A Novel Biomarker for Staging Human Prostate Adenocarcinoma: Overexpression of Matriptase with Concomitant Loss of its Inhibitor, Hepatocyte Growth Factor Activator Inhibitor-1

Mohammad Saleem,1 Vaqar Mustafa Adhami,1 Weixiong Zhong, B. Jack Longley,1 Chen-Yong Lin,4 Robert B. Dickson,4 Shannon Reagan-Shaw,1 David F. Jarrard,3 and Hasan Mukhtar1

Departments of 1Dermatology, 2Pathology and Laboratory Medicine, and 3Surgery, University of Wisconsin, Madison, Wisconsin; and 4Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC

Abstract

Background: Matriptase, a type II transmembrane serine protease is involved in angiogenesis, degradation of extracellular matrix, and in the progression of some epithelial cancers. Here, we establish the clinical significance of matriptase and its inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1), during the progression of human prostate cancer (CaP).

Methods: The expression patterns of matriptase and HAI-1 were determined in primary cultures of normal human prostate epithelial (NHPE) cells, human CaP cells LNCaP, DU-145, CWR22Rv1, and PC-3, and in tissue samples of 172 patients with normal prostate, benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), and adenocarcinoma of different tumor grades.

Results: The protein and mRNA levels of matriptase were significantly higher in all carcinoma cells as compared with NHPE cells. Conversely, all CaP cells exhibited a reduced expression of HAI-1 as compared with NHPE cells. A progressive increase in the protein levels of matriptase was observed with increasing tumor grade in CaP specimens as compared with normal and BPH tissue specimens. Tissue samples of normal prostate exhibited a high constitutive protein level of HAI-1 compared with BPH and low-grade cancer with a progressive loss with increasing tumor grade.

Conclusion: The increased expression of matriptase and loss of HAI-1 may be an important event during the progression of CaP in humans. We suggest that the ratio of these two gene products may serve as a promising biomarker for CaP progression and a potential marker for establishing the efficacy of therapeutic and chemopreventive interventions.


Introduction

Prostate cancer (CaP) is the most common visceral cancer diagnosed in men; it is the second leading cause of cancer-related deaths in males in the U.S. and in the Western world (1). The lack of effective therapies for advanced CaP reflects, to a large extent, the paucity of knowledge about the molecular pathways involved in CaP development. Thus, the identification of new predictive biomarkers will be important for improving clinical management, leading to improved survival of patients with CaP. Such molecular targets, especially those that are indicative of invasiveness of the disease, will also be excellent candidate targets for staging the disease and establishing the effectiveness of therapeutic and chemopreventive intervention of CaP (2, 3).

Among the critical pathologic processes that occur during the progression and metastasis of CaP is the breakdown of extracellular matrix (ECM) and interstitial stroma (4). An array of ECM-degrading proteases have been identified and implicated in cancer invasion and metastasis (5). The serine proteases with conserved residues of histidine, aspartate, and serine constitute a novel family of transmembrane enzymes involved in numerous biological processes, including activation of growth and angiogenic factors, and in the degradation of ECM components (6). The high expression of serine and other proteases in CaP are reported to be correlated with poor survival of the patients (7, 8). Because of a high level of serine protease expression in several human solid tumors, these enzymes have recently been proposed as potential diagnostic, prognostic, and/or therapeutic targets for several human tumors (9).

Matriptase, a type II transmembrane protein also known as tumor-associated differentially expressed gene-15 (TADG-15), is a serine protease and has been detected in various tissues of epithelial origin along with many cell lines in vitro and has been characterized as an activator of tumor cell invasion and metastases (10, 11). Matriptase has been shown to be overexpressed during tumor growth, invasion, and progression of breast, cervical, and ovarian cancer of humans (12-18). Virtually nothing is known about the function of matriptase in the prostate, with the exception of a study that identified a serine protease, MT-SP1, identical to matriptase in the prostatic cell line PC-3 (19). In addition, a recent study has shown that the specific inhibition of matriptase in CaP cells inhibits tumorigenesis in an athymic nude mouse model (20). The hepatocyte growth factor activator inhibitor-1 (HAI-1), a Kunitz-type serine protease inhibitor found predominantly in the columnar epithelium of many tissue types including breast, stomach, lung, kidney, prostate, and uterus, has been shown to modulate the activity of matriptase in several cancer types (10, 12, 21-24). HAI-1 has been shown to suppress the growth and motility of carcinoma cells by inhibiting the matriptase activity (12, 13).

