Preoperative Blood Reverse Transcriptase-PCR Assays for Prostate-specific Antigen and Human Glandular Kallikrein for Prediction of Prostate Cancer Progression after Radical Prostatectomy


ABSTRACT

The association of the results reverse transcription-PCR (RT-PCR) assays for prostate-specific antigen (PSA) with prostate cancer stage and progression remains controversial. We have previously shown that RT-PCR amplification of a native human glandular kallikrein fragment (hK2-L), but not a splice-variant hK2 transcript (hK2-U), performed preoperatively on peripheral blood before radical prostatectomy is an independent predictor of metastases to lymph nodes [K. M. Slawin, et al., Cancer Res., 60: 7142–7148, 2000]. We evaluated the ability of preoperative peripheral blood RT-PCR for PSA and hK2 to predict prostate cancer progression after surgery. The study group consisted of 224 consecutive patients who underwent radical prostatectomy for clinically localized disease (median follow-up: 52.9 months). Also evaluated were 14 healthy men and 5 patients with hormone-naive, prostate cancer metastatic to bone. RT-PCR for PSA, hK2-L, and hK2-U were positive in 24, 25, and 26%, respectively, of prostatectomy patients; 88, 71, and 86%, respectively, of patients with metastases to bones; 7, 14, and 36%, respectively, of healthy men. Preoperative RT-PCR-hK2-L positivity was associated with higher final Gleason sum (P = 0.028), with an increased risk of metastases to lymph nodes (P = 0.019) and with overall and aggressive disease progression (P = 0.0231 and P = 0.028, respectively). Preoperative blood RT-PCR-PSA and RT-PCR-hK2-U were not associated with characteristics or outcomes of prostate cancer. Preoperative blood RT-PCR for PSA and hK2-U are neither therapy-guiding staging tools nor prognostic indicators in patients with clinically localized prostate cancer. In contrast, preoperative RT-PCR-hK2-L status is associated with established markers of aggressive prostate cancer.

INTRODUCTION

RT-PCR is a highly sensitive technique to detect small quantities of mRNA within the peripheral blood or within other body fluids or tissues. RT-PCR is superior to cytology and immunological approaches in sensitivity and specificity for detecting disseminated cells in peripheral blood. The presence of tumor cells in the circulation, however, does not necessarily indicate that clinically significant metastasis has occurred. On the other hand, foci of metastatic prostate cancer detectable by conventional modalities (e.g., histology, bone scan, or computed tomography scan) are almost always associated with biologically significant advanced disease. Newer, ultrasensitive assays, like RT-PCR, that can identify smaller numbers of cells, place the burden squarely on investigators to demonstrate the clinical and biological significance of a positive result.

Some studies have demonstrated a significant predictive value of RT-PCR for PSA or for prostate-specific membrane antigen for both pathologic stage of prostate cancer (1–4) and progression-free survival (5, 6) with these assays, but the majority have failed to demonstrate any clinically significant role for them (7–9). In addition, the limited follow-up time and the formidable technical problems regarding sample processing and handling that may underlie these conflicting results (10), questions regarding the specificity of some of these markers for prostate cancer cells, and the potentially variable biological and clinical potential of the cells detected by these assays have lessened the initial enthusiasm for their clinical applicability (11–14).

hK2 is an androgen-regulated protein that has an ~80% amino acid sequence identity with PSA (15) and is expressed almost exclusively in prostatic epithelial cells. Although the expression of PSA has been found to be reduced in higher grade and presumably more biologically active disease, (16, 17) the expression of hK2 has been found to increase gradually from benign epithelium to prostatic intraepithelial neoplasia and to prostate cancer (17). Furthermore, hK2 expression was directly associated with the Gleason grade of the primary tumor, and foci of prostate cancer metastatic to the lymph nodes have been found to demonstrate the highest level of expression (18). We have previously described a highly sensitive and specific RT-PCR assay for hK2 mRNA (19) that we designed to amplify differentially two previously described splice variants of the hKLK2 gene (20). When the assay was performed on peripheral blood of patients with clinically localized prostate cancer before a radical prostatectomy, the native hK2-amplified fragment (hK2-L), but not the splice variant fragment (hK2-U), was an independent predictor of metastases to regional lymph nodes. On the basis of these observations, we hypothesized that RT-PCR for hK2, but not PSA, would detect circulating cells that might indicate the presence of occult metastatic disease in patients undergoing radical prostatectomy and that therefore would be associated with prostate cancer progression despite effective control of local disease. To determine the relationship between preoperative peripheral blood RT-PCR for PSA, hK2-L, and hK2-U and the risk for prostate cancer progression, we studied a large consecutive cohort of patients with clinically localized prostate cancer who underwent radical prostatectomy and who had long-term follow-up.

