Higher Expression of the Androgen-Regulated Gene PSA/HK3 mRNA in Prostate Cancer Tissues Predicts Biochemical Recurrence-Free Survival


Abstract

Purpose: Alterations of the androgen receptor (AR)-mediated signaling through numerous mechanisms are increasingly recognized in prostate cancer (CaP) progression. We hypothesized that the assessment of well-defined AR transcriptional targets (e.g., PSA/HK3 mRNA) in CaP tissues will provide in vivo readout of AR dysfunctions. Moreover, quantitative expression features of PSA/HK3 mRNA in prostate tumor cells may serve as a prognostic indicator of disease progression.

Experimental Design: Paired benign and malignant epithelial cells (242 specimens) were obtained from laser capture microdissection of frozen OCT-embedded tissue sections prepared from radical prostatectomy specimens of 121 patients. Quantitative expression of PSA/HK3 mRNA in the matched malignant and benign cells was analyzed by real-time reverse transcription-PCR.

Results: CaP cells express significantly lower PSA/HK3 mRNA levels than matched benign cells ($P = 0.0133$). Moreover, low PSA/HK3 mRNA expression in malignant cells was associated with increased risk of biochemical recurrence ($P = 0.0217$), as well as with time to recurrence ($P = 0.0371$), in patients with intermediate preoperative serum prostate-specific antigen levels (2-10 ng/mL). The expression of androgen-dependent genes in clinical samples correlates with each other in patients with higher expression of PSA/HK3 mRNA but not in patients with lower expression of PSA/HK3 mRNA reflecting AR pathway dysfunction.

Conclusions: Our study has unraveled a novel prognostic utility of quantitative measurements of PSA/HK3 mRNA reflecting AR transcriptional activity in CaP cells, which is independent of serum prostate-specific antigen. It also has potential in stratifying subsets of patients exhibiting progressive disease associated with dampened AR transcriptional functions who may be targeted by tailored therapeutic strategies.

Defects in androgen receptor (AR)-mediated signaling are increasingly highlighted for potentially causal roles in prostate cancer (CaP) progression (1, 2). Our laboratory has been evaluating AR transcriptional targets, which have promise in defining the role of AR dysfunctions in CaP and in providing novel biomarkers and therapeutic targets during CaP progression (3–6). Despite impressive research progress in the area of AR signaling in CaP cells and the identification of numerous androgen-regulated genes, we have yet to realize the translation of these findings into streamlined measurements of the of AR dysfunctions, which can be applied to CaP patients. Because AR can be altered via numerous mechanisms (physiologic changes in the male hormone, testosterone, AR mutations, AR amplifications, defects of AR coactivators and corepressors, and aberrant cross-talk with other protumorigenic signaling pathways), the net effect of one or more of these changes is reflected in defective transcription factor functions of the AR. Therefore, measurement of expression of carefully selected AR transcriptional targets should provide information on the in vivo status of the AR in CaP cells. This information will help identify patients based on AR signal amplitude and may provide new ways of managing and treating these patients.

Whereas serum prostate-specific antigen (PSA) protein measurements have been a staple of the urologic oncology literature since the late 1980s, tissue PSA protein and mRNA expression have been little studied (7, 8). Given the widespread use of serum PSA in CaP screening, and its role in predicting treatment outcome, it is surprising that tissue PSA mRNA or...
protein expression has not undergone greater investigation. Some such attempts have been made, however, with one example identifying a correlation between PSA/HK3 mRNA expression within biopsy specimens and CaP-specific mortality (9). Although prior studies assessing tissue PSA have focused on immunohistochemistry or RIA for detecting protein, and in situ hybridization for detecting RNA, real-time reverse transcription-PCR (RT-PCR) provides the more sensitive and quantitative means of detecting mRNA (10–13). Serum PSA has previously been shown to be unrelated to tissue protein or mRNA levels (10). If tissue PSA/HK3 mRNA expression were to provide prognostic information independent of serum PSA, this finding could improve the ability to counsel patients about outcomes.

