Annals of Oncology Advance Access published August 9, 2008

original article

Annals of Oncology
doi:10.1093/annonc/mdn556

Hepatocyte growth factor activator inhibitor-2 (HAI-2) is a favorable prognosis marker and inhibits cell growth through the apoptotic pathway in cervical cancer

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Received 11 April 2008; revised 10 July 2008; accepted 15 July 2008

Background: In light of the poor prognosis for cervical cancer, research continues into the development of innovative and efficacious treatment modalities for this disease. We investigated the role of hepatocyte growth factor activator inhibitor-2 (HAI-2) and evaluated its clinical importance in cervical cancer.
Patients and methods: HAI-2 expression was examined in cervical cancer specimens (n = 52) by immunohistochemistry. We further attempted to investigate the biological functions and inhibitory effects of HAI-2 using human papillomavirus (HPV) 16 type SiHa and HPV 18 type HeLa cervical cancer cell lines.
Results: There were significant correlations between HAI-2 expression and stage (P = 0.017), lymph node metastasis (P = 0.005) and ovarian metastasis (P = 0.038). Low HAI-2 expression was a significant predictor for a poor prognosis compared with high HAI-2 expression (disease-free survival rate, P = 0.016; overall survival rate, P = 0.021). After transient transfection into the SiHa and HeLa cell lines, HAI-2 showed potential inhibitory effects mediated by reductions in hepsin and matriptase expression, which led to apoptosis by increasing the level of Bak and reducing the level of Bcl-2.
Conclusions: The present findings indicate that low HAI-2 expression in cervical cancer may be associated with a poor prognosis. We propose that HAI-2 may represent a therapeutic target for the treatment of cervical cancer.
Key words: apoptosis, cervical cancer, favorable prognosis marker, HAI-2

introduction

Cervical cancer is the second most common cancer in women worldwide, with 250 000 new cases diagnosed each year [1]. Cervical cancer is the most important manifestation of genital human papillomavirus (HPV) infection, and these viruses encode the E6 and E7 oncogenes involved in cervical cancer [2]. Due to the poor prognosis of cervical cancer, several studies have been carried out to develop more effective treatments for this disease. Along with surgical, radiation and chemotherapeutic regimens, gene therapy has emerged as one of the leading contenders in the treatment armamentarium. It is now widely accepted that new approaches for the treatment of cervical cancer are pivotal to further improve the prognosis of this disease.

The metastatic process involves degradation of the extracellular matrix (ECM), including interstitial basement membranes, by proteinases, which facilitates cell detachment followed by local and systemic spreading. Hepatocyte growth factor (HGF) is a multifunctional growth factor that is secreted by mesenchymal cells in the liver as an inactive single-chain propeptide and normally remains in this form associated with the ECM [3]. It induces the proliferation, motility and morphogenesis of epithelial and endothelial cell types via a high-affinity receptor tyrosine kinase, c-Met [4, 5]. c-Met is known to play important roles in the migration and invasion of tumor cells. Overexpression of c-Met in cervical cancer is associated with cancer progression, and high c-Met expression is correlated with poor survival [6, 7].

The inactive secreted HGF propeptide is cleaved by HGF activator (HGFA) to achieve its active form. HGFA is regulated by two inhibitors, HGFA inhibitor type I (HAI-1) and type II (HAI-2), which are both type 1 transmembrane proteins with two Kunitz-type serine protease domains in their extracellular portions [8–11]. Complementary DNA (cDNA) cloning of HAI-2 revealed that this protein is identical to placental bikunin [12]. The two types of HAI identified so far, HAI-1 and HAI-2, both show cell surface expression on epithelial cells. HAI-1 appears to be the cognate inhibitor of HGFA and matriptase [13, 14], and both HAI-1 and HAI-2 inhibit HGFA and hepsin. HAI-2 is a more

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efficient inhibitor of hepsin [15] and displays a broader inhibitory spectrum [9, 16] than HAI-1. To date, several studies on HAI-2 expression in tumor tissues have been published. Although initial studies showed that HAI-2 expression was upregulated in pancreatic cancer [11], other studies demonstrated marked downregulation in glioblastoma, hepatocellular carcinoma and renal carcinoma, which was partly due to hypermethylation of the HAI-2 promoter region [17–19]. However, the issues of whether HAI-2 is correlated with cervical cancer and its mechanisms of action in this disease remain unclear. In the current study, we examined whether HAI-2 protein expression is correlated with the clinicopathological characteristics of patients with cervical cancer. The main aim of the study was to determine whether HAI-2 protein could be used as a favorable prognosis marker for patients with cervical cancer. Our data clearly indicated that overexpression of HAI-2 inhibited cell growth and promoted apoptosis. Thus, we propose that HAI-2 may represent a potential target for gene therapy of cervical cancer.

