis from HAI-1 overexpression remains unclear. In this report, we observed significant inhibition which resulted in apoptosis by HAI-1, as shown in Fig. 2A and B. Furthermore, HAI-1 was likely to regulate the early apoptosis process. To trace the steps in the apoptotic cascade, we evaluated the activation of pro- and anti-apoptotic protein (28-30). HAI-1 was able to down-regulate both Bcl-2 and Bcl-xL, thereby inducing apoptosis (Fig. 3A).

The E6 and E7 proteins of HPV immortalize, at least in part, the inactivation of the tumor suppressor proteins p53 and PDZ domain-containing protein and RB protein (22-25). These tumor suppressor proteins also regulate the expression of pro- and anti-angiogenic factors by cells. For this reason, experiments were conducted to determine the role of HAI-1 on p53, PDZ domain-containing protein (hDlg, MUGG-1 and MAGI-3) and RB proteins. The expression of PDZ domain-containing protein (MUGG-1 and MAGI-3) was down-regulated in the cells transiently transfected with HAI-1 (Fig. 3A). However, HAI-1 was not correlated with the levels of the p53, hDlg and RB protein (data not shown). Overall, HAI-1 shows potential inhibitory effects mediated by reduction of hepsin, matriptase and prostatic, which leads to apoptosis through a reduction in the level of Bcl-2, Bcl-xL, MUPP-1 and MAGI-3.

In clinical case, prostate, breast and gastric cancer has been reported that the reduced expression of HAI-1 is possibly involved in the progression of these cancers (18-20). The current study, the immunohistochemistry of cervical cancers from chemo-naïve patients showed decreased HAI-1 protein expression that increased with stage of cancer, tumor size, stromal invasion, vaginal invasion, parametrial invasion, lymph-node metastasis and LVS involvement. Interestingly, a low HAI-1 immunostaining pattern was significantly associated with poor prognosis in cervical cancer (Fig. 5A and B). These findings all indicate that HAI-1 protein could be an important tool for identifying patients with a favorable prognostic marker of cervical cancer.

In summary, these studies have revealed a critical role for HAI-1 in the disruption of the basement membrane of cervical cancer. These findings identify HAI-1 as a favorable prognostic marker for patients and could be considered as a

therapeutic target for certain approaches to the treatment of cervical cancer.

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