In this study, to develop an in vivo readout of AR functional status in CaP cells, we have been pursuing parallel quantitative measurements of various AR-regulated genes in carefully isolated benign and tumor cells of more than 200 specimens. Quantitative expression analyses of AR and androgen-regulated genes (PSA/HK3, PMEPA1, and PCA3), as well as androgen-independent genes (AMACR and LTF) representing more than 2,000 data points, suggest that PSA/HK3 mRNA and similar androgen-regulated genes reflect in vivo functional status of the AR. We chose to focus on HK3/PSA mRNA as it is one of the most robust direct transcriptional targets of AR and is easily detectable in CaP cells (14). We report here novel observations suggesting that malignant cells express significantly lower PSA/HK3 mRNA levels than matched benign cells. Moreover, decreased expression of PSA/HK3 mRNA in CaP tissue, independent of serum PSA, predicts greater odds of biochemical recurrence, and it associates with time to recurrence, in patients with intermediate preoperative serum PSA levels (2-10 ng/mL).

### Materials and Methods

The prostate tissue specimens used in this study were obtained under an Institutional Review Board–approved protocol at Walter Reed Army Medical Center. Laser microdissected cells were evaluated from benign and neoplastic epithelium of 121 men who had undergone a radical prostatectomy as their primary treatment for CaP. All patients were hormone naive. A chart of specimen processing, microdissection, and quantitative RT-PCR work flow is presented in Fig. 1A.

Benign and malignant prostate epithelial cells (consisting of secretory cells with nuclear anaplasia evidenced by the presence of nuclei that are larger and more varied in size and shape than those of adjacent, obviously benign glands) were laser capture microdissected by a pathologist from OCT medium-embedded and H&E-stained frozen prostate sections of radical prostatectomy specimens (5-10,000 cells per one sample). The MicroRNA kit (Stratagene) was used for RNA isolation from laser capture microdissected specimens. The RNA was quantified with RiboGreen dye (Molecular Probes) and a VersaFluor fluorimeter (Bio-Rad). The integrity of the total RNA isolated from laser capture microdissected samples was determined by Bioanalyzer 2100 (Agilent Technologies) as well as by assaying for a housekeeping gene (GAPDH) expression in the same tube for each quantitative RT-PCR (Taqman) measurement. Real-time quantitative RT-PCR (Taqman) was done as previously described (15, 16). Total RNA was converted to cDNA (Sensiscript, Qiagen) and Taqman-based quantitative RT-PCR provided quantitative analysis of gene expression (ABI 7700, PE Applied Biosystems). The Taqman primers and probe for PSA/HK3 were as follows: 5′-CCCCACTGCTACAGAGAAAAA-3′ (forward), 5′-GACGCGGTCTGGAGAGGCT-3′ (reverse), and 6FAM-ACACAGCGCAGTATCTCCAGTGCC-TAMRA (probe) (probe). The Taqman probe/primers were designed to overlap the exon 2-exon 3 border of PSA/HK3 that is present in all isoforms except for isoform 6, which is a truncated form expressing no functional protein (it has only exons 1 and 2). Taqman primers and probe were as follows: PMEPA1, 5′-CAGTATCCCCGAGCTGCT-3′ (forward), 5′-TGGATGCTAACAACTCAGCCTCC-3′ (reverse), and 6FAM-AGGCCAGACGTCTCCTCCGGAAAC-TAMRA (probe); LFT, 5′-CCATGCCCCGAGATAT-3′ (forward), 5′-AGGGTTGTGGGTTCCAGCTG-3′ (reverse), and 6FAM-ACCAACAGGTAAATMTG-BNFQ (probe); PCA3, 5′-CACATTCGCCCGCCATAT-3′ (forward), 5′-CCGCCAGGCTCAGCTGAT-3′ (reverse), and TET-GGAAGCGACAGATCCGGTGAGAAATG-TAMRA (probe); and AR, 5′-GCTGTCATCATGAGCAGTATCTCCAGTGCC-TAMRA (probe) (probe). The Taqman primers and probe for AMACR were “Assay-on-Demand” (PE Applied Biosystems).

The expression of GAPDH served as an endogenous control. Target gene expression in each sample was normalized to GAPDH. A negative control was provided by RNA samples without reverse transcription. The standard thermal cycling conditions were the following: 95°C for 10 min, 50 cycles at 95°C for 15 s, and 60°C for 1 min. Results were plotted as average Cj (threshold cycle) values of duplicate samples. The relative gene expression level was presented as ΔCj tumor versus matched benign cells, where ΔCj means normalized Ct value of target genes to GAPDH.