patients and methods

patients and tissues

The patient population consisted of 52 patients presenting with 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 neo-adjuvant chemotherapy were excluded from this study. 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 classified according to the World Health Organization classification as follows: 27 squamous cell carcinomas, 18 adenocarcinomas and seven adenosquamous carcinomas. Clinical staging was assessed based on the FIGO staging system as follows: 22 stage IB cancers, three stage IIA cancers and 27 stage IIB cancers. The median age at the time of surgery was 46 years (range 27–66 years). Patients with lymph node metastasis, parametrial involvement, deep stromal invasion or marked lymph-vascular space involvement were treated with adjuvant external whole-pelvic irradiation or chemoradiation ($n = 19$) or chemotherapy ($n = 11$). The disease-free survival (DFS) and overall survival (OS) rates were defined as the intervals 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 31 July 2006, and the median duration of the follow-up was 77 months (range 4–146).

immunohistochemical analysis and staining evaluation

Formalin-fixed paraffin-embedded 4-μm sections 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. Next, the sections were incubated with an anti-HAI-2 primary antibody 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-2 staining in the tumors were classified into three groups by scoring the percentages of positively stained cells: 2, strong staining, >50% of cells; 1, moderate staining, 10%–50% of cells and 0, weak staining, <10% of cells. Two independent examiners with no prior knowledge of the patients' clinical data conducted the microscopic evaluations. Controversial cases were evaluated using a conference microscope.

cell culture, media and generation of transfectants

The HPV 18 type HeLa (ATCC no. CCL-2) and HPV 16 type SiHa cancer cell lines examined were derived from human cervical carcinomas. The SiHa and HeLa cell lines 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 of 5% CO_2 in air. A cDNA encoding the whole coding region of HAI-2 was constructed by PCR using a full-length HAI-2 cDNA as a template. The primers used for HAI-2 were 5′-AGCTCTAGAGCC ATGGCGCAGCTGTGCGG-3′ and 5′-TTAGTC-GACTCACAGGACATATGTGTTCTTC-3′. The restriction sites *XBa*I and *Sal*I present in the primers were used for subcloning into the pCIneo expression vector (Promega, Madison, WI) to generate the expression vector pCI-HAI-2. The HAI-2 cDNA expression vector was transfected into the two cervical cancer cell lines using the TransFast transfection reagent (Promega, Madison, WI). 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 separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The polyclonal and monoclonal antibodies used for immunoblotting were as follows: anti-HAI-2, anti-matriptase, anti-Bcl-2 and anti-Bak (Santa Cruz Biotechnology, Santa Cruz, CA); anti-hepsin (Cayman Chemical, Ann Arbor, MI) and anti-β-actin (Sigma Chemical Co., St Louis, MO). The working dilution of all the primary antibodies was 1 : 1000. After incubation of the membranes with appropriate secondary antibodies, antigen–antibody complexes were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

cell viability assay

To examine the cell viabilities after transient transfection of the HAI-2 vector into the SiHa and HeLa cervical cancer cell lines, SYTO 10 green fluorescent nucleic acid stain and Dead Red (ethidium homodimer-2) nucleic acid stain (Live/Dead® Reduced Biohazard Viability/Cytotoxicity Kit; Invitrogen, Eugene, OR) were added to each well and incubated for 15 min. Cell fluorescence was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Matrigel invasion assay

To investigate differences in the invasive abilities of cells expressing HAI-2, we used BioCoat Matrigel Invasion Chambers (BD Bioscience, Bedford, MA). SiHa and HeLa cervical cancer cells with or without transient transfection of the HAI-2 vector were incubated *in situ* with 10 μg/ml of DiI (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 inserts and mounted on slides, and the numbers of invading cells were counted under a microscope. The Matrigel assays were carried out in triplicate.

