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# Receptor for Hyaluronic Acid-mediated Motility (RHAMM) Is Associated With Prostate Cancer Migration and Poor Prognosis

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**Abstract.** *Background/Aim: Hyaluronic acid (HA) is a large glycosaminoglycan composed of an extracellular matrix. The HA-rich microenvironment and receptors of HA have been suggested to play roles in cancer progression. The biological and clinical significance of receptor for HA-mediated motility (RHAMM), known as CD168 in prostate cancer (PC) remains unknown. This study aimed to investigate the expression of RHAMM, as well as its functional and clinical relevance in PC. Materials and Methods: HA concentration and RHAMM mRNA expression were examined in 3 PC cell lines (LNCaP, PC3 and DU145). We investigated the effect of HA and RHAMM on the migratory ability of PC cells using a transwell migration assay. Immunohistochemistry was also used to evaluate the RHAMM expression pattern in pre-treatment tissue samples from 99 patients with metastatic hormone-sensitive PC (HSPC) who received androgen deprivation therapy (ADT). Results: HA was secreted in all cultured PC cell lines. Among the total HA, low-molecular-weight HA (LMW-HA) (<100 kDa) was detected all examined cell lines. The number of migration cells was significantly increased by adding LMW-HA. RHAMM mRNA expression was increased in DU145 cells. Knockdown of RHAMM using small-interfering RNA resulted in decreased cell migration. Immunohistochemical analysis revealed strong RHAMM expression in 31 (31.3%) patients with metastatic HSPC. A strong RHAMM expression was significantly associated with short ADT duration and poor survival in univariate and multivariate analyses. Conclusion: The size of HA is important in terms of PC progression. LMW-HA and RHAMM enhanced PC cell migration. RHAMM could be used as a novel prognostic marker in patients with metastatic HSPC.*

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*Key Words:* Prostate cancer, hyaluronic acid, receptor for hyaluronic acid-mediated motility, migration, prognosis.



Androgen deprivation therapy (ADT) is a gold-standard treatment for patients with metastatic prostate cancer (PC). Metastatic hormone-sensitive PC (HSPC) progresses to castration-resistant PC (CRPC) despite a favorable initial ADT response. Recently, guidelines recommended a combination of abiraterone, apalutamide, or enzalutamide with ADT as first-line therapy for patients with metastatic HSPC (1, 2). However, a large retrospective study using real-world data in the United States revealed that approximately 43% of patients received ADT alone as first-line therapy for metastatic HSPC (3). Additionally, we previously demonstrated that some patients with metastatic HSPC achieved long-term survival under ADT alone (4). Therefore, biomarkers to effectively predict primary ADT response are required.

The microenvironment surrounding the tumor cells plays an important role in cancer progression. Hyaluronic acid (HA) is a large glycosaminoglycan composed of an extracellular matrix. A previous study revealed that HA-rich stroma promotes tumor progression in various cancer cells (5-7). HA was detected relatively frequently and abundantly

in PC tissue (8). However, the relationship between PC cells and HA concentration has rarely been reported.

The receptor for HA-mediated motility (RHAMM), also known as CD168, is a specific HA receptor. Turley *et al*. (9, 10) were the first to identify this receptor as a soluble binding partner of HA that is directly involved in tumor cell locomotion by the regulation of *Ras* oncogene. HA binding to RHAMM stimulates various signal cascades on the cell surface (11). Previous studies have reported that RHAMM overexpression was detected in several solid malignant tumors and was associated with the invasion, progression and poor prognosis of mammary carcinoma, pancreatic ductal adenocarcinoma, gastric cancer, lung cancer, and colorectal cancer (12-16). The functional significance of RHAMM has yet to be comprehensively elucidated in PC, although the association between RHAMM expression and CRPC was previously studied (17-19).

This study aimed to evaluate the concentration of HA and mRNA levels of *RHAMM* in PC cell lines and elucidate the effect of HA and RHAMM on the migratory ability of PC cells. Additionally, we assessed the impact of RHAMM expression on oncological outcomes of patients with metastatic HSPC who received ADT.

## Materials and Methods

*Cell cultures*. Three cell lines, LNCaP (androgen-sensitive cell line derived from human PC), PC3 and DU145 (androgen-independent cell lines derived from human PC) were used in this study. PC cell lines were cultured in RPMI1640 medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin in a 5% $CO_2$ incubator at 37˚C.