### Statistical analysis

Clinical and pathologic characteristics of the study sample were examined, including age at time of surgery, diagnostic PSA (ng/mL), race (Caucasian and other versus African-American), family history (yes versus no), pathologic T stage (pT2 versus pT3, we refer to 1997 tumor-node-metastasis staging system for
pathologic stage), postoperative Gleason sum (2-6 and 7 versus 8-10), margin status (positive versus negative), PSA recurrence (yes versus no), and follow-up time. PSA recurrence was defined as a single postoperative PSA of ≥0.2 ng/mL 2 months after surgery. Follow-up time was calculated as time from surgery to PSA recurrence for patients who experienced this event as opposed to time from surgery to last follow-up visit within the study period for those who did not experience PSA recurrence.

Frequencies are reported for categorical patient features, whereas measures of central tendency and dispersion are reported for continuous patient features. Univariate Cox proportional hazards analysis was used to identify factors that are independently associated with odds of PSA recurrence over time among men with serum PSA of 2 to 10 ng/mL. Kaplan-Meier unadjusted survival analysis curves were used to examine time to PSA recurrence across quintiles of tissue PSA/HK3 mRNA expression. Due to distinct separation of quintiles 1 to 2 from quintiles 3 to 5 in Kaplan-Meier analysis, as well as strong skewing in continuously measured tissue PSA/HK3 mRNA expression data, a dichotomy of quintiles 1 to 2 versus quintiles 3 to 5 was created for multivariate analysis. Multivariate Cox proportional hazards analysis was carried out to examine odds of PSA recurrence over time in men with serum PSA of 2 to 10 ng/mL. Hazards odds ratios and corresponding confidence intervals and P values are reported. Finally, Pearson correlation analysis was used to determine associations between mRNA expression of selected genes, including AR, PCA3, PMEPA1, AMACR, and LTF, in tumor cells in two groups of patients: those with low tissue PSA/HK3 mRNA expression and those with high tissue expression. AMACR and LTF were selected as "controls" because both are non–AR regulated and, therefore, not expected to be associated with tissue PSA/HK3 expression. Unadjusted regression tree analysis was used to determine the dichotomy of tissue PSA/HK3 mRNA expression into groups of "low" and "high," modeling PSA recurrence as the dependent outcome.

Results

RNA specimens from laser capture microdissection--derived benign and malignant prostate epithelial cells of 121 CaP patients were evaluated for PSA/HK3 mRNA expression. Patient demographic and clinicopathologic characteristics are seen in Supplementary Tables S1 and S2. Tissue PSA/HK3 mRNA expression was compared with serum PSA protein expression via Spearman correlation analysis (n = 109), finding no significant relationship (P = 0.9635). Paired t test assessment showed tissue PSA/HK3 mRNA expression to be significantly lower (P = 0.0133) in malignant prostate epithelial cells than benign prostate epithelial cells (n = 121).

To study patients with intermediate serum PSA levels, further analysis was limited to those patients with serum PSA from 2 to 10 ng/mL (n = 79). The distribution of biochemical recurrence and PSA/HK3 mRNA expression in tumor cells of CaP patients is presented in Fig. 1B. The expression of tissue PSA/HK3 mRNA in tumor cells of biochemical recurrence-free patients