In the current study, we provide evidence for the first time for overexpression of matriptase with concomitant loss of HAI-1 in human CaP cells and tissue specimens and show that this...
correlates with the clinical stages of human CaP. We propose a role for matriptase and HAI-1 protein in CaP development and suggest their potential use as a biomarker in the clinical management of CaP.

Materials and Methods

Primary Cell Culture. Prostate tissues were obtained under an approved Institutional Review Board protocol from men (ages 44-66) undergoing cystoprostatectomy for bladder cancer at the University of Wisconsin Hospital and Clinics. Histology confirmed that no bladder or prostate cancer was present in the prostate tissue harvested for our studies. Prostate epithelial cultures were established, as described previously (25).

Normal human prostate epithelial (NHPE) cells were maintained in Ham’s F-12 media (Invitrogen, Carlsbad, CA) supplemented with 0.25 units/mL regular insulin, 1 µg/mL hydrocortisone, 5 µg/mL human transferrin, 2.7 mg/mL dextrose, 0.1 mmol/L nonessential amino acids, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L l-glutamine, 10 ng/mL cholera toxin, 25 µg/mL bovine pituitary extract, and 1% fetal bovine serum.

Cell Lines. PC-3, CWR22Rv1, DU-145, and LNCaP cancer cells were obtained from American Type Culture Collection (Manassas, VA). PC-3, CWR22Rv1, and LNCaP cancer cells were cultured in RPMI 1640 (American Type Culture Collection), whereas DU-145 cells were grown in EMEM. The cells were cultured in a humidified atmosphere of 95% air and 5% CO2 in an incubator at 37°C.

Figure 1. Expression of (A) matriptase and (B) HAI-1 protein in NHPE and prostate cancer cells LNCaP, CWR22Rv1, DU145, and PC-3 by Western blotting. Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody. The histogram indicates the relative density of the bands normalized to β-actin. Columns, mean of relative densities; bars, ± SE; NS, nonsignificant.

Figure 2. Expression of (A) matriptase and (B) HAI-1 mRNA in NHPE and prostate cancer cells LNCaP, CWR22Rv1, DU145, and PC-3 by RT-PCR. Equal loading of RT-PCR product was confirmed by GAPDH as an internal control. The histogram indicates the relative density of bands normalized to GAPDH. Columns, mean of relative densities; bars, ± SE; NS, nonsignificant.
Monoclonal Antibodies. Anti-matriptase (M32) and anti-HAI-1(M19) monoclonal antibodies were developed in the Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC. The development and specificity of monoclonal antibodies has been previously described (11, 26).

Immunohistochemistry. Paraffin-embedded sections of human prostate tissues of 172 patients with normal, benign prostate hyperplasia (BPH), PIN, and adenocarcinoma were obtained from the Department of Pathology, University of Wisconsin Hospital, Madison, WI and Cybrdi Corporation (Gaithersburg, MD). Immunohistochemical staining was done using an automated Benchmark immunostainer (Ventana Medical Systems, Tucson, AZ). After antigen retrieval, thick-paraffin-embedded sections (5 μm) were dewaxed, rehydrated, and endogenous peroxidase activity was blocked. Sections were washed in water and PBS and were blocked in blocking buffer (2% goat serum/5% bovine serum albumin in PBS) for 30 minutes followed by incubation with primary antibody of matriptase-specific monoclonal antibody M32 (IgG1), and HAI-1-specific monoclonal antibody clone M19 (IgG1), at the dilution of 1:50 for 1 hour at room temperature. A negative control was included, in which prostate tissues were incubated with normal mouse IgG1 replacing the anti-HAI-1 or matriptase monoclonal antibody. In addition, we also included positive and negative tissue controls using the breast carcinoma specimens to show the specificity of the antibodies and quality of the immunohistochemical staining in our study. After incubation in the primary antibody, sections were washed twice in PBS to remove unbound antibody, followed by incubation for 2 hours at room temperature with appropriate horseradish peroxidase–conjugated secondary antibody. Immunoreactive complexes were detected using 3,3'-diaminobenzidene (Dako Corp., Carpinteria, CA). Slides were then counterstained in hematoxylin, mounted in crystal mount, and coverslipped in 50:50 xylene/Permount. Sections were visualized on a Zeiss-Axiophot DM HT microscope. Images were captured with an attached camera linked to a computer.