MTS assay and fluorescein isothiocyanate-conjugated annexin V

The effects of HAI-2 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-2 vector for 12, 24, 36 or 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, Hercules, CA). In addition, apoptosis was measured by staining with fluorescein isothiocyanate (FITC)-conjugated annexin V using a MEBCYTO Apoptosis

Kit (MBL, Nagoya, Japan) according to the manufacturer's recommendations and flow cytometric analysis.

cell growth in monolayers

Cells were plated at a density of 3×10^4 cells/well in six-well plates containing DMEM supplemented with 10% FBS. The cell numbers were counted in triplicate after 2, 4, 6, 8 and 10 days of culture using a hemocytometer to assess cell proliferation.

cell growth in soft agar

A cell suspension (1×10^4 cells/well) in 1 ml of 0.2% Noble agar DMEM containing 10% FBS was overlaid onto 35-mm dishes containing a 0.5% agar base. Colonies of >0.2 mm in diameter were counted on day 21 of culture. The soft agar assays were carried out in triplicate.

statistical analysis

Statistical analyses were carried out using the Mann–Whitney *U* test for comparisons with controls and one-factor analysis of variance followed by Fisher's protected least significance difference test for all pairwise comparisons. The analyses were carried out using the software package Stat View version 5.0 (Abacus Concepts, Berkeley, CA). Differences were considered significant at $P < 0.05$.

results

immunoassays

HAI-2 expression in human cervical cancer specimens was examined by immunoassays. Representative staining patterns of HAI-2 are shown in Figure 1. Weak HAI-2 staining was observed in three cases (5.8%), moderate HAI-2 staining in 25 cases (48.1%) and strong HAI-2 staining in 24 cases (46.1%).

Table 1 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, significant associations were found between HAI-2 expression and clinicopathological parameters such as stage ($P = 0.017$), lymph node metastasis ($P = 0.005$) and ovarian metastasis ($P = 0.038$), while no associations were detected for age, histological type, tumor size, stromal invasion, vaginal invasion, parametrial invasion or lymph-vascular involvement (Mann–Whitney *U* test, $P < 0.05$).

univariate survival analysis

The results of univariate survival analyses of the other variables are shown in Table 2. Overall, HAI-2 was most significantly identified in DFS and OS analyses as a prognostic factor for cervical cancer using the log-rank test. The DFS and OS curves of the 52 cervical cancer patients according to their HAI-2 expression status are shown in Figure 2A and B, respectively. The DFS and OS rates of patients exhibiting high HAI-2 expression (score of 2) were significantly higher than those of patients exhibiting low HAI-2 expression (scores of 0 or 1) ($P = 0.016$ and 0.021, respectively, Mann–Whitney *U* test).

western blot assay

The functional roles of HAI-2 were examined by transient transfection of a HAI-2 vector followed by western blotting (Figure 3A). Transient transfection of the HAI-2 vector into SiHa and HeLa cells resulted in significant increases in HAI-2 expression. The levels of hepsin expression were decreased following transient transfection of the HAI-2 vector into SiHa and HeLa cells, and the decrease was significantly greater in SiHa cells than in HeLa cells. The level of matriptase expression was significantly decreased by transient transfection of the HAI-2 vector into HeLa cells. Matriptase expression was not detected in SiHa cells without or with transient transfection of the HAI-2 vector.

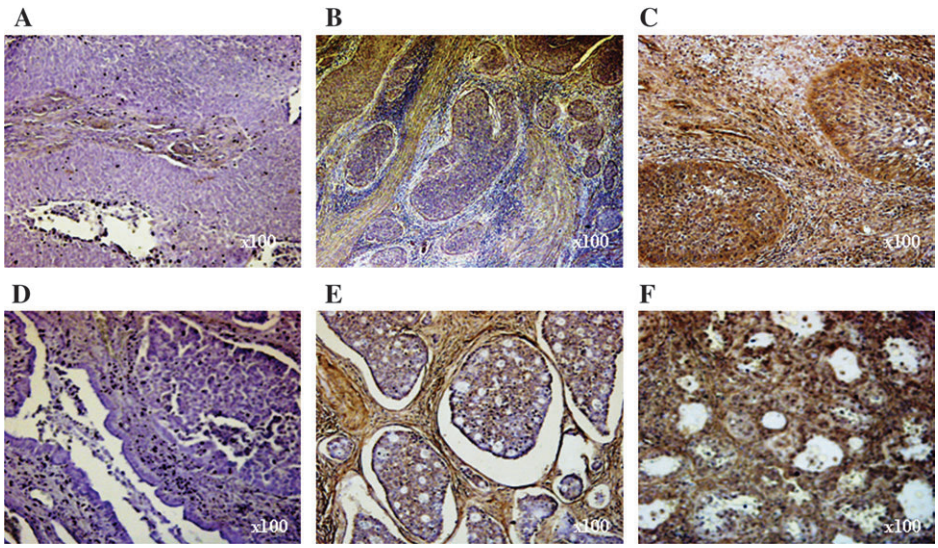


Figure 1. Representative immunostaining patterns of an hepatocyte growth factor activator inhibitor-2 (HAI-2). (A) Weak HAI-2 staining in a squamous cell carcinoma. (B) Moderate HAI-2 staining in a squamous cell carcinoma. (C) Strong HAI-2 staining in a squamous cell carcinoma. (D) Weak HAI-2 staining in an adenosquamous cell carcinoma. (E) Moderate HAI-2 staining in an adenosquamous cell carcinoma. (F) Strong HAI-2 staining in an adenocarcinoma. The relative strengths of the HAI-2 immunohistochemical staining were assessed qualitatively.

Table 1. Association between HAI-2 and clinicopathological factors in cervical cancer

Variable	No. of cases	HAI-2 score (mean ± SD)	*P* value[a]
Age (years)			0.865
<50	33	1.39 ± 0.61	
≥50	19	1.42 ± 0.61	
FIGO stage			0.017[a]
I	22	1.63 ± 0.49	
II	30	1.23 ± 0.63	
Histological type			0.637
Non-SCC	25	1.36 ± 0.70	
SCC	27	1.44 ± 0.51	
Tumor size (cm)			0.153
≤4	41	1.46 ± 0.56	
>4	11	1.18 ± 0.60	
Stromal invasion			0.149
≤2/3	27	1.52 ± 0.57	
>2/3	25	1.28 ± 0.61	
Vaginal invasion			1
Negative	42	1.40 ± 0.59	
Positive	10	1.40 ± 0.70	
Parametrial invasion			0.956
Negative	37	1.41 ± 0.58	
Positive	15	1.40 ± 0.63	
Lymph node metastasis			0.005[a]
Negative	42	1.50 ± 0.59	
Positive	10	1.00 ± 0.47	
LVS involvement			0.515
Negative	26	1.46 ± 0.58	
Positive	26	1.35 ± 0.63	
Ovarian metastasis			0.038[a]
Negative	49	1.44 ± 0.61	
Positive	3	0.67 ± 0.58	

[a]Mann–Whitney *U* test.

HAI-2, hepatocyte growth factor activator inhibitor-2; FIGO, International Federation of Gynecology and Obstetrics; SCC, squamous cell carcinoma; LVS, lymph-vascular space.

Table 2. DFS and OS analysis of prognostic factor using the log-rank test in cervical cancer

Variable	No. of cases	Estimated 5-year DFS (%)	*P* value	Estimated 5-year OS (%)	*P* value[a]
Age (years)			0.109		0.211
<50	33	90.9		90.9	
≥50	19	73.7		78.9	
FIGO stage			0.072		0.112
I	22	95.4		95.4	
II	30	76.7		80	
Histological type			0.373		0.188
Non-SCC	25	80		80	
SCC	27	88.9		92.6	
Tumor size (cm)			0.050[a]		0.029[a]
≤4	41	90.2		92.7	
>4	11	63.6		63.6	
Stromal invasion			0.019[a]		0.039[a]
≤2/3	27	96.3		96.3	
>2/3	25	72		76	
Vaginal invasion			0.484		0.411
Negative	42	85.7		88.1	
Positive	10	80		80	
Parametrial invasion			0.156		0.095
Negative	37	89.1		91.9	
Positive	15	73.3		73.3	
Lymph node metastasis			0.035[a]		0.121
Negative	42	90.5		90.5	
Positive	10	60		70	
LVS involvement			0.033[a]		0.024[a]
Negative	26	96.2		96.2	
Positive	26	80.8		76.9	
Ovarian metastasis			0.061		0.341
Negative	49	87.8		87.8	
Positive	3	33.3		66.6	
HAI-2			0.010[a]		0.023[a]
Low (0–1)	23	69.6		73.9	
High (2)	29	96.6		96.6	

[a]Mann–Whitney *U* test.

DFS, disease-free survival; OS, overall survival; FIGO, International Federation of Gynecology and Obstetrics; LVS, lymph-vascular space; HAI-2, hepatocyte growth factor activator inhibitor-2.

cell viability assay

The viabilities of SiHa and HeLa cells were evaluated after transient transfection of the HAI-2 vector. The percentages of viable cells were decreased to 81.8% and 72.7% of the control cell viabilities at 48 h after transient transfection of the HAI-2 vector into SiHa and HeLa cells, respectively. Furthermore, cell death was increased following transient transfection of the HAI-2 vector into SiHa and HeLa cells (Figure 3B).

Matrigel invasion assay

We assessed the motility and invasiveness of SiHa and HeLa cells overexpressing HAI-2 using BioCoat Matrigel Invasion Chambers. Cells were plated on the upper surface of each membrane and cultured for 48 h. Next, the cells invading to the bottom side of each membrane were stained and counted. The percentages of cells reaching the bottom of the filter were decreased to 31.8% and 25.8% at 48 h after transient transfection of the HAI-2 vector into SiHa and HeLa cells, respectively (Figure 4A).

MTS and FITC-conjugated annexin V assays

Previous reports have shown that HAI-2 acts during apoptosis in ovarian cancer [20, 21]. We used MTS and FITC-conjugated annexin V assays to examine apoptosis after transient transfection of the HAI-2 vector into SiHa and HeLa cervical cancer cell lines. Transient transfection of the HAI-2 vector resulted in significant cell growth inhibition of these cell lines, as evaluated by the MTS assay (Figure 4B). Furthermore, flow cytometric data revealed that transient transfection of the HAI-2 vector for 48 h increased the FITC-annexin V-positive and propidium iodide-positive signals (Figure 4C), indicating that HAI-2 induced apoptosis in these cell lines. HAI-2 increased the total percentages of apoptotic and necrotic cells to

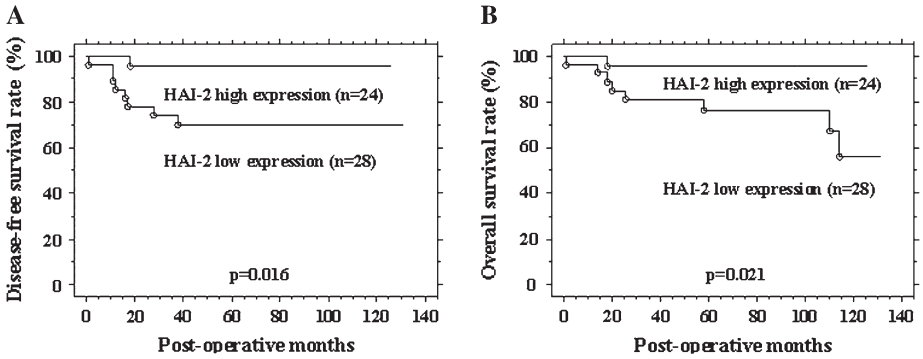


Figure 2. Kaplan–Meier plots for the disease-free (A) and overall survival (B) rates of the 52 patients with cervical cancer according to their hepatocyte growth factor activator inhibitor-2 (HAI-2) expression status. Low HAI-2 expression, score 0 or 1; high HAI-2 expression, score 2.

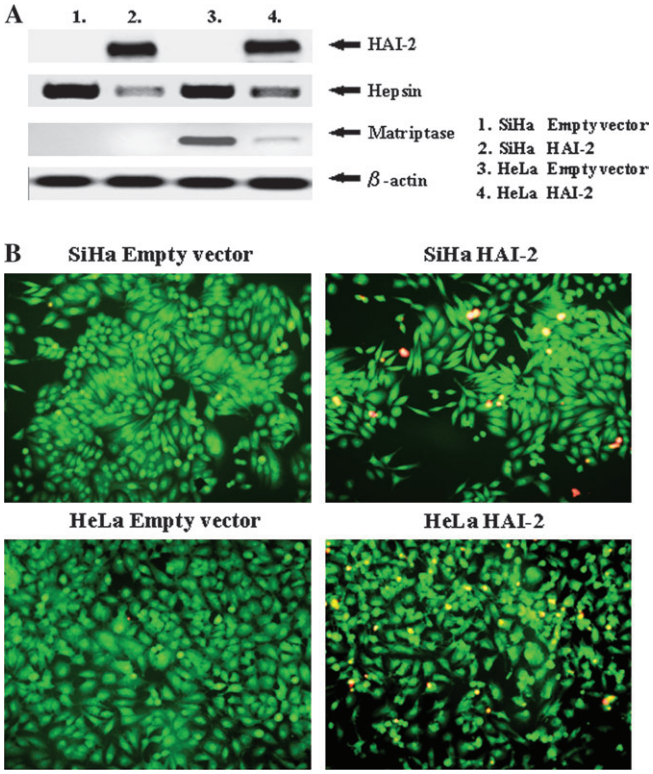


Figure 3. Effects of transient transfection of an hepatocyte growth factor activator inhibitor-2 (HAI-2) vector into SiHa and HeLa cervical cancer cells for 48 h. (A) Western blot analysis of the HAI-2, hepsin and matriptase expression levels after transient transfection of the HAI-2 vector into SiHa and HeLa cervical cancer cells for 48 h. β-Actin expression was analyzed as a loading control in the same blot. (B) The cell viabilities of SiHa and HeLa cervical cancer cells after transient transfection with the HAI-2 vector were evaluated using a fluorescence microscope.

34.85% and 43.41% compared with the control percentages of 7.10% and 8.24% for the SiHa and HeLa cell lines, respectively.

We also investigated the effects of HAI-2 overexpression on the protein levels of apoptotic molecules, namely the antiapoptotic Bcl-2 and proapoptotic Bak. As shown in Figure 5A, the level of Bak protein expression was upregulated while that of Bcl-2 protein expression was downregulated in the SiHa and HeLa cell lines following transient transfection of the HAI-2 vector.

cell growth in monolayers and anchorage-independent cell growth in soft agar

The effects of HAI-2 overexpression on cell proliferation were analyzed using transient transfection of the HAI-2 vector into the SiHa and HeLa cell lines. Significant inhibitory effects of HAI-2 on the cell growth were observed following transient transfection of the HAI-2 vector into both SiHa and HeLa cells compared with the control cells ($P < 0.05$; Figure 5B). Furthermore, colony formation assays following transient transfection of the HAI-2 vector into SiHa and HeLa cells revealed significantly reduced numbers of colonies compared with control cells ($P < 0.01$; Figure 5C).

discussion

This is the first study to examine HAI-2 protein expression in human cervical cancer specimens. HAI-2 is a Kunitz-type serine protease inhibitor that has a broad inhibitory spectrum against serine proteases. It has been reported that reduced expression of HAI-2 is possibly involved in disease progression in glioblastoma, hepatocellular carcinoma and renal carcinoma [17–19]. In the current study, we examined whether HAI-2 protein expression was correlated with clinicopathological characteristics in patients with cervical cancer. Immunohistochemistry of cervical cancer specimens from chemonaive patients revealed decreased HAI-2 protein expression with increasing stage, lymph node metastasis and ovarian metastasis. Interestingly, a low level of HAI-2 immunostaining was significantly associated with a poor prognosis in cervical cancer. Taken together, these findings indicate that HAI-2 protein could be an important factor for identifying cervical cancer patients with a favorable prognosis. However, the mechanism of action of HAI-2 in cervical cancer remains unclear.

HAI-2 is an endogenous inhibitor of matriptase and hepsin. Matriptase and hepsin are both type 2 transmembrane proteins with an extracellular serine proteinase domain and show enhanced expression in a variety of tumor tissues [22].

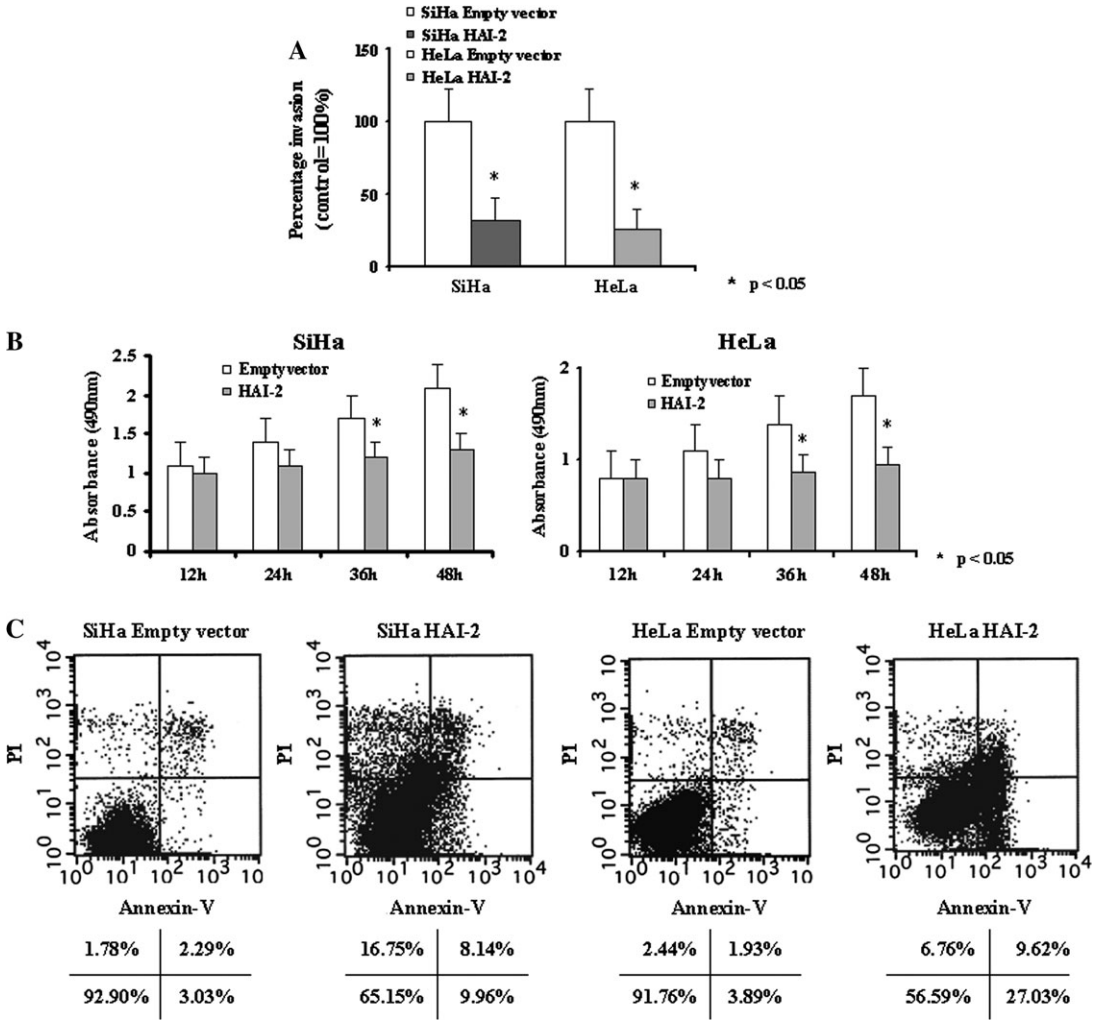
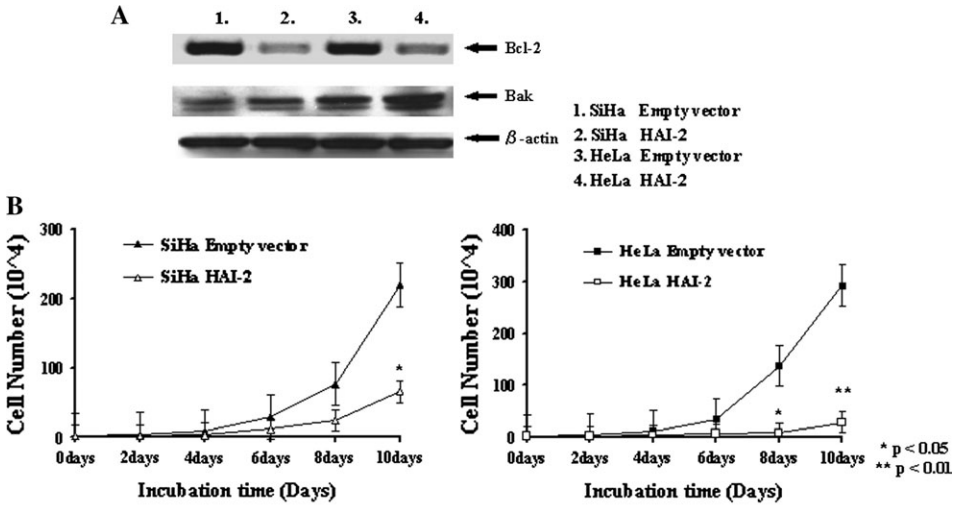