![Fig. 2. A, lower PSA/HK3 mRNA expression in prostate tumor cells correlates with increased risk of biochemical recurrence: Kaplan-Meier survival estimation curve for time to PSA recurrence-free survival by tumor tissue PSA/HK3 mRNA quintiles among patients with serum PSA of 2 to 10 ng/mL. Quintiles are ordered in such a way that quintile 1 refers to the highest and quintile 5 to the lowest PSA/HK3 expression (n = 76). B, prognostic features of PSA/HK3 mRNA readout in CaP tissue: schematic representation of AR-regulated PSA/HK3 mRNA expression in tumor cells of CaP patients. Top, high expression of tumor PSA/HK3 mRNA, which associates with biochemical recurrence-free survival; bottom, patients with low expression of PSA/HK3 mRNA, reflecting alteration of AR signaling in tumor cell microenvironment, which may lead to increased chance of biochemical recurrence after radical prostatectomy.](www.aacrjournals.org)
was significantly higher than in patients with biochemical recurrence \((P = 0.0062, \text{ Student's } t \text{ test})\). PSA/HK3 mRNA expression in benign epithelial cells did not show such correlation. This cohort was divided into quintiles based on tissue \(\text{PSA/HK3} \text{ mRNA expression in tumor cells and compared with respect to time to biochemical relapse. As seen in Fig. 2A, unadjusted Kaplan-Meier analysis showed improved biochemical survival for patients with the highest \(\text{PSA/HK3} \text{ mRNA expression (quintiles 1 and 2; } P = 0.0217\). Univariate Cox proportional hazards analysis was done, finding postoperative Gleason sum \((P = 0.0115\) and tumor \(\text{PSA/HK3} \text{ mRNA expression } (P = 0.0129\) predictive for biochemical recurrence \((n = 78; \text{ Supplementary Table S3}\). Moreover, when subjected to multivariate analysis, only tumor \(\text{PSA/HK3} \text{ mRNA } (P = 0.0371\) and postoperative Gleason grade \((P = 0.0194\) were predictive of PSA recurrence \((P = 0.04095\). The mean tissue \(\text{PSA/HK3} \text{ mRNA expression did not differ across Gleason scores categorized as 2 to 6, 7, and 8 to 10 } (P = 0.4095)\).

To investigate the relationship of tumor \(\text{PSA/HK3} \text{ mRNA expression to the other AR-regulated genes, regression tree analysis was used to dichotomize tissue \(\text{PSA/HK3} \text{ into high and low values. Quantitative expression levels of AR and androgen-dependent genes } \text{PMEPA1} (17) \text{ and } \text{PCA3/DD3} (18) \text{ and androgen-independent genes } \text{LTF} (19) \text{ and } \text{AMACR} (20) \text{ in tumor epithelial cells were compared with one another at high and low tumor } \text{PSA/HK3} \text{ expression levels. Although not androgen dependent, } \text{AR} \text{ was also included in this analysis as it is a master regulator of the androgen signaling pathway. As shown in Supplementary Table S4, the androgen-dependent genes exhibited a significant correlation in patients with high tissue } \text{PSA/HK3} \text{ mRNA expression but no such relationship existed in patients with low tissue } \text{PSA} \text{ levels. In contrast, the androgen-independent genes did not exhibit correlation to any level of tissue } \text{PSA/HK3} \text{ mRNA.}