MATERIALS AND METHODS

hK2 and PSA cDNA. A full-length human hK2 cDNA clone was used as PCR template for hK2. Previous analysis of this clone demonstrated a 70-bp deletion beginning at position 150, and an additional 40 bp of intron 4 associated with a previously reported splice variant of the hK2 gene (21). Plasmid DNA was purified using the Qiagen-Plasmid Miniprep kit (Qiagen, Inc., Valencia, CA). The cloned full-length human PSA cDNA, which was used as a PCR template for PSA, was obtained as a gift from Dr. Robert L. Vessella (Department of Urology, University of Washington, Seattle, WA).
Patient Selection and Sample Acquisition. Two hundred twenty-four consecutive patients undergoing radical prostatectomy performed by various surgeons for clinically localized prostate cancer (cT1-2) at The Methodist Hospital, Houston, Texas, between November 9, 1994, and November 2, 1995, were evaluated. Institutional Review Board-approved informed consent for the collection of clinical data, as well as serum and prostatic tissue samples, was obtained for all patients. Peripheral blood specimens were collected into Vacutainer CPT 8-ml tubes, containing 1 ml of 0.1 m sodium citrate anticoagulant (Becton Dickinson, Franklin Lakes, NJ) on the morning of the day of surgery, and at least 4 weeks after prostate biopsy or other urological procedures (e.g., cystoscopy). No patient was treated with either neoadjuvant hormonal or radiation therapy before radical prostatectomy. Serum PSA was measured by the Hybritech Tandem-R assay (Hybritech, Inc., San Diego, CA). Clinical data were recorded by the surgeon, according to the 1992 Tumor-Node-Metastasis system. The mean patient age in this study was 61.8 ± 7.2 years (median 63.0, range 40 to 76 years). The mean preoperative PSA was 9.05 ± 6.6 ng/ml (median 7.5, range 0.2 to 51.0 ng/ml).

Control Patients. Control patients included those with documented metastatic prostate cancer and healthy male subjects without prostate cancer. Blood specimens from 8 patients with bone-scan-proven metastatic prostate cancer were used. None of these patients had hormonal or radiation therapy before plasma collection. The negative control group consisted of 14 subjects who had no prior history of any cancer or chronic disease, a normal digital rectal examination, and a normal PSA level (<0.2 ng/ml). A staging biopsy was performed in 37 of 38 patients who had no prior hormonal or radiation therapy before prostatectomy. These patients were unlikely to have clinically detectable prostate cancer or to develop it over the next 10 years (22).

Pathological Examination. All radical prostatectomy specimens were examined at our institution by a single pathologist (T. M. W.) who was blinded to clinical outcome. Standard pelvic lymph node dissection was performed in each patient. Sections of all lymph nodes were frozen and examined at the time of surgery for the presence of micrometastases. Frozen lymph node tissue remaining was then fixed in formalin and embedded in paraffin. Paraffin sections from each lymph node were stained with H&E and microscopically examined for the presence of micrometastases. The radical prostatectomy specimens were processed by whole-mount technique, and pathological parameters were evaluated as described previously (23). Total tumor volume was computed by computerized planimetry from the whole-mount sections from 119 of 224 prostatectomy patients (24).

Postoperative Follow-up. Patients generally were scheduled to have a digital rectal examination and serum PSA evaluation postoperatively every 3 months for the first year, semianually from the second through the fifth year, and annually thereafter. Of 224 patients who underwent radical prostatectomy, 46 experienced prostate cancer progression (21%). Eight patients (4%) had lymph node-positive disease at the time of radical prostatectomy, and surgery was halted before prostate removal. These patients were categorized as having disease progression from the day after surgery. Biochemical progression was defined as a sustained elevation on two or more occasions of PSA level after surgery for the presence of micrometastases. Frozen lymph node tissue remaining was then fixed in formalin and embedded in paraffin. Paraffin sections from each lymph node were stained with H&E and microscopically examined for the presence of micrometastases. The radical prostatectomy specimens were processed by whole-mount technique, and pathological parameters were evaluated as described previously (23). Total tumor volume was computed by computerized planimetry from the whole-mount sections from 119 of 224 prostatectomy patients (24).