Histopathologic Grading of Prostate Cancer Specimens. The Gleason system and the WHO grading system were used for evaluation of levels of matriptase and HAI-1 in the prostatic tissue by immunoperoxidase staining. Prostate adenocarcinoma was first graded in Gleason patterns 1, 2, 3, 4, and 5. The intensity of immunoperoxidase staining for matriptase and HAI-1 was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) based on the Gleason patterns. The primary Gleason pattern and the secondary Gleason

Figure 3. Immunostaining for matriptase and HAI-1 in representative specimens of positive and negative controls. A negative control in which CaP tissues were incubated with normal mouse IgG replacing the anti-HAI-1 or matriptase monoclonal antibody (top). Positive controls for matriptase (middle) and HAI-1 (bottom) using the breast carcinoma specimens to show the specificity of the antibodies and quality of the immunohistochemical staining. The breast cancer specimens showed that both HAI-1 and matriptase were only present in the ductal and lobular epithelial cells, whereas the stroma was negative for both markers. Arrows, staining for matriptase and HAI-1 staining in the ductal and lobular epithelial cells. Original magnification, ×10; insets, ×40.
pattern were added to arrive at a Gleason score, ranging from 2 (1 + 1) to 10 (5 + 5). The Gleason scoring system was then converted to the WHO grading system as grade 1 (well differentiated prostatic adenocarcinoma) corresponding to Gleason score 5 to 6, and grade 3 (poorly differentiated or undifferentiated adenocarcinoma) corresponding to Gleason score 7 to 10.

**Western Blot Analysis.** Cell lysates were prepared in cold lysis buffer [0.05 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na3VO4, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA). The lysate was collected, cleared by centrifugation, and the supernatant aliquoted and stored at −80°C. The protein content in the lysates was measured by bichinonic acid protein assay (Piecer, Rockford, IL), as per the manufacturers’ protocol. For Western blot analysis, 40 μg protein was resolved over 12% Tris-glycine polyacrylamide gels (Novex, Carlsbad, CA) under nonreducing conditions, transferred onto nitrocellulose membranes and subsequently incubated in blocking buffer [5% nonfat dry milk/1% Tween 20; in 20 mmol/L TBS (pH 7.6)] for 2 hours. The blots were incubated with the appropriate primary (human reactive matriptase and HAI-1), washed and incubated with appropriate secondary horseradish peroxidase–conjugated antibody (Promega, Madison, WI). The blots were visualized by chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ). The blots were analyzed for the presence of the matriptase and HAI-1 protein and the expression levels were estimated using densitometry

**Statistical Analysis.** All measures were summarized as means ± SE. Graphical summaries of the distribution of measures were made using box plots. Measures were examined for the appropriateness of a normality assumption by density estimation (data not shown). Associations of categorical variables were evaluated using Fisher’s exact test. Sample correlations were estimated using Spearman’s rank correlation. All tests were two-sided and conducted at the α = 0.05 significance level. All statistical analyses were done with the S-plus, Professional Version 6.2 software (Insightful Corp., Seattle, WA).

**Results**

**Matriptase and HAI-1 Expression in NHPE and CaP Cells**

Matriptase and HAI-1 protein expression in NHPE and CaP cells. As an attempt towards identifying the expression of matriptase and HAI-1 in CaP progression, we first measured protein expression levels by immunoblot analysis in several human prostate carcinoma cell lines, LNCaP, CWR22Rv1, DU-145, of the cDNA were subjected to PCR in PCR buffer [20 mmol/L Tris-HCl (pH 8.4) containing 50 mmol/L KCl], 1.5 mmol/L MgCl2, 0.2 mmol/L deoxynucleotide triphosphate, 2.5 units of Platinum Taq DNA polymerase and 0.2 μmol/L of each primer for matriptase (forward, 5′-TATCGCTACTTCTGCTAGT-3′; reverse, 5′-GATCTTGTGCGTGTTCGTGC-GAC-3′) and HAI-1 (forward, 5′-AGACTATCGCTCCATCAACA-3′; reverse, 5′-AGCTGCAAGGCACGCT-GTAT-3′) obtained from Integrated DNA Technologies, Inc. (Coralville, IA) in a total volume of 25 μL. A constitutively expressed GAPDH gene (forward, 5′-ATGCCATACCATCATCTTCCAGGAG-3′; reverse, 5′-GATTGCATATGCCTTGGAGGCTTG-3′) was coamplified to confirm the equal loading of the cDNA. Fifteen-microliter aliquots of PCR products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining under UV transilluminator.

**Table 2. Expression of matriptase and HAI-1 in normal human prostate, BPH, PIN, and adenocarcinoma specimens**

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Matriptase staining intensity</th>
<th>HAI-1 staining intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>None</td>
</tr>
<tr>
<td>Carcinoma (all grades)</td>
<td>42</td>
<td>2</td>
</tr>
<tr>
<td>Grade 1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Grade 2</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Grade 3</td>
<td>24</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: The expression of matriptase and HAI-1 was evaluated as staining of the tissue as none (0), weak (1), moderate (2), and strong (3). Fisher exact test was used to examine the association between staining intensity and tumor grade (for tumor specimen only).

*P < 0.05 was considered significant.
Immunohistochemical Analysis of Matriptase and HAI-1 Protein in Normal, BPH, PIN, and CaP Specimens. In the next series of experiments, we used immunoperoxidase to determine matriptase and HAI-1 protein expression in specimens of normal, BPH, PIN, and CaP representing all tumor grades. These specimens were obtained as tissue array slides from Cybrdi Corporation. In the first experiment, a total of 88 samples were obtained out of which 48 were evaluated for matriptase expression and 40 samples were evaluated for HAI-1 expression. Breast carcinoma specimens were used as positive controls to show the specificity of the antibodies and the quality of the immunohistochemical staining. The breast carcinoma specimens showed that matriptase expression was present in the ductal and lobular epithelial cells, whereas the stroma was negative for matriptase (Fig. 3). These results are consistent with the results in the literature (12). The staining intensity in tissue specimens were scored on a scale of 0 to 3.

Scoring pattern for matriptase in tissue specimens. Based on the scoring patterns, a significant difference was observed in matriptase protein expression between cancer tissues and normal tissues (Table 1). The staining for matriptase protein was moderate to strong in CaP specimens as compared with normal prostate specimens which exhibited either none or weak staining (Table 1). In a total of 12 specimens of normal (n = 6) and BPH (n = 6), the staining for matriptase was weak in 1 (8%), moderate in 3 (25%) and negative in the remaining 8 (67%) specimens (Table 1). Staining for matriptase protein in PIN specimens (n = 4) showed moderate staining in 1 (25%) specimen and weak staining in the remaining 3 (75%) specimens (Table 1). In BPH and PIN specimens, staining for matriptase was either weak or moderate as compared with normal prostate, suggesting a possible role of matriptase during progression of CaP because BPH and PIN conditions are considered as high-risk factors for CaP development. Staining for matriptase in stromal cells was either occasional or negative in normal as well as cancer specimens.