*HA concentration evaluation*. After 48 h of cell culture, the culture supernatant was collected. The HA concentration in the culture supernatant was measured using the Quantikine ELISA Hyaluronan Immunoassay (R&D Systems Inc., Minneapolis, MN, USA) (20).

*Cell migration assay*. The transwell cell migration assay was used for determining the cell migration ability using cell culture inserts equipped with a filter membrane containing 8 μm pores (BD Biosciences, Franklin Lakes, NJ, USA), as described previously (21). The lower chamber was filled with RPMI1640 containing 10% FBS. The upper chamber was filled with $2.0\times10^4$ cells in the RPMI1640 containing 1% FBS. After 24 h incubation, cells on the bottom surface of the membrane were counted.

*Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)*. Total RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Real-time mRNA expression of *RHAMM* and a housekeeping gene (*GAPDH*) for control was analyzed using TaqMan@ Gene Expression Assays and Step One Plus real-time RT-PCR system (Thermo Fisher Scientific Inc.) according to the previously described method for pancreatic cancer by Cheng *et al*. (13). The assay numbers for these genes were as follows: Hs00234864_m1 (*RHAAM*) and Hs02786 624_g1 (GAPDH).

*RHAAM siRNA knockdown*. The small-interfering RNA targeting for *RHAAM* [ON-TARGETplus Human HMMR (3161) siRNA SMARTpool L-010409-00-0005] and control siRNA (ON-TARGETplus Control siRNA Non-targeting siRNA #1 D-001810-01-05) (GE Healthcare, Buckinghamshire, UK) were used for knockdown assay (21). Cells were transfected with 100 nM siRNA using DharmaFECT 1 Transfection Reagent (GE Healthcare) for 48 h of treatment.

*Patients*. This study retrospectively reviewed 104 consecutive patients with metastatic HSPC who had undergone conventional ADT at the University of Occupational and Environmental Health (UOEH; Kitakyushu, Japan) from August 2005 to August 2018. Only patients with histologically-confirmed prostatic adenocarcinoma were included. The analysis included 99 patients after the exclusion of 5 patients due to incomplete clinical data. We collected pre-treatment tissue samples from trans-rectal prostate biopsy for diagnosis. The clinical tumor stage was defined according to the tumor, lymph nodes, and metastasis (TNM) staging system (22). The patient's clinical information and follow-up data were retrieved from the medical records of our department. The present study protocol was approved by the UOEH Institutional Review Board (approval no. UOEHCRB19-050).

*Immunohistochemical staining of RHAMM*. Immunohistochemistry studies were carried out using the polymer-based detection with antibodies conjugated to an enzyme labeled dextran chain for antibody-bridge labelling (EnVision; Dako, Glostrup, Denmark). Nuclear counterstaining was performed with hematoxylin. Formalin fixed paraffin embedded tissue blocks were cut at 4-$\mu$m unstained sections, they were deparaffinized in xylene, and hydrated in ethanol. Sections were blocked for the endogenous peroxidase activity by incubating in 10% $H_2O_2$ for 5 min. Subsequently, sections were rinsed and incubated with a primary antibody targeting RHAMM (anti-CD168 antibodies, ab124729, Abcam, Cambridge, MA, USA) at a dilution of 1: 50 overnight, followed by the second antibody-peroxidase-linked polymers. Sections were examined under the light microscopy. At first, we scanned each section at low power for entire fields (original magnification: ×40) using tumor and non-tumor tissues, respectively. Then the tumor region at a high-power field (original magnification: ×200) was observed to grade the neoplastic cells by the Gleason system (Gleason score of 6-10) (23) and determine the distribution. The normal prostate gland was used as the positive control for RHAMM immunohistochemistry.

The immunoreactivity degree for RHAMM was assessed according to the previously described scoring method by Cheng *et al*. (24) with a minor modification, which combined intensity and percentage of positivity. The entire staining intensity was graded (0-3+) and was calculated by multiplying the percentage of RHAMM positive cells in the Gleason score (*e.g*., 0×100% = 0; +1×50%=50; and +3×100%=300). Therefore, each specimen was evaluated with a staining score between 0 and 600. Finally, we divided all patients into two groups to simply classify the staining pattern as follows: weak expression (scores of 0-300) or strong expression (scores of 301-600). All immunohistochemical slides were scored by two independent researchers (A.M. and H.N.) who were blinded to the clinical data.

*Statistical analysis*. All statistical analyses were performed using EZR version 1.40 (Easy R, Vienna, Austria). Differences between

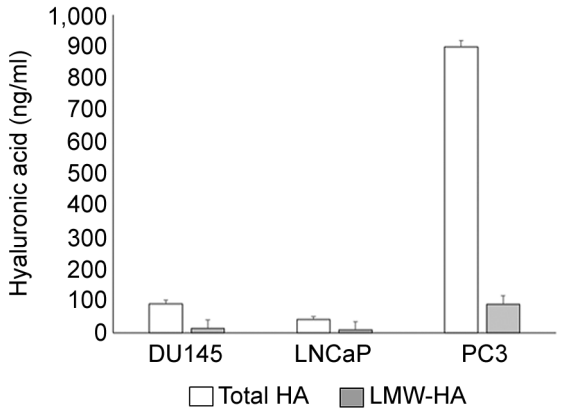


Figure 1. *Hyaluronic acid levels in DU145, LNCAP and PC3 cell lines as measured by the Quantikine ELISA Hyaluronan Immunoassay. HA: Hyaluronic acid; LMW-HA: low-molecular-weight hyaluronic acid.*

categorical variables were examined by the Fisher's exact test, whereas continuous variables were compared using the Mann–Whitney *U*-test. Time to prostate-specific antigen (PSA) progression and overall survival (OS) were evaluated using the Kaplan–Meier method and log-rank test. The associations between the clinicopathological factors and time to mortality were assessed using the Cox proportional hazard models. A *p*-values of <0.05 was considered to indicate statistical significance.

## Results

*HA levels in PC cells*. HA was secreted in all PC cell lines (Figure 1). Low-molecular-weight HA (LMW-HA) (<100 kDa) was also detected in each cell line among the total HA.

*Effects of adding HA on PC cells*. We examined the changes in DU145 cell migration by adding exogenous high-molecular-weight HA (HMW-HA) (>1,000 kDa) and LMW-HA. The number of migration cells was significantly increased by adding HMW-HA, and more robustly by adding LMW-HA ($p<0.01$; Figure 2).

*Effects of RHAMM expression and migration in PC cells*. Real-time RT-PCR showed higher expression of the *RHAMM* mRNA in the DU145 cells compared to LNCAP and PC3 cells (Figure 3). Then, we used siRNA to knock-down *RHAMM* expression in DU145. The transwell migration assay showed that *RHAMM* knockdown significantly decreased the DU145 cell migration compared to the control (non-target siRNA transfection) ($p<0.01$; Figure 4).

*Immunohistochemical analysis of RHAMM in PC tissues*. RHAMM expression was predominantly found in the cytomembrane and cytoplasm of tumor cells (Figure 5). Of the 99 specimens examined, 68 (68.7%) revealed weak RHAMM expression, while 31 (31.3%) revealed strong expression according to the scoring method.

*Association between RHAMM expression and prognosis in patients with metastatic HSPC*. The clinicopathological characteristics of patients stratified by RHAMM expression are summarized in Table I. No significant differences were found between the groups in terms of age, PSA levels, Gleason score, clinical tumor stage, node status, presence of visceral metastasis and number of bone metastasis.

The median follow-up time was 37 months [interquartile range (IQR)=21-61], during which 71 (71.7%) patients experienced PSA progression with median time of 14 (IQR=9-29) months, whereas 52 (52.5%) died from PC. A strong RHAMM expression was significantly associated with a shorter time to PSA progression after ADT initiation, as shown in Figure 6A ($p<0.001$). The median time to progression of patients with RHAMM-weak and -strong expression groups was 29 and 10 months, respectively. Patients in the RHAMM-strong expression group had poorer OS values compared to those in the RHAMM-weak expression group ($p<0.001$; Figure 6B). The median OS of RHAMM-weak and -strong expression groups was 87 and 23 months, respectively.

Table II presents the results of univariate and multivariate Cox proportional hazards regression analyses predicting OS after adjusting for clinicopathological characteristics. Accordingly, RHAMM expression was identified as an independent predictor of OS (HR=5.97; 95% CI=3.05-11.70; $p<0.001$). Moreover, the time to PSA progression on ADT was significantly associated with survival outcomes.

## Discussion

RHAMM exerts distinct functions that potentiate tumor progression in human malignancies (12-16), but the role of RHAMM in PC remains unknown. The current study was conducted using cell migration assay and immunohistochemistry to assess the influence of the HA-RHAMM pathway on oncological PC outcomes. Our study indicated that RHAMM contributes to PC cell migration and clarified the correlation between the RHAMM expression pattern and the survival of patients with metastatic HSPC.

This study revealed that HA, including LMW-HA, was secreted from cultured PC cells. Interestingly, the addition of LMW-HA to cultured PC cells more robustly increased their migratory ability than addition of HMW-HA. Several studies have shown that LMW-HA promotes cancer growth and metastasis in pancreatic and breast cancer (7, 25). Our results suggest that the size of HA is important in terms of its effect on PC progression. Aaltomaa *et al*. (26) reported that HA expression was associated with PSA recurrence in localized PC. Further, Lipponen *et al*. (27) revealed that a high HA

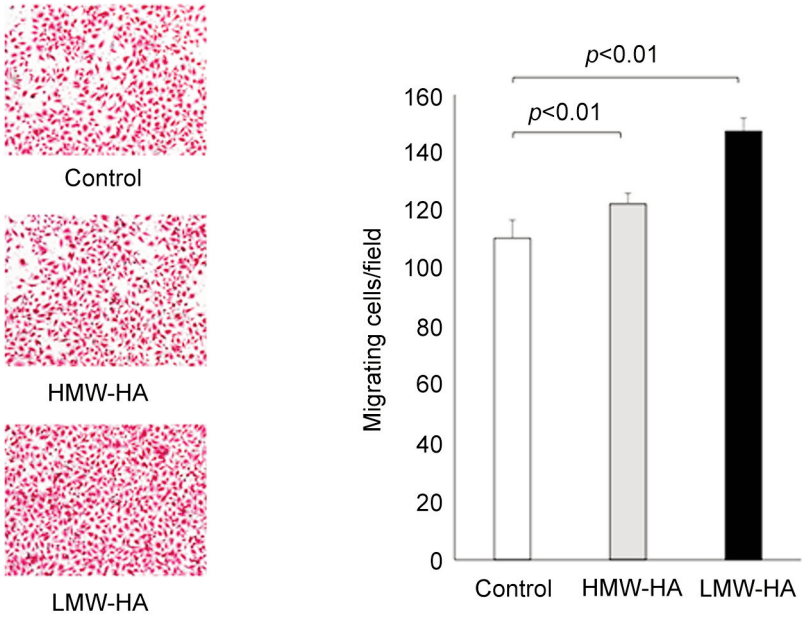


Figure 2. *Effects of HMW-HA and LMW-HA on the migration of prostate cancer cells in DU145 as assessed by the transwell migration assay. HMW-HA: High-molecular-weight hyaluronic acid; LMW-HA: low-molecular-weight hyaluronic acid.*

level in the tumor stroma predicted metastasis and poor survival outcome.

RHAMM is a multifunctional protein that controls differential response to injury contexts (28). Normal prostatic tissue dose not express RHAMM (19). RHAMM expression was not an independent prognostic factor although previous studies have shown increased RHAMM expression in PC (18, 19). Most RHAMM expression analyses revealed that elevated RHAMM expression was associated with worse prognostic factor in many cancers, including breast cancer (29), oral squamous cell carcinoma (30), ovarian cancer (31), lung cancer (15), colon cancer (16), gastric cancer (14), and pancreatic cancer (13). Our study demonstrated that RHAMM overexpression enhances the malignant potential of PC. Previous studies revealed that the migratory ability of RHAMM was particularly evident in various breast cancer cell lines (12, 32, 33). The binding of HA to RHAMM induced focal adhesion kinase and enhances the mitogen-activated protein kinase family (Ras-MEK-ERK signaling pathway) in various cancers (34). This complex could stimulate these kinase cascades and promotes cell migration and invasion. Additionally, activated RHAMM controls expression of genes involved in cell motility such as PAI-1 and MMP-9, which results in cell migration (11). Interestingly, RHAMM forms an intracellular complex with BRCA1 and BRCA2 (28) and may therefore contribute to a higher risk of PC.

Our findings suggest that the inhibition of HA synthesis may be a straightforward therapeutic target for PC. Blocking the HA-RHAMM signaling pathway may be a potent

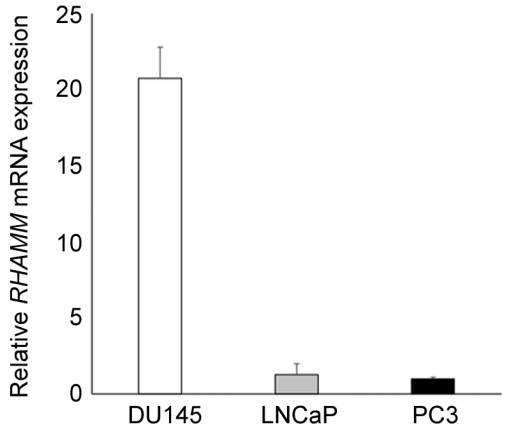


Figure 3. *Real-time RT-PCR analysis validated RHAMM mRNA expression in DU145, LNCAP and PC3 cell lines. RT-PCR: Reverse transcription polymerase chain reaction; RHAMM: receptor for hyaluronic acid-mediated motility.*

inhibitor to suppress PC progression. Recently, Yates *et al.* (35) demonstrated that 4-methylumbelliferone, which inhibits HA synthesis, had significant efficacy against tumor growth and metastasis in the PC model.

The present study revealed a significant difference in time to PSA progression between strong and weak RHAMM expression in patients who received conventional ADT alone. Korkes *et al.* (19) showed that significant RHAMM expression occurs as early as one month after ADT and

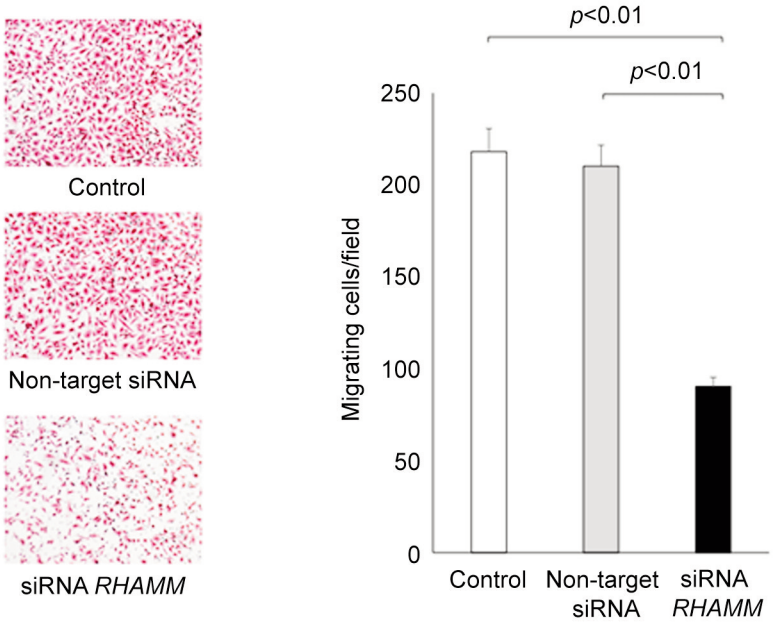


Figure 4. *Knockdown of RHAMM expression decreased migration in DU145 cells as shown by a transwell migration assay. RHAMM: Receptor for hyaluronic acid-mediated motility; siRNA: small-interfering ribonucleic acid.*

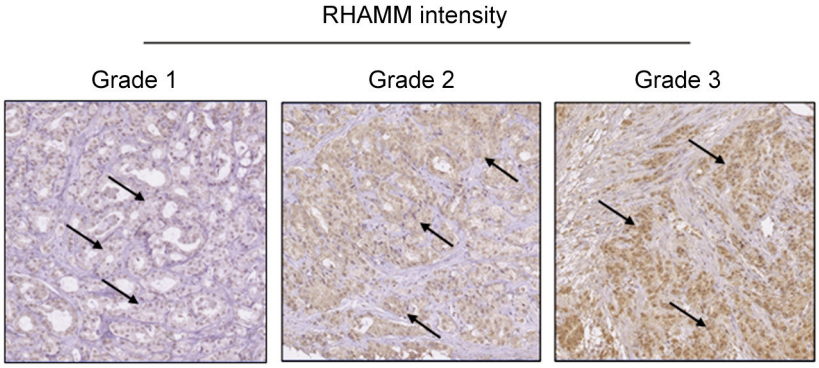


Figure 5. *Representative immunohistochemistry for RHAMM staining (arrows) in prostate cancer tissues. RHAMM: Receptor for hyaluronic acid-mediated motility.*

progressively increased with ADT duration. RHAMM could be a novel marker predicting the ADT response duration in patients with metastatic HSPC. Therefore, further investigations comparing the effects of upfront combination therapies with ADT to ADT alone according to RHAMM expression are imperative. Conversely, conventional ADT may be sufficient for the weak RHAMM expression.

This study has several limitations. First, we could not investigate the relationship between RHAMM and androgen receptor (AR). Therefore, it is important task to indicate that RHAMM regulates AR expression in androgen-dependent PC cell lines. Lin *et al*. (17) showed that overexpressed RHAMM bound to AR. Second, we were unable to perform quantitative immunohistochemical analyses regarding localized PC and CRPC. Gust *et al*. (18) showed that the protein level of RHAMM was highest in metastatic PC compared to localized PC. Third, this study did not assess a role of CD44, a known cancer stem cell marker and another major receptor for HA. Evaluating the interactions between RHAMM and CD44 is required to further validate our results shortly.

The HA-RHAMM pathway was associated with an aggressive PC phenotype. The size of HA is important in terms of PC progression. LMW-HA enhanced PC cell migration compared to HMW-HA. To our best knowledge, for the first time, we revealed that knockdown of *RHAMM* expression using siRNA resulted in decreased PC cell

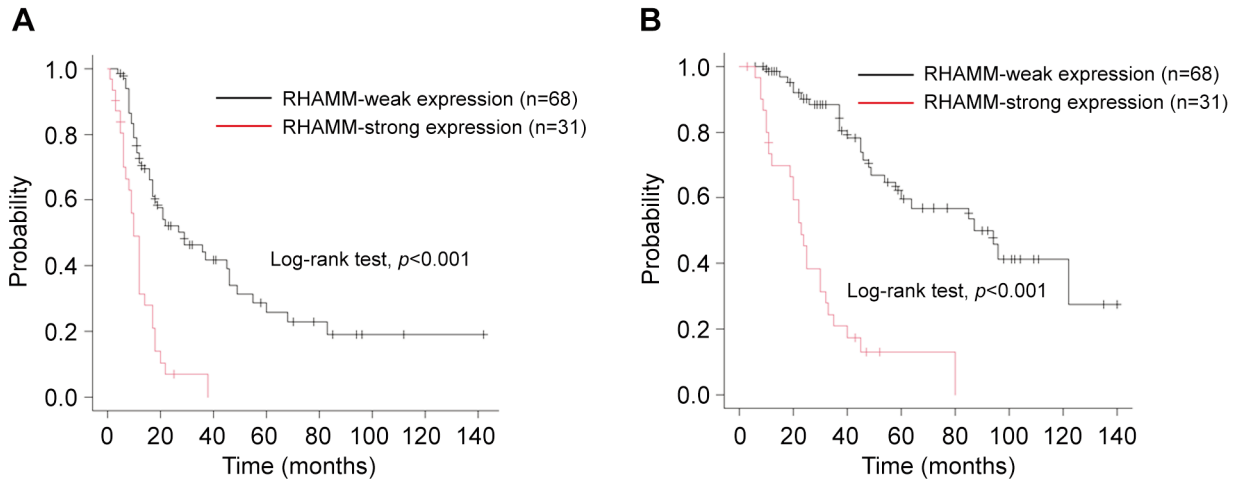


Figure 6. *Kaplan–Meier curves showing the prognostic value of RHAMM expression for time to PSA progression (A) and overall survival (B) after ADT initiation. RHAMM: Receptor for hyaluronic acid-mediated motility; PSA: prostate-specific antigen; ADT: androgen deprivation therapy.*

Table I. *Relationship between RHAMM expression and patient characteristics.*

| Variable | | RHAMM weak n=68 | RHAMM strong n=31 | *p*-Value |
|---|---|---|---|---|
| Age, years | Median (IQR) | 73 (68-78) | 75 (67-80) | 0.475 |
| PSA level at diagnosis (ng/ml) | Median (IQR) | 170.2 (72.3-603.5) | 141 (62.7-519.6) | 0.692 |
| Gleason score, n (%) | <8 | 10 (14.7) | 1 (3.2) | 0.165 |
| | ≥8 | 58 (85.3) | 30 (96.8) | |
| Tumor stage, n (%) | <cT3 | 20 (29.4) | 6 (19.4) | 0.335 |
| | ≥cT3 | 48 (70.6) | 25 (80.6) | |
| Lymph node metastasis, n (%) | Negative | 29 (42.6) | 13 (40.9) | 1.000 |
| | Positive | 39 (57.4) | 18 (59.1) | |
| Visceral metastasis, n (%) | Negative | 55 (80.9) | 26 (83.9) | 0.787 |
| | Positive | 13 (19.1) | 5 (16.1) | |
| Bone metastases, n (%) | <4 | 22 (32.4) | 9 (29.1) | 0.818 |
| | ≥4 | 46 (67.6) | 22 (70.9) | |

PSA: Prostate-specific antigen; IQR: interquartile range; RHAMM: receptor for hyaluronic acid motility.

Table II. *Results of univariate and multivariate analyses for overall survival in patients after ADT.*

| Variable | Comparison | Univariate | | Multivariate | |
|---|---|---|---|---|---|
| | | HR (95% CI) | *p*-Value | HR (95% CI) | *p*-Value |
| Age, years | ≥74 *vs.* <74 | 1.42 (0.82-2.44) | 0.212 | 1.01 (0.56-1.82) | 0.981 |
| PSA level at diagnosis (ng/ml) | ≥170 *vs.* <170 | 1.13 (0.66-1.95) | 0.658 | 0.98 (0.53-1.81) | 0.958 |
| Gleason score | ≥8 *vs.* <8 | 2.42 (0.94-6.25) | 0.067 | 1.46 (0.50-4.23) | 0.488 |
| Tumor stage | ≥cT3 *vs.* <cT3 | 1.42 (0.74-2.72) | 0.292 | 1.55 (0.74-3.27) | 0.245 |
| Lymph node metastasis | Presence *vs.* absence | 1.94 (1.08-3.50) | 0.028 | 1.83 (0.94-3.53) | 0.074 |
| Visceral metastasis | Presence *vs.* absence | 0.85 (0.40-1.81) | 0.673 | 0.78 (0.32-1.91) | 0.588 |
| Bone metastases | ≥4 *vs.* <4 | 1.86 (0.97-3.57) | 0.061 | 1.66 (0.79-3.52) | 0.183 |
| Time to PSA progression on ADT, months | <14 *vs.* ≥14 | 5.03 (2.75-9.21) | <0.001 | 4.03 (2.05-7.91) | <0.001 |
| RHAMM expression | Strong *vs.* weak | 6.42 (3.49-11.81) | <0.001 | 5.97 (3.05-11.70) | <0.001 |

PSA: Prostate-specific antingen; ADT: androgen deprivation therapy; RHAMM: receptor for hyaluronic acid motility; HR: hazard ratio; CI: confidence interval.

migration. We believe that our study provides a better understanding of the significance of RHAMM in terms of the biological behavior of PC.

In conclusion, increased RHAMM expression was associated with higher malignant potential of PC. RHAMM overexpression could be an independent predictor of survival in patients with metastatic HSPC who received ADT.

## Conflicts of Interest

The Authors declare no competing interests in relation to this study.

## Authors' Contributions

AM: Conceptualization, investigation, data curation, formal analysis and writing of the original manuscript. YK: investigation and writing of the original manuscript. HN: pathologic assessment and reviewing. SK: conceptualization and reviewing. YH: data curation. NS and KH a: supervision. NF: reviewing and supervision. All Authors discussed, verified, and approved the final version of the manuscript.

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