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RESULTS

Assay Performance. To determine the detection limit of the assay, we performed serial dilutions of the plasmid PSA cDNA, yielding 0–100 copies/reaction. Our PCR assay consistently detected as few as 5 copies of the plasmid-containing PSA cDNA (Fig. 1). Serial dilution of LNCaP cells into immortalized human lymphoblasts demonstrated that the lower limit of detection for our RT-PCR assay for PSA was at least one LNCaP cell diluted in 10^6 lymphoblasts (Fig. 2). RT-PCR-PSA assay amplifies a 710-bp fragment specific for the PSA cDNA. We have previously shown that our PCR assay for hK2 amplifies hK2 cDNA appropriately but not PSA cDNA, demonstrating the absence of cross-reactivity with PSA using this assay (19). In addition, our PCR assay reliably detected as few as five copies of the plasmid containing hK2 cDNA with lower copy numbers occasionally yielding positive results. Serial dilution of LNCaP cells into immortalized human lymphoblasts at ratios of 1:10^3 to 1:10^6 demonstrated...
Association of RT-PCR for PSA, hK2-L, and hK2-U with Prostate Cancer Progression. Overall, 21% of patients (46 of 224) had cancer progression with a median postoperative follow-up of 52.9 months (range, 1.3–72.8). The overall PSA progression-free survival was 86.6 ± 2.3% (probability ± SE) at 3 years and 80.2 ± 2.9% (SE) at 5 years. Using the log-rank test, we found that patients with a positive preoperative RT-PCR-hK2-L assay had a significantly increased probability of PSA-progression ($P = 0.0231$; Fig. 3A). However, RT-PCR-PSA and RT-PCR-hK2-U were not associated with prostate cancer progression ($P = 0.8931$ and $P = 0.0651$, respectively; Fig. 3, B and C). Only biopsy Gleason sum was associated with prostate cancer progression (hazard risk ratio, 2.790; 95% confidence interval, 1.481–5.254; $P < 0.001$) in a multivariate model that adjusted for the effects of preoperative RT-PCR-hK2-L ($P = 0.108$), preoperative PSA ($P = 0.331$), and clinical stage ($P = 0.742$).

Association of RT-PCR for PSA, hK2-L, and hK2-U with Features of Aggressive Prostate Cancer Progression. Of 46 radical prostatectomy patients whose disease progressed, 8 patients had lymph node-positive disease at the time of radical prostatectomy. Twenty-seven patients were categorized as having features of nonaggressive prostate cancer progression because their PSA doubling times were $>10$ months ($n = 21$; median 25.1, range 13.3–317.4) and/or because they achieved a complete response to local salvage radiation therapy ($n = 9$). Nineteen patients were categorized as having features of aggressive failure because of the results of a metastatic work-up (pathological lymph node involvement, positive

\[ P = 0.490, \text{respectively}. \]

However, RT-PCR-hK2-L ($P = 0.008$) but not RT-PCR-hK2-U ($P = 0.237$) and RT-PCR-PSA ($P = 0.958$) were predictors of lymph node involvement. In a multivariate model that included preoperative PSA, clinical stage, and biopsy Gleason sum and RT-PCR-hK2-L, only biopsy Gleason sum (hazard risk ratio, 3.28; 95% confidence interval, 1.28–8.63; $P = 0.012$) and RT-PCR-hK2-L (hazard risk ratio, 5.32; 95% CI, 1.21–9.40; $P = 0.019$) were predictors of pelvic lymph node metastases.

Detection of PSA, hK2-L, and hK2-U mRNA in Peripheral Blood Samples from Normal Control Subjects and Metastatic Prostate Cancer Patients. The specificity of the RT-PCR assays was tested on the peripheral blood specimens of 14 healthy control subjects. As previously reported, five of these specimens (58%) tested positive for hK2-U and two (14%) tested positive for hK2-L (19). However, RT-PCR-PSA was positive in only one of these specimens (7%).

We also performed RT-PCR assays on peripheral samples from 8 patients with metastatic disease. As previously reported, six (85.7%) of eight specimens tested positive for hK2-U and five (71%) for hK2-L (19). RT-PCR-PSA was positive in seven of eight specimens (88%).

Assessment of RT-PCR for PSA, hK2-L, and hK2-U with Clinical and Pathological Characteristics. RT-PCR for PSA, hK2-L, and hK2-U were positive in 24, 25, and 26% of prostatectomy patients, respectively (Table 1). RT-PCR-PSA assay results were concordant with those of RT-PCR-hK2-L in 67% of the cases (151 of 224, $P = 0.073$) and with those of RT-PCR-hK2-U in 63% of the cases (144 of 224, $P = 0.480$). RT-PCR-hK2-L results and RT-PCR-hK2-U results were concordant in 88% of cases (197 of 224, $P < 0.001$).

Clinical and pathological characteristics of 224 prostatectomy patients and association with preoperative peripheral blood RT-PCR assay results for PSA, hK2-L, and hK2-U are shown in Table 1. RT-PCR-hK2-L positivity was associated with final pathologic Gleason sum $\geq 7$ ($P = 0.028$) and lymph node metastases ($P = 0.004$). There was no association between RT-PCR assay results for hK2-L, hK2-U, or PSA and patient’s age at the time of radical prostatectomy, preoperative PSA level, preoperative PSA density, or prostatectomy tumor volume (all $P > 0.05$). In univariate logistic regression analyses, neither RT-PCR-hK2-L, RT-PCR-hK2-U, nor RT-PCR-PSA was a predictor of organ-confined disease ($P = 0.487, P = 0.675$, and $P = 0.490$, respectively). However, RT-PCR-hK2-L ($P = 0.008$) but not RT-PCR-hK2-U ($P = 0.237$) and RT-PCR-PSA ($P = 0.958$) were predictors of lymph node involvement. In a multivariate model that included preoperative PSA, clinical stage, and biopsy Gleason sum and RT-PCR-hK2-L, only biopsy Gleason sum (hazard risk ratio, 3.28; 95% confidence interval, 1.28–8.63; $P = 0.012$) and RT-PCR-hK2-L (hazard risk ratio, 5.32; 95% CI, 1.21–9.40; $P = 0.019$) were predictors of pelvic lymph node metastases.

**Fig. 1** Determination of detection limit of PCR for PSA cDNA. Dilution experiments carried out with 100, 50, 20, 10, 5, 1, 0.1, 0.01, and 0 copies of the plasmid PSA cDNA. RT-PCR assay results are reproducibly sensitive to at least five copies of PSA cDNA. The lane marked M is molecular weight marker.

**Fig. 2** Determination of lower detection limit of this RT-PCR assay for PSA mRNA in prostate cancer cells. RNA was isolated from serially diluted mixtures of human prostate carcinoma LNCaP cells and American Type Culture Collection RPMI 7666 immortalized human lymphoblasts. The RT-PCR assay was sensitive to at least one LNCaP cell in a background of $10^8$ lymphoblasts. The lane marked M represents molecular weight standards.
bone, or Prostascint scan; n = 9) since their PSA doubling times were <10 months (n = 14; median 7.4, range 2.3–8.6), and/or because they failed to respond to local salvage radiation therapy (n = 9). Preoperative RT-PCR-hK2-L was positive in 6 patients who developed prostate cancer progression with nonaggressive features and 10 patients with aggressive features (22 versus 58%, \( P = 0.028 \)). However, RT-PCR-PSA and RT-PCR-hK2-U were not associated with the features of prostate cancer progression (\( P = 0.694 \) and \( P = 0.188 \), respectively).

**DISCUSSION**

We previously designed a highly sensitive and specific RT-PCR assay for the detection of prostate cancer cells expressing hK2 mRNA, which can differentiate between amplification of the native hK2 transcript (hK2-L), which encodes for the full-length hK2 protein, and an alternate spliced transcript (hK2-U), which contains an additional 37 nucleotides downstream from the native splice donor site (19). In this study, we found that preoperative peripheral blood RT-PCR-hK2-L was associated with pathologic Gleason sum and metastases to regional lymph nodes, with the latter association remaining after controlling for preoperative PSA, biopsy Gleason sum, and clinical stage. Neither preoperative blood RT-PCR for hK2-U nor PSA was associated with any clinical or pathological characteristics of prostate cancer or clinical outcome. For patients whose disease progressed, a positive preoperative RT-PCR-hK2-L assay was associated with disease failure with aggressive features.

In addition to confirming our previous finding that RT-PCR-hK2-L is an independent predictor of metastases to regional lymph nodes, we found that RT-PCR-hK2-L positivity was associated with higher prostatectomy Gleason sum. Prostatectomy Gleason sum has consistently been shown to be a strong independent predictor of prostate cancer progression (28–31). Our confirmed finding of an association with lymph node disease is also clinically important because most, if not all, patients with histopathologically evident pelvic lymph node involvement will fail local therapy, eventually developing distant metastases and experiencing clinical disease progression, regardless of success in eradicating local disease (29–31). However, although these associations between preoperative RT-PCR-hK2-L and pathological features are important, an association with occult metastases that can lead to disease progression in patients treated effectively for clinically localized disease would be more useful for managing patients with prostate cancer (32).

Whereas in a univariate analysis, preoperative RT-PCR-hK2-L was a predictor of disease progression after surgery, when controlling for preoperative PSA level, biopsy Gleason sum, and clinical stage, this association was no longer significant. The pathogenesis and natural history of prostate cancer progression is multifaceted, including local failure because of residual local disease present after radical prostatectomy, occult metastatic nodal or distant metastatic disease present at the time of surgery, and some combination of these. These forms of recurrent disease have variable progression rates with regard to metastases and eventual death. Many patients who experience biochemical progression after radical prostatectomy will not develop clinically evident metastases, nor will they die of prostate cancer (33, 34). The absence of predictive value of RT-PCR-hK2-L for disease progression after surgery may be attributable to a lack of association of RT-PCR-hK2-L with local and/or biologically more indolent disease. Thus, in addition to the relatively low progression rate (21%), the high rate of patients with features of nonaggressive failure (59%) in this dataset might have limited the statistical power of our study for detecting a predictive effect of RT-PCR-hK2-L on overall prostate cancer progression. An improved ability to predict the likelihood of clinical disease progression to metastases would have a greater clinical impact on managing prostate cancer patients. We found that preoperative RT-PCR-hK2-L was associated with features of aggressive prostate cancer progression, defined by a PSA doubling time of <10 months (33, 34), the failure to respond to salvage local radiation therapy (26), and/or a positive metastatic work up. These parameters

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**Table 1** Correlation between results of preoperative peripheral blood RT-PCR for PSA and hK2 assays with categorical clinical and pathologic characteristics of 224 patients undergoing radical prostatectomy for clinically localized prostate cancer

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<tr>
<th><strong>No. of patients (%)</strong></th>
<th><strong>RT-PCR-PSA</strong></th>
<th><strong>RT-PCR-hK2-L</strong></th>
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<td><strong>RP</strong> seminal vesicle involvement</td>
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<td><strong>RP</strong> Gleason sum</td>
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<td><strong>RP</strong> DNA ploidy**</td>
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<td>Aneuploid</td>
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\( P \) Fisher’s exact test.

* RP, radical prostatectomy.

* Radical prostatectomy extraprostatic extension, seminal vesicle involvement, margin status, and Gleason sum were unavailable for 5 patients, who did not undergo a prostatectomy because of grossly positive pelvic lymph nodes at the time of surgery.

* Radical prostatectomy DNA ploidy was unavailable for 36 patients.
have all been associated with an increased risk of developing overt metastases (33, 34). This suggests an association of RT-PCR-hK2-L with occult metastatic disease present at the time of radical prostatectomy. Ideally, after additional confirmation of these results, preoperative RT-PCR-hK2-L could be included in appropriate nomograms that distinguish between the more and less aggressive forms of cancer recurrence, and the likely effectiveness of adjuvant and salvage therapy.