Scoring pattern for HAI-1 in tissue specimens. Based on the scoring patterns, a significant difference in HAI-1 protein expression was also observed between normal prostate and CaP specimens (Table 1). Among the total sample size of 22 (normal, n = 6; BPH, n = 16) specimens, 19 (86%) specimens
exhibited weak to moderate staining for HAI-1 protein, whereas 3 (14%) specimens did not exhibit any HAI-1 protein staining (Table 1). The staining for HAI-1 protein in PIN specimens \((n = 5)\) showed weak staining in 2 (40%) and no staining in the remaining 3 (60%) specimens (Table 1). The staining for HAI-1 protein was generally moderate in normal prostate specimens and weak to moderate in BPH specimens (Table 1). However, in sharp contrast, CaP specimens exhibited weak or no staining suggesting a loss of HAI-1 protein during progression of CaP (Table 1).

Scoring pattern of matriptase and HAI-1 in pair-matched CaP specimens. Because we observed a significant difference in staining pattern for matriptase and HAI-1 protein between normal and CaP specimens, we next asked whether the overexpression of matriptase and loss of its inhibitor HAI-1 protein, was a general phenomenon that occurs during the progression of CaP. To achieve this objective, we examined the staining pattern for matriptase and HAI-1 proteins in 42 pair-matched specimens. These samples were obtained as tissue array slide from CaP patients at University Hospitals (University of Wisconsin-Madison). As is evident from the data, the scoring pattern for matriptase protein was moderate to strong in all grades of CaP specimens, being mostly moderate in grades 1 to 2 carcinomas and generally strong in grade 3 carcinomas (Table 2). In sharp contrast, the scoring pattern for HAI-1 staining in pair-matched samples was weak to moderate in all grades of CaP specimens being generally moderate in grades 1 to 2 carcinomas and mostly weak in grade 3 carcinomas (Table 2).

Average scoring pattern of matriptase in CaP specimens. The box plots of the cumulative data (48 specimens + 42 pair-matched specimens) for matriptase protein expression exhibited a wide

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**Figure 5.** Immunostaining for matriptase in representative specimens of (A) normal, BPH, PIN (B) CaP specimens of tumor grades 1 to 3, and (C) in specimens with adjacent normal and cancerous regions. CaP specimens were assigned tumor grades on the basis of Gleason pattern and Gleason score as described in Materials and Methods. Immunoreactive matriptase protein was observed in a coarsely granular pattern in cell cytoplasms of epithelial cells of BPH and of grade 1, grade 2 and grade 3 prostatic adenocarcinoma. There was minimal staining of occasional stromal cells. Matriptase expression was weak in normal and moderate to strong in BPH, PIN and advanced CaP specimens. ➔, staining for matriptase in cancer regions; ←, staining for matriptase in normal regions. Original magnification, ×10; insets, ×40.
interspecimen variation in cancer specimens, compared with the normal tissues and revealed a significant difference in the level of protein expression between normal and CaP tissues (Fig. 4A). The average score for matriptase protein in normal prostate tissues was $0.25 \pm 0.02$ (mean $\pm$ SE; $n = 6$) and was significantly higher, $2.5 \pm 0.2$ (mean $\pm$ SE; $n = 44$) in high-grade cancer specimens (grade 3), a 5.5-fold increase ($P < 0.001$; Fig. 4A). Although the staining for matriptase in BPH specimens was higher than normal, it was significantly lower ($0.70 \pm 0.05$; mean $\pm$ SE; $n = 6$, $P < 0.001$), compared with CaP tissues.

Average scoring pattern for HAI-1 protein in CaP specimens. The box plots (cumulative data of 40 specimens + 42 pair-matched specimens) for HAI-1 protein indicated a wide interindividual...
variation in cancer specimens compared with normal tissue (Fig. 4B). The average scoring pattern for HAI-1 protein was 2.8 ± 0.15 (mean ± SE; n = 6) in normal and 1.8 ± 0.08 (mean ± SE; n = 16) in BPH specimens, however, the scoring pattern was significantly reduced to 1.0 ± 0.05 (mean ± SE; n = 27) in grade 3 cancer specimens (P < 0.05). These data show that prostate tissue significantly loses the inhibitory protein HAI-1 with the onset of CaP and is almost completely lost at the highly advanced stages of the disease (Fig. 4B).

