Detection of Metastatic Prostate Cancer Using a Splice Variant-specific Reverse Transcriptase-Polymerase Chain Reaction Assay for Human Glandular Kallikrein

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ABSTRACT

We developed a highly sensitive splice variant-specific reverse transcriptase-PCR (RT-PCR) assay for human glandular kallikrein (hK2) mRNA and tested its ability to detect metastatic disease in men with clinically localized prostate cancer. An RT-PCR assay using primers spanning intron IV and including a significant portion of the 3′ untranslated region of the hKIL2 gene, with maximum nonhomology to both hK1 and hK3, was developed. The limit of detection of the assay was five copies of hK2 cDNA and one LNCaP cell in 10^6 lymphoblasts. RT-PCR-hK2 was performed on preoperative peripheral blood specimens from 228 consecutive radical prostatectomy patients as well as 7 metastatic prostate cancer patients and 14 healthy men without prostate cancer. This new RT-PCR-hK2 assay amplifies two distinct fragments. The larger fragment (hK2-U) is approximately 680 bp in length and corresponds to the amplified product of a previously reported splice variant in the splice donor site of intron IV in the hK2L gene. The smaller fragment (hK2-L) is ~643 bp in length and corresponds to the amplified product of the native hK2 mRNA. Whereas the RT-PCR-hK2-L assay was positive in 71% of our patients with metastatic prostate cancer, 14% of healthy control men also tested positive. By univariate (P = 0.028) and multivariate (P = 0.0269) analysis, which controlled for preoperative PSA, clinical stage, and biopsy Gleason score, RT-PCR-hK2-L status added prognostic information to the prediction of lymph node-positive disease. We have developed a new RT-PCR assay which demonstrates a high sensitivity for detecting hK2 mRNA. Preoperative RT-PCR-hK2-L status helps predict pathological lymph node positivity in patients with clinically localized prostate cancer.

INTRODUCTION

Through the widespread use of PSA-based screening, the number of men diagnosed and treated for clinically localized prostate cancer over the past decade has dramatically increased (1). Concomitantly, the incidence of clinical metastatic disease at presentation has dropped considerably in concert with an overall decrease in prostate cancer mortality (2). Despite the significant rate of long-term cancer control afforded patients with clinically localized prostate cancer treated with radical prostatectomy or radiation therapy, ~30% of these patients will fail treatment, as evidenced by a detectable or rising PSA that often is attributable to early dissemination of microscopic metastatic disease (3). Conventional staging modalities such as bone scan and CT scan have a limited role in staging patients with clinically localized prostate cancer because of their poor performance in detecting early, low-volume metastases (4, 5). Preoperative nomograms that consider established markers like PSA, clinical stage, and biopsy Gleason Score can provide an estimate of the risk of nodal metastases or disease recurrence, but are still imperfect for determining the pathological stage or prognosis in individual patients (6, 7). Preoperative identification of patients with metastatic disease would be helpful in sparing men from the morbidity of a radical prostatectomy that would be ineffective or for selecting patients best suited for clinical trials of neoadjuvant or adjuvant therapy.

The RT-PCR is a powerful and highly sensitive tool that has the ability to detect small numbers of prostatic cells disseminated within the peripheral blood or within other body fluids or tissues. Whereas some studies have demonstrated a significant predictive value of RT-PCR for PSA or RT-PCR for prostate-specific membrane antigen for both pathological stage (8–11) and progression-free survival (12, 13) with these assays, the majority have failed to demonstrate any clinically significant role (Refs. 14–16; most recent review, Ref. 17). In addition to the formidable technical problems regarding sample processing and handling that may underlie these conflicting results (18), questions regarding the specificity of these markers for prostate cancer cells and the potentially variable biological potential of the cells detected by these assays have also limited the clinical utility of these assays (19–22). Whereas foci of metastatic prostate cancer detectable by conventional modalities (e.g., bone scan or computed tomography scan) are almost always associated with biologically significant advanced disease, the development of ultrasensitive techniques that can identify minute numbers of cells, like RT-PCