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ORIGINAL ARTICLE

Expression of hepatocyte growth factor activator inhibitor-1 (HAI-1) gene in prostate cancer: Clinical and biological significance

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Summary

Purpose: *Hepatocyte growth factor activator inhibitor type-1 (HAI-1) is an integral-membrane proteinase inhibitor. Some studies have shown that HAI-1 as a matriptase inhibitor that plays a significant role in regulating cancer progression and metastasis. In this study, we attempted to clarify whether the levels of HAI-1 could be a useful marker in patients with prostate cancer (Pca).*

Methods: *HAI-1 protein was evaluated by immunohistochemistry (IHC) and HAI-1 mRNA was evaluated by reverse transcription–polymerase chain reaction (RT-PCR) in 48 patients with Pca and 20 patients with benign prostate hyperplasia (BPH). The association between HAI-1 and clinicopathological features and survival were analyzed.*

Results: *A high level of HAI-1 protein and mRNA expression was detected in BPH compared to Pca specimens. The HAI-1 expression inversely correlated with Gleason score and pathological stage ($p<0.05$). It was significantly stronger in N_0M_0 tumors than in N+ or M+ tumors ($p<0.05$). Furthermore, low HAI-1 expression was a significant predictor for poor prognosis when compared with high HAI-1 expression (disease-free survival/DFS rate, $p=0.0487$; overall survival/OS rate; $p=0.0492$).*

Conclusion: *The results of the present study identified HAI-1 as a favorable prognostic marker for Pca and may indicate that HAI-1 could be a therapeutic target for the treatment of this malignancy.*

Key words: *hepatocyte growth factor activator inhibitor type-1(HAI-1), metastasis, prognosis, prostate cancer*

Introduction

Pca is the most common nondermatologic cancer in men (29% of all cancers) in the United States. Besides lung cancer, Pca is the second cause of cancer-related deaths (9% of all cancer deaths) in males in the United States [1]. Pca is so highly variable that it is relatively hard to diagnose and its prognosis using the current diagnostic and prognostic indicators varies greatly. For example, the Pca prognosis is unreliable considering only the primary tumor size and histology, as these characteristics can not reflect its invasiveness [2]. Similarly, serum prostate specific antigen (PSA) levels can increases in non-cancerous conditions such as BPH and prostatitis [3,4]. Thus, molecular markers for Pca identification and prognosis are required.

HAI-1, encoded by the serine protease inhibitor Kunitz type 1(SPINT1) gene, is a type I transmembrane serine protease inhibitor that was initially identified as a potent inhibitor of hepatocyte growth factor activator (HGFA) [5,6]. HAI-1 is able to form a complex with active HGFA and may function to temporarily sequester HGFA to the cell surface and serve as a reservoir for HGFA [7]. The shed form of HAI-1 is active in inhibiting HGFA, matriptase, hepsin and prostasin, all of which are pro-HGF activators.

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 and metastasis

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[8,9]. In our study, we investigated the expression of HAI-1 in prostatic tissues and Pca, and analyzed the relationship between HAI-1 expression with several related clinicopathological factors.

Methods

Patients and tissue preparation

Patients

A retrospective analysis was carried out on 48 patients with untreated Pca (age range 53–76 years, median 62) and 20 patients with BPH (age range 57–78 years, median 67) between 2008 and 2010 in the Departments of Urology of the Gongli Hospitals (Shanghai, P.R. China). Informed consent was obtained from all patients. All specimens were handled and made anonymous according to the ethical and legal standards. Diagnosis was confirmed pathologically by transrectal ultrasonography (TRUS)-guided systematic biopsy of the prostate or radical prostatectomy with no chemotherapy or hormonal therapy before surgery. BPH tissues were from transurethral resection of the prostate in patients with BPH.

Tissue preparation

All specimens were evaluated with respect to histological grade and subtypes by two pathologists. The clinical stages were determined according to the Jewett staging system [10], and the histological grade was determined by the classification of General Rule for Clinical and Pathological Studies on Prostatic Cancer [11]. Out of the 48 patients with Pca, 21 had clinical stage T2, 14 stage T3, and 3 stage T4. For each case, 4 different tumor sites and the non-neoplastic prostate were studied. Half of the resected tissues were stored at -80°C for RT-PCR and the other half for pathological examination and immunohistochemistry.

Immunohistochemistry

For immunohistochemical staining of HAI-1 protein, the sections were deparaffinized in xylene and rehydrated in a graded series of alcohols. To remove the endogenous peroxidase activity, sections were treated with freshly prepared 0.3% hydrogen peroxide in methanol for 20 min at room temperature. Nonspecific antibody binding was then blocked using 5% normal goat serum in phosphate-buffered saline (PBS) for 1 h. A goat polyclonal antibody that reacts with human HAI-1 (Santa Cruz Biotechnology,Inc., Santa Cruz, Calif., USA) was used in this study. The sections were then incubated for 1 h at room temperature with the primary antibody diluted 1: 300 in PBS. After 3 washes with PBS, sections were incubated for 30 min at room temperature with rabbit anti-goat serum (RayBiotech, Norcross, Ga.,USA), diluted 1: 1,000 in PBS and rinsed 3 times with PBS. The resultant immune peroxidase complexes were developed in 0.5% 3,3-diaminobenzidine hydrochloride (DAB; Sigma, Saint Louis, Mo., USA) in PBS. The sections were then counterstained with hematoxylin. An additional staining without primary antibody served as negative control. This study was approved by the Ethical Committee of the Gongli Hospitals.

Immunohistochemical staining results were read separately by 2 pathologists, who were blinded to the grade or other clinical parameters of an individual case. The number of HAI-1-expressing tumor cells was estimated as a percentage of the final number of cells per section and scored in 4 categories: 0: <10%; 1: 10–25%; 2: 25–50%; 3: >50%. The intensity of staining was graded as equivalent to the negative control (–), weak (+), moderate (++), or strong (+++). Then the score of staining percentage and intensity of stained cells was combined: a score of 0 (–) was score 0, of 1 (+) was score 1, of 2 (++) was score 2, and a score of 3 (+++) was score 3.

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA extraction

Fresh prostate tissue samples were obtained from patients who had undergone biopsy or radical prostatectomy. Total RNA was extracted using TRIzolTM (Invitrogen, Carlsbad, CA, USA). Briefly, the samples were minced and lysed in the TRIzolTM reagent. Subsequently, a 1/5 volume of chloroform was added and the mixture was agitated vigorously for 15 sec and then maintained on ice for 5 min. The suspension was centrifuged at 12,000 rpm at 4°C for 15 min. The upper aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol, and then maintained on ice for 5 min. The resulting RNA was precipitated and pelleted by centrifugation at 12,000 rpm at 4°C for 15 min. The RNA pellets were washed with 75% ethanol by vortexing and subsequent centrifugation at 12,000 rpm at 4°C for 5 min. The pellets were dried briefly and dissolved in diethyl-pyrocarbonate(DEPC)-treated RNase-free solution. Total RNA was quantified by measuring the absorbance at 260 nm.

Reverse transcription of RNA

The prepared RNA was reverse transcribed into complementary DNA (cDNA) in a 20µl volume containing 5×RT reaction buffer plus 0.005 M DTT, 1 mM of each dNTP, 20 U RNase inhibitor, 50 µM oligo (dT) primer, 2µg total RNA and 200 U of DiaStar™ RTase (SolGent, Daejeon, South Korea). The mixture was then incubated at 50°C for 50 min and then at 70°C for 10 min.

PCR

All subsequent quantification steps were performed according to the manufacturer's instructions. The PCR primer pairs used for cDNA amplification were as follows: 5'-CAGCAGTGCCTCGAGTCTTGTC-3' (sense) and

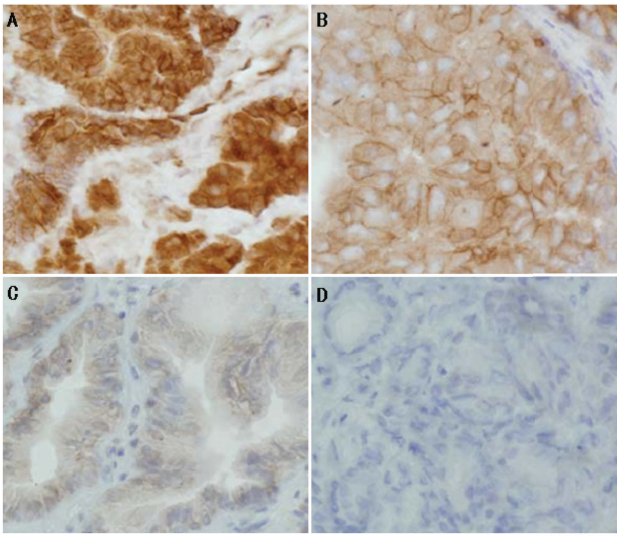

Figure 1. Immunohistochemical staining for HAI-1 in prostatic tissues (×400). **A:** strong (+++) expression in high-grade Pca. **B:** moderate (++) expression in medium-grade Pca. **C:** weak (+) expression in low-grade Pca. **D:** negative (-) expression in low-grade Pca.

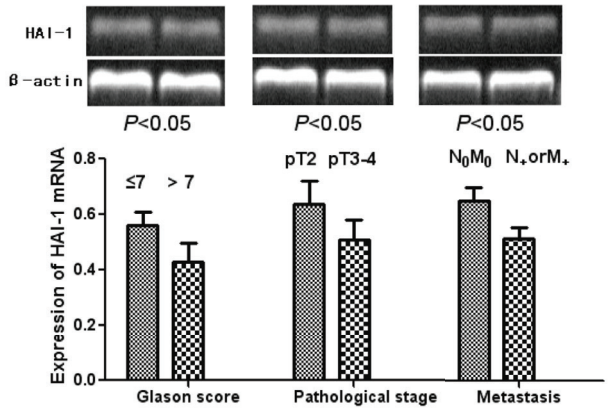

Figure 2. RT-PCR analyses of HAI-1 gene in 48 Pca samples. β-actin was used to normalize the HAI-1 mRNA.

5'-GATGGCTACCACCACCACAATG-3' (antisense) for human HAI-1; 5'-GGGGAGCCAAAAGGGTCATCATCT-3' (sense) and 5'-GAGGGGCCATCCACAGTCTTCT-3' (antisense) for human β-actin. A typical 20 µll one-tube PCR reaction contained 1 µl cDNA (sample) or serially diluted standard cDNA. PCR amplifications were performed in separate tubes for 32 cycles (10 sec at 95°C; 5 sec at 57°C and 15 sec at 72°C) using PCR Master mixtures specific for HAI-1 gene. To normalize the amplified products in each sample, we used β-actin as a quantitative internal control. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel. The mRNA expression levels of HAI-1 were presented as a ratio to that of β-actin, and the relative expression levels were calculated.

Statistics

Two sample t–test, chi-square test, Gehan-Breslow-Wilcoxon test and Kaplan-Meier method were used for statistical analyses. Statistical differences with $p<0.05$ were considered significant. Data were analyzed by SAS 8.2 software package (SAS Institute Inc., Cary, N.C., USA).

Results

HAI-1 expression decreased with increasing clinical stage in patients with Pca

We examined the HAI-1 protein level of prostatic tissues in 20 BPH patients and 48 Pca patients by IHC. BPH showed intense HAI-1 expression in the cytoplasm, while Pca showed decreased HAI-1 staining and a pattern of progressively reduced HAI-1 levels with increasing tumor grades (Figure1 A-C). A lack of HAI-1 staining in metastatic PCa was observed (Figure 1D).

The pathological stages and histological Gleason score are presented in Table 1. Out of the 20 BPH tissues, 17 (85%) were scored as positive

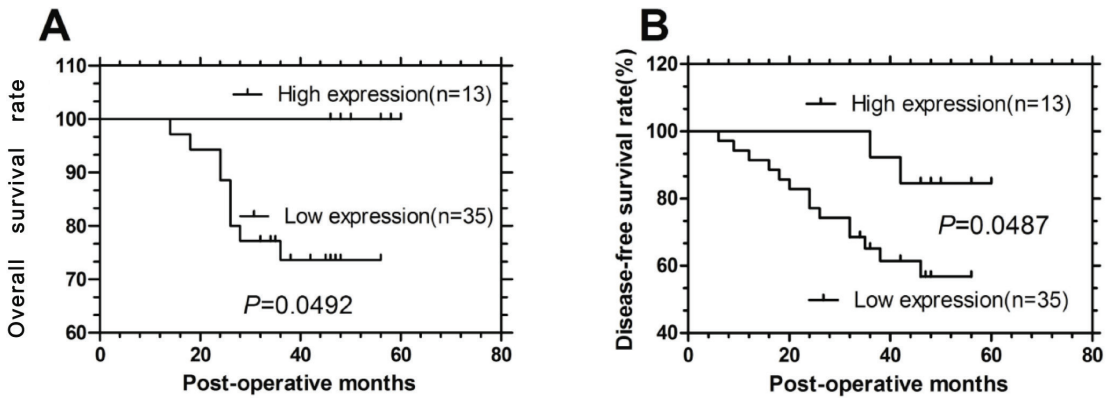

Figure 3. Kaplan-Meier plots for overall survival **(A)** and disease-free survival **(B)** of the 48 patients with Pca according to their HAI-1 expression status. Low HAI-1 expression, score 0-1; high HAI-1 expression, score 2-3.

Table 1. Association of HAI-1 protein with the clinicopathological features of PCa patients

Variables	*N*	*IHC Score*				x^2	*p-value*
		0	*1*	*2*	*3*		
Histology						13.36	<0.005
BPH	20	3	3	5	9		
Pca	48	23	12	8	5		
Gleason score						12.59	<0.01
≤7	36	13	11	7	5		
>7	12	10	1	1	0		
Pathological stage						15.61	<0.005
pT2	21	5	7	5	4		
pT3-4	27	18	5	3	1		
Metastasis						11.53	<0.01
N_0M_0	25	8	7	6	4		
N_+ or M_+	23	15	5	2	1		

Table 2. Association of HAI-1 mRNA expression in Pca and BPH tissue

Variables	*N*	*HAI-1 mRNA Mean±SEM*	*p-value*
Histology			<0.01
BPH	20	0.6718±0.0516	
Pca	48	0.5482±0.0313	
Gleason score			<0.05
≤7	9	0.5613±0.0461	
>7	23	0.4984±0.0667	
Pathological stage			<0.05
pT2	21	0.6375±0.0817	
pT3-4	27	0.5291±0.0698	
Metastasis			<0.05
N_0M_0	25	0.6482±0.0497	
N_+ or M_+	23	0.5345±0.0388	

SEM: standard error of the mean

for HAI-1 (weak in 3, moderate in 5 and strong in 9), and 3 were scored as negative for HAI-1. Out of the 48 Pca tissues, 25 (52.08%) were scored as positive for HAI-1 (weak in 12, moderate in 8 and strong in 5), and 23 were scored as negative. Representative examples of HAI-1 tumor staining of the various histological grades are shown in Figure 1. The expression of HAI-1 protein in patients with BPH was significantly higher than in those with Pca (p<0.005). It was also higher in patients with lower Gleason score (≤7) and in earlier stage (T2). The expression was much higher in Pca with N_0M_0 than in Pca with N+ or M+ (p<0.01).

HAI-1 mRNA level decreased with increasing clinical stage in patients with Pca

HAI-1 mRNA level was tested in 48 cases with Pca and 20 cases with BPH by RT-PCR. The HAI-1 mRNA values in Pca and BPH tissue samples were normalized to β-actin mRNA (Table 2). The expression of HAI-1 mRNA was significantly higher in BPH compared with Pca cases (0.6718±0.0516 vs 0.5482±0.0313, p<0.01), it was significantly stronger in lower than in higher Gleason score (p<0.05), and the mean HAI-1 mRNA level was significantly higher in pT2 than in pT3-4 tumors (0.6375±0.0817 vs 0.5291±0.0698, p<0.05). Furthermore, HAI-1 expression was significantly stronger in N_0M_0 tumors than in N+ or M+ tumors (0.6482±0.0497 vs 0.5345±0.0388, p<0.05) (Figure 2).

Overall and disease-free survival analysis

HAI-1 was significant in the OS and DFS analysis of prognostic factors using the Gehan-Breslow-Wilcoxon test in Pca. Figure 3 shows OS and DFS curves of 48 patients with Pca, according to HAI-1 expression status. The OS and DFS rates of patients exhibiting high HAI-1 expression (score 2-3) were significantly higher compared to patients exhibiting low HAI-1 expression (score 0-1) (p=0.0492 and p=0.0487, respectively).

Discussion

HAI-1 is a Kunitz-type serine protease inhibitor that has a broad inhibitory spectrum against serine proteases, such as plasmin, trypsin, tissue and plasma kallikreins. These serine protease inhibitors are type I transmembrane glycoproteins that contain two extracellular Kunitz type inhibitory domains. They likely have important roles in cellular homeostasis and their dysregulated activities. To date, two studies on HAI-1 expression in Pca tissues have been published [12,13]. However, few studies have examined the association between the expression of HAI-1 and clinical features in patients with PCa.

In the current study, we compared the expression of HAI-1 in patients with PCa according to clinical features, including Gleason score, stage and metastasis by immunohistochemistry and RT-PCR. Immunohistochemistry on Pca tissues showed that HAI-1 expression was associated with Gleason score and pathological stage. RT-PCR showed that the expression of HAI-1 mRNA was significantly higher in BPH than in Pca, and

it was also significantly stronger in lower Gleason grade and pT2 cancer than in higher Gleason grade and pT3-4 cancer ($p<0.05$). Furthermore, HAI-1 expression was significantly stronger in N_0M_0 tumors than in N_+ or M_+ tumors. Moreover, the low HAI-1 expression was a significant predictor of poor prognosis compared with high HAI-1 expression (OS and DFS rates: p=0.0492, p=0.0487, respectively) (Figure 3). These findings indicate that HAI-1 could be an important negative correlation marker in progression and metastasis in Pca.

Our results coincide with a report by Saleem et al. [14], in which tissue samples from a normal prostate exhibited a high constitutive protein level of HAI-1 compared with BPH and low grade Pca. Previous studies indicated that reduced expression of HAI-1 is likely involved in the progression of prostate, breast, gastric and gynaecological cancer [11,14-17]. In breast cancer, inactivation of HAI-1 and its homologous protein HAI-2 significantly increased HGF mediated breast cancer cell migration and invasion [18]. Pca cells, after loss of HAI-1, showed increased invasiveness and cellular motility *in vitro* [19]. Engineered overexpression of HAI-1 in glioblastoma cells reduced the invasiveness *in vitro* [19]. Fukushima et al. investigated the effect of HAI-1 knockdown on the experimental pulmonary metastasis of the human pancreatic carcinoma cell line SUIT-2 and showed that loss of HAI-1 enhanced the metastatic pulmonary colonization [20]. In a study HAI-1 showed potential inhibitory effects on cell proliferation, migration and cellular invasion by reducing the matriptase and hepsin expression in endometrial cancer [21]. These results suggest that HAI-1 may play an important role in the progression of cancer.

The mechanism of HAI-1 in tumorigenesis is not clear. Further studies based on the therapeutic applications of HAI-1 for metastasis prevention are required. Andrew et al. [13] reduced the expression of HAI-1 in both PC-3 and DU-145 cell lines using hammerhead ribozyme technology, and found that the Pca cells had a significantly increased invasiveness *in vitro* together with an increase in cellular motility after losing HAI-1. Nakamura et al. found that HAI-1 prohibited the disease progression by inhibiting cell proliferation, migration and invasion in uterine leiomyosarcoma [22]. Keiichiro et al. [23] showed that the total apoptotic and necrotic cells increased to 47.80 and 59.64% as compared with the control of 27.96 and 9.80% for SiHa and HeLa cells respectively by transfection of the HAI-1 vector.

Conclusion

Our study demonstrated that HAI-1 protein expression and mRNA level are significantly decreased in Pca patients. With the Gleason score and clinical stage increased, the expression of HAI-1 decreased. Furthermore, HAI-1 expression was significantly stronger in N_0M_0 tumors than in N+ or M+ tumors. In summary, these findings identify HAI-1 as a favorable prognostic molecular marker, and could be considered as a potent therapeutic target for Pca.

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