Figure 4. (A) Matrigel invasion assays after transient transfection of an hepatocyte growth factor activator inhibitor-2 (HAI-2) vector into SiHa and HeLa cervical cancer cells. Following incubation, the membranes were removed from the inserts and mounted on slides. The numbers of invading cells were counted under a microscope. The Matrigel assays were carried out in triplicate. (B) MTS assays of cell proliferation of SiHa and HeLa cervical cancer cells after transient transfection of the HAI-2 vector for 12, 24, 36 or 48 h. The assays were carried out for quadruplicate transfection experiments. (C) Representative flow cytometric data for apoptosis of SiHa and HeLa cervical cancer cells after transient transfection of the HAI-2 vector.

Matriptase has been proposed to initiate signaling and proteolytic cascades through its ability to activate prourokinase and protease-activated receptor 2 [23, 24], while hepsin is thought to be involved in activating the coagulation cascade [25]. In the present study, we found that hepsin and matriptase were significantly inhibited by HAI-2 in cervical cancer cell lines. Several previous reports have also shown that HAI-2 acts during apoptosis [20, 21]. Indeed, HAI-2 was found to induce apoptosis in the cervical cancer cell lines examined in the present study. To trace the steps in the apoptotic cascade, we evaluated the activation of the proapoptotic protein Bak and antiapoptotic protein Bcl-2 [26, 27]. HAI-2 was found to upregulate Bak protein expression and downregulate Bcl-2 expression in the cervical cancer cell lines examined, thereby inducing apoptosis. Overall, HAI-2 shows potential inhibitory effects mediated by reductions in hepsin and matriptase expression, which lead to apoptosis by increasing the level of Bak and reducing the level of Bcl-2.

Cervical cancer is the most important manifestation of genital HPV infection, and these viruses encode the E6 and E7 oncogenes, which are essential for malignant transformation as well as maintenance of the malignant phenotype of cervical cancer [2]. The E6 and E7 proteins of HPV become immortalized, at least in part, and prevent inactivation of the tumor suppressor protein p53 and retinoblastoma protein (RB) [28]. These tumor suppressor proteins also regulate the expression of proangiogenic and antiangiogenic factors by cells. For this reason, experiments were conducted to determine the effects of HAI-2 on the p53 and RB proteins. However, HAI-2 was not correlated with the levels of the p53 tumor suppressor protein or cell cycle regulatory RB protein (data not shown).

In summary, *in vitro* and *in vivo* studies have revealed a critical role for HAI-2 in disruption of the basement membrane in cervical cancer. The present findings identify HAI-2 as a favorable prognostic marker for cervical cancer. We



C

Colony formation on soft agar of HAI-2 transfectants and empty vector-transfected SiHa and HeLa cervical cancer cells

Cell line		Colonies/dish (mean/SD)***	Inhibitory effect (%)
SiHa	Empty Vector	213.8 ± 43.8	0
	HAI-2	54.2 ± 10.2	74.5 **
HeLa	Empty Vector	20.5 ± 3.8	0
	HAI-2	1.5 ± 0.5	92.7**

*** Numbers represent data from triplicate experiments ** p < 0.01

Figure 5. (A) Western blotting analysis of the Bak and Bcl-2 expression levels after transient transfection of the hepatocyte growth factor activator inhibitor-2 (HAI-2) vector into SiHa and HeLa cervical cancer cells for 48 h. β-Actin expression was analyzed as a loading control in the same blot. (B) After transient transfection of the HAI-2 vector into SiHa and HeLa cervical cancer cells, the cell growth in monolayers was evaluated after culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 2, 4, 6, 8 and 10 days. Numbers represent the data from triplicate experiments. (C) Colony formation on soft agar of SiHa and HeLa cervical cancer cells after transient transfection of the HAI-2 vector. The data from triplicate experiments are shown.

propose that HAI-2 could represent a therapeutic target for the treatment of cervical cancer.

acknowledgements

We are grateful to Dr H. Kataoka for providing the HAI-2 protein and to the Department of Section of Oncopathology and Regenerative Biology (Department of Pathology, Faculty of Medicine, University of Miyazaki, Japan) for providing the full-length HAI-2 cDNA construct encoding the HAI-2 protein.

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