Discussion

To develop \textit{in vivo} functional readout of AR dysfunctions in CaP cells, we have used quantitative RT-PCR to assay \textit{PSA/HK3} mRNA expression in tumor and benign cells of patient specimens. Similar to prior studies, there is no relationship between tissue \(\text{PSA/HK3} \text{ mRNA and serum PSA measurements } (10-13). Although Stege et al. (11) showed an inverse relationship between tissue PSA and serum PSA, they used fine-needle aspiration techniques to assess the tumor PSA expression. The lack of correlation between tissue and serum PSA measurements is clinically significant in that tissue \(\text{PSA/HK3} \text{ mRNA expression, as it is presented here, predicts biochemical outcome independent of serum PSA for those patients with serum PSA between 2 and 10 ng/mL. This novel observation was shown in both univariate and multivariate analysis } (\text{ Table 1; Supplementary Table S3). The predictive ability of tumor } \text{PSA/HK3} \text{ mRNA expression was further shown in Kaplan-Meier analysis with lower tumor } \text{PSA/HK3} \text{ mRNA expression correlating with increased risk of biochemical recurrence } (\text{ Fig. 2A). Relatively lower tumor } \text{PSA/HK3} \text{ mRNA may reflect alterations in androgen signaling in the tumor cell microenvironment, which might associate with progressive disease } (\text{ see model in Fig. 2B). These findings warrant further investigation in both pathologic and in biopsy specimens. In the setting of prostate biopsy, tumor } \text{PSA/HK3} \text{ mRNA measurement may provide another clue to distinguish which patients could be safely offered watchful waiting, whereas in the postprostatectomy setting it could dictate follow-up schedules or need for adjuvant therapy. We also showed that malignant prostate epithelial cells express significantly less } \text{PSA/HK3} \text{ mRNA than benign cells from the same patient, which has been suggested based on } \textit{in situ} \text{ hybridization studies before } (21, 22). Although the genesis of this difference in PSA expression is unknown, it identifies a molecular alteration in tumor cells and perhaps suggests for alterations of AR-mediated signaling leading to cell differentiation--associated change, as PSA is an AR-regulated prostate-specific differentiation marker. Of note is that recent reports support our intriguing observations of decreased AR signaling with CaP progression } (23, 24). The fact that lower tissue \(\text{PSA/HK3} \text{ mRNA expression is associated with malignant cells and that within that malignant population it is associated with increased risk of biochemical recurrence suggests that
tumor cells. A direct comparison of the PSA protein level readout in the tumor cells more closely than a PSA mRNA expression readout reflects the in vivo transcriptional activity in the tumor cells. Therefore, it is a more accurate estimate of the AR status in the tumor cells. A direct comparison of AR and PSA/HK3 mRNA levels in tumor cells of a cohort of CaP patients (n = 186) by Spearman correlation analysis revealed no significant correlation (R = 0.17; P = 0.078), suggesting that AR mRNA levels do not correlate well with AR protein transcriptional factor activity, which is reflected in tissue PSA/HK3 mRNA levels. To further investigate the molecular changes indicated by the tissue PSA/HK3 mRNA, we have also investigated other androgen-dependent genes. An alteration in the AR-regulated gene network was suggested by the fact that the expression levels of androgen-dependent genes and AR itself were significantly related to each other at higher, but not at lower, tissue PSA/HK3 mRNA levels. In contrast, androgen-independent genes did not exhibit any relationship to the tissue PSA/HK3 mRNA expression. Androgen pathway dysregulation is increasingly highlighted in CaP progression (2, 14). Our results show that the expression of genes within the androgen-dependent pathway is dysregulated within tumor cells with low tissue PSA/HK3 mRNA. Given the ability of tumor PSA/HK3 mRNA levels to predict outcome, this study supports our hypothesis for role for AR dysfunction in dictating the behavior of CaP even in the early stages. These promising results must be interpreted with caution in the context of our study population. The applicability of our findings to a general population must be determined independently.

Unlike in breast cancer where estrogen receptor protein status in primary tumor is effectively used in making therapeutic and prognostic decisions (25–27), AR protein expression status does not seem to be useful in CaP, likely because many factors besides its expression level affect AR activity. Although AR expression can be detected throughout the progression of CaP, it is heterogeneous and changes over time. Several studies have indicated that AR expression is reduced in poorly differentiated areas with higher Gleason score (2, 28–30). A signature of attenuated AR function in late stage, especially in metastatic CaP in human specimens (23), as well as in a xenograft model system (24) was noted recently. In contrast, others found that higher AR expression, amplification, or activity is associated with higher clinical stage, higher Gleason score, decreased PSA recurrence-survival, and metastasis (1, 31–33). Part of the reason for this controversy is the inherent heterogeneity of AR expression in the prostate (especially in late stages and androgen-independent, metastatic CaP) and the semiquantitative nature of immunohistochemical evaluations (30, 34).

Initially, CaP development is driven by the AR pathway (1, 2, 28). However, frequent alterations of AR structure and function are well recognized during CaP progression. AR can be altered via numerous mechanisms (as described in Introduction). The net effect of these changes is reflected in the defective transcription factor functions of the AR. Therefore, measurement of the expression of carefully selected AR downstream targets, such as PSA/HK3, provides information on the in vivo functional status of AR in CaP cells. This information may help in stratifying patients based on AR signal amplitude and may provide new ways of managing and treating these patients.

In conclusion, we show here that, for radical prostatectomy patients with preoperative serum PSA between 2 and 10 ng/mL, tumor PSA/HK3 mRNA expression, as a marker of in vivo AR function, is predictive of biochemical recurrence. This finding makes tissue PSA/HK3 mRNA a compelling target for future investigation in predicting CaP outcome. Thus, our study has unraveled a novel prognostic utility of quantitative measurements of PSA/HK3 mRNA in CaP cells and this strategy may also help in stratifying subset of patients exhibiting progressive disease associating with dampened AR transcriptional functions. Moreover, quantitative features of PSA/HK3 mRNA expression in CaP cells may help not only to assess disease progression but also to offer new targeted therapeutic strategies for this group of patients at high risk of progression.

References


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