Our assay for PSA was highly sensitive as demonstrated by a reliable detection of five copies of PSA cDNA and at least one LNCaP cell in $10^6$ cultured lymphoblasts, but less sensitive than our assay for hK2, which reliably detected five copies of hK2 cDNA and at least one LNCaP cell in $10^9$ cultured lymphoblasts. Our RT-PCR assay was clinically more sensitive for detecting PSA-expressing cells in patients with metastatic disease (7 of 8 patients positive, 88%) than the average of peripheral blood assays used in previous studies (53%, range 13–100%; most recent review Ref. 35). Only one of 14 control specimens (7%) obtained from men at low risk for harboring prostate cancer tested positive for PSA, which is in range of false positives reported in the literature (35). In this same group of patients, we have previously reported that 14 and 36% demonstrated a positive result with a highly sensitive RT-PCR for hK2-L and hK2-U, respectively, that has been reported to detect at least one LNCaP cell diluted in $10^9$ lymphoblasts (19). Twenty-four percent of the peripheral blood specimens obtained from 224 patients with clinically localized prostate cancer tested positive for RT-PCR-PSA, a rate that is in accordance with that found in previous studies (35). The consistent results with positive and negative controls in each assayed cohort, and the close correspondence in the *in vitro* and clinical sensitivity and specificity of our RT-PCR-PSA assay with results from the literature, support the broad applicability of our negative clinical associations using RT-PCR-PSA assays and confirm the negative results of most other previous studies.

Neither RT-PCR for PSA nor hK2-U was associated with any clinical or pathological characteristics or clinical outcome of patients with clinically localized disease undergoing radical prostatectomy and long-term follow-up. We have previously reported the lack of association of RT-PCR-hK2-U with pathological characteristics, including lymph node involvement (19). RT-PCR for the alternate spliced transcript (hK2-U), which contains an additional 37 nucleotides downstream from the native splice donor site in intron IV, is predicted to encode a truncated and, presumably nonfunctional, version of the hK2 protein, although no such species of hK2 protein has been identified *in vivo*. Although more than two dozen studies using a large variety of different procedures have investigated the value of RT-PCR of peripheral blood PSA in predicting final pathological stage of patients undergoing radical prostatectomy, only two groups of researchers showed that the presence of PSA-expressing cells correlated with both capsular penetration and positive surgical margins (1, 36–38). Other studies have not found a statistically significant correlation between blood RT-PCR-PSA positivity and clinical or pathological characteristics (35). Follow-up studies have found that preoperative RT-PCR for PSA is associated with an increased risk of biochemical failure by one group (2, 5), although we and others did not (9, 39). The causes underlying these conflicting results are difficult to establish because the variability in patient population, RT-PCR protocols, specimen acquisition, and verification of product all could potentially alter results significantly. Using serial specimens, we and others have found that most patients with a preoperative RT-PCR-PSA assay result positive convert to a negative result after prostate removal, suggesting that these circulating cells generally are clinically insignificant (9). Illegitimate basal expression of the targeted marker mRNA in nonprostatic cells, down-regulation of PSA mRNA in high-grade tumor cells, and the high level of expression of this mRNA in normal primary prostatic and nonprostatic epithelial cell all diminish PSA as a target for RT-PCR assays in this clinical setting (12–14, 40–42). On the other hand, RT-PCR detection of the native hK2

Fig. 3. Kaplan-Meier estimates of PSA progression-free probability for the 224 patients with clinically localized prostate cancer treated with radical prostatectomy stratified by results of the preoperative (A) RT-PCR-hK2-L, (B) RT-PCR-hK2-U, or (C) RT-PCR-PSA assays.
transcript combines the use of targeting a prostate-specific marker with a potential marker of biologically more aggressive disease. Our data support these assumptions and establish a framework for further study of hK2 as both a marker and a therapeutic target in patients with prostate cancer. In conclusion, in our experience, neither RT-PCR for PSA nor hK2-U is a useful staging tool for guiding therapy or prognosticating outcome in patients with clinically localized prostate cancer, and therefore they have limited clinical utility. In contrast, our results suggest that RT-PCR-hK2-L is associated with established markers of biologically and clinically aggressive prostate cancer, potentially improving our ability to predict metastasis to regional lymph nodes, and disease progression. We also found an association of RT-PCR-hK2-L with prostate cancer progression with aggressive features, suggesting its association with the presence of occult small volume metastases. Prospective studies using a more objective assay (e.g., real-time quantitative PCR format) are required to confirm these results to construct a potentially better prognostic nomogram that incorporates additional validated biomarkers (43–45) of early metastases and aggressive disease progression and to establish recommendations for adjuvant and salvage therapies.

REFERENCES

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