Staining pattern of matriptase protein. Figure 5A and B shows the immunoreactive matriptase protein in a coarsely granular pattern in the cytoplasm of epithelial cells of normal, BPH, PIN, and of grade 1 (Gleason pattern 2, Gleason scores 5-6) to grade 3 (Gleason pattern 4, Gleason scores 7-10) prostatic adenocarcinoma. The staining for matriptase was highly positive in epithelial cells of CaP specimens (cumulative sample size) and was negative in epithelial cells of normal prostate specimens. A cumulative analysis of all CaP specimens (n = 74) suggested higher levels of matriptase expression, with moderate to strong staining in 66 (90%) specimens, weak staining in 3 (4%) specimens, and no staining in 5 (6%) specimens (Tables 1 and 2). Because PIN has been identified as the most significant risk factor for CaP development and the expression of matriptase protein was found to be increased in both PIN and CaP specimens, a strong link could be suggested between the expression of matriptase protein and the development of human CaP.

The staining pattern of matriptase protein was further compared in grade 1, grade 2, and grade 3 CaP specimens. The mean matriptase expression was 1.75 ± 0.15 (mean ± SE; n = 6) in grade 1 specimens, 2.0 ± 0.20 (mean ± SE; n = 24) in grade 2 specimens, and 2.7 ± 0.15 (mean ± SE; n = 44) in grade 3 specimens indicating a progressive increase of matriptase as the disease progresses from lower to higher stages (Fig. 5B). In specimens belonging to grade 1 and grade 2

Figure 6. Immunostaining for HAI-1 protein in representative specimens of normal (A), BPH, PIN, CaP of Gleason patterns 2 to 4 (B), and in specimens with adjacent normal and cancerous regions (C). CaP specimens were assigned tumor grades on the basis of Gleason pattern and Gleason score as described in Materials and Methods. Staining for HAI-1 was positive in the cytoplasm of the stromal cells of normal and BPH but was progressively lost in CaP specimens. →, cancerous region; ↔, normal regions. HAI-1 protein expression was lost in both cancer as well as adjacent normal regions in CaP specimens of Gleason pattern 4. Original magnification, ×10; insets, ×40.
CaP ($n = 30$), 27 specimens exhibited moderate to strong staining for matriptase protein whereas no staining was observed in 3 specimens (Tables 1 and 2). Similarly, in grade 3 CaP specimens ($n = 44$), 39 (89%) exhibited moderate to strong staining for matriptase protein, 3 (7%) exhibited weak staining, and the remaining 2 (4%) specimens showed no staining (Tables 1 and 2). These data show a progressive increase of matriptase protein expression corresponding with increasing tumor grade in human CaP.

Next, we evaluated the staining pattern of matriptase protein in the tissues which had normal regions and cancer regions adjacent to each other in the same specimen. In these tissues, the scoring pattern for matriptase in the normal region was 0, whereas it was significantly higher (2-3) in cancer

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**Figure 6 Continued.**
regions (Fig. 5C). In these tissues, matriptase protein expression was undetectable in normal regions adjacent to cancer regions, whereas its expression was significantly high in all grades of cancer of all specimens (Fig. 5C).

**Staining pattern of HAI-1 protein.** Normal glandular epithelial cells were generally negative for HAI-1 protein (with some occasional epithelial staining); however, intense cytoplasmic staining for HAI-1 was observed in stromal cells in these specimens (Fig. 6A). The normal stromal cells which were found to be positive for HAI-1 staining are most likely to be smooth muscle cells. Furthermore, staining for HAI-1 was found to be positive in the stromal cell cytoplasm of BPH specimens and less staining was observed in PIN specimens (Fig. 6A). Among grade 1 specimens (Gleason pattern 2, Gleason scores 2-4), some specimens exhibited a minimal apical staining for HAI-1 in stromal cells, however, all specimens belonging to grade 2 (Gleason pattern 3, Gleason scores 5-6) and grade 3 (Gleason pattern 4, Gleason scores 7-10) category, exhibited a low cytoplasmic staining in stromal cells.

Figure 6A and B shows the HAI-1 protein staining in normal, BPH, PIN, and CaP specimens. The staining for HAI-1 protein in CaP tissues (cumulative sample size; Tables 1 and 2) was strong in 2 (3%), moderate in 21 (26%), weak in 29 (35%), and negative in 30 (36%) specimens (Tables 1 and 2). The staining pattern of HAI-1 protein was further compared in grade 1, grade 2, and grade 3 CaP specimens (Fig. 6B). Contrary to matriptase expression, a progressive loss of HAI-1 protein was observed in CaP specimens with increasing tumor grade (Table 1). Analysis of the cumulative samples (Tables 1 and 2) suggested that in CaP specimens belonging to grade 1 (n = 6) and grade 2 (n = 22), the staining for HAI-1 protein was strong in 2 (4%), moderate in 9 (16%), weak in 11 (20%), and the remaining 6 (11%) specimens did not exhibit staining. In grade 3 CaP specimens (n = 27) moderate staining was observed in 1 (2%), weak in 8 (14%) and the remaining 18 (33%) specimens did not exhibit staining (Tables 1 and 2).

Next, we evaluated the staining pattern of HAI-1 protein in CaP tissues with cancerous as well as adjacent normal regions (Fig. 6C). Interestingly, HAI-1 protein expression was lost in both cancer as well as adjacent normal regions in CaP specimens of Gleason pattern 4 (Fig. 6C).

**Statistical Analysis.** We next examined the association between matriptase and HAI-1 protein expression in BPH and CaP tissues. The Spearman correlation between matriptase and HAI-1 was found to be -0.46 (P < 0.001) based on total specimens (Tables 1 and 2). This analysis indicates an inverse correlation between matriptase and HAI-1 protein expression during the progression of human CaP.

**Discussion**

One important consideration in the intervention and prevention of CaP is the development of surrogate end point biomarker(s) that can be correlated with the staging of disease along the course of tumor development. The expression levels of matriptase and HAI-1 genes, which have been proposed to be involved in tumor development and metastases for some cancers, were examined in normal and prostate carcinoma cell lines along with tissue specimens of normal, BPH, PIN, and malignant prostate tissues. The strong expression of matriptase in prostate carcinoma cell lines and in malignant tissues of the human prostate, as compared with a very low constitutive level in normal prostate cells and tissues, indicate that this gene product may be involved in the progression of human prostate adenocarcinoma. In contrast, HAI-1 protein expression was significantly lower in highly aggressive prostate carcinoma PC-3 cells, as compared with LNCaP and CWR22Rv1 cells. In addition, the expression of matriptase inhibitor HAI-1 protein was highly diminished or absent in the high-grade malignant prostate tissues, as compared with normal prostate tissues, and a progressive loss of HAI-1 expression was observed during the progression of disease from low-grade to high-grade CaP. The important finding from our study is that the expression of matriptase protein increases significantly with a concomitant loss of its inhibitor HAI-1 with progressive stages of CaP development in humans. This observation suggests that the matriptase/HAI-1 complex could be employed as a predictive biomarker in human CaP development.

Metastatic spread of CaP to distant sites is regarded as the major cause of CaP-related deaths in humans (4). Tumor cells acquire this increased invasive potential by a complex pathway, which include decreased cell substrate attachment and cell-cell adhesion, as well as increased cell motility (4). To successfully complete the complex invasion and metastatic process, degradation of the ECM including the basement membrane and interstitial stroma, is required for tumor cells to migrate through anatomic barriers and to invade distant tissues (4, 28). In this process, the proteolysis of ECM is thought to be one of the most critical steps. High levels of proteolytic activities have been implicated in neoplastic progression (11, 29). Serine proteases have been implicated in the degradation of ECM in various types of cancers and have been proposed as potential diagnostic and therapeutic targets for human tumors (5, 6, 30). It is known that two members of the serine protease family, prostate-specific antigen and human kallikrein 2, serve as important prostate carcinoma biomarkers (6, 31).

Matriptase is a trypsin-like type II membrane serine protease that was first isolated from human breast milk (10, 32). It has a multidomain structure, containing a putative amino-terminal transmembrane region, a sperm protein, enterokinase, an agrin (SEA) domain, two complement subcomponents (C1r/C1s), urchin embryonic growth factor, and bone morphogenetic protein (CUB) domains, four low-density lipoprotein receptor class A repeats, and a carboxyl-terminal serine protease domain (11, 13). The human gene for matriptase is located within chromosome 11q24-25 and is likely to be the human homologue of the mouse serine protease epitphin, based on its high degree of sequence identity and chromosomal location (12, 33).

Matriptase has a widespread expression in normal epithelial tissues including the skin, thymic stroma, gastrointestinal tract, kidney, lung, seminal vesicle, and mammary gland (19). High levels of matriptase have been reported in a variety of carcinomas such as ovarian, cervical, breast, and gastric cancer (12, 21). It has been shown to be a potential activator of key molecules associated with tumor invasion and metastasis (16). For example, when activated by matriptase, urokinase plasminogen activator and the protease-activated receptor-2, leads to the degradation of the ECM and activation of other protease systems involved in the spread of cancer cells (16, 34). Matriptase has also been reported to convert hepatocyte growth factor/scatter factor to its active form, resulting in the scattering of tumor cells (35). Therefore, matriptase has been suggested to play an important role in the growth and/or invasion of human cancers via its ability to activate pro-urokinase plasminogen activator and pro-hepatocyte growth factor. In the current study, moderate to strong expression of matriptase was observed in cancer tissue, which increased with tumor grade.

The activity of matriptase has been reported to be modulated by HAI-1, a Kunitz-type inhibitor (21). HAI-1 has been shown to colocalize with matriptase and prevent the undesired proteolysis in breast cancer cells (11, 32). It has been shown that HAI-1 protein expression in human primary colorectal carcinomas is decreased significantly in cells within colon carcinomas relative to adjacent normal mucosa or adenomas.
(35). Recently, the loss of HAI-1 expression has been implicated in the progression of renal cell and colorectal carcinoma in human patients (22, 36). Earlier studies have implicated HAI-1 protein in the pathogenesis of human hepatocellular carcinoma (22). It has been reported that the invasive nature of several cancer cell lines of breast, prostate, colon, bladder, liver, lung and pancreas is correlated to the degree of HAI-1 protein expression (10, 12, 21, 22). It is important to emphasize here that in the current study, diminution of HAI-1 expression was observed in CaP specimens of all grades. Although HAI-1 protein and mRNA expression was observed in NHPF cells, its expression was observed to be mostly strong in stromal cells than in epithelial cells in CaP specimens. Additional studies with a large sample size of various tumor grades are required to establish a significant inverse correlation between matriptase and HAI-1 protein expression during CaP progression. Similarly, further studies are warranted to validate the role of ratio of matriptase to HAI-1 as a predictive potential predictive indicator for human CaP development.

In conclusion, this study indicates that the enhancement of matriptase and diminution of HAI-1 expression during the progressive stages of CaP may be associated with increasing biological aggressiveness of the disease. Thus, the combined analysis of matriptase and HAI-1 in tissue biopsies may serve as a prognostic marker in cases of prostate adenocarcinoma and may ultimately lead to monitoring therapeutic response during CaP treatment protocols and for establishing efficacy of CaP chemopreventive regimens.

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In conclusion, this study indicates that the enhancement of matriptase and diminution of HAI-1 expression during the progressive stages of CaP may be associated with increasing biological aggressiveness of the disease. Thus, the combined analysis of matriptase and HAI-1 in tissue biopsies may serve as a prognostic marker in cases of prostate adenocarcinoma and may ultimately lead to monitoring therapeutic response during CaP treatment protocols and for establishing efficacy of CaP chemopreventive regimens.

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A Novel Biomarker for Staging Human Prostate Adenocarcinoma: Overexpression of Matriptase with Concomitant Loss of its Inhibitor, Hepatocyte Growth Factor Activator Inhibitor-1

Mohammad Saleem, Vaqar Mustafa Adhami, Weixiong Zhong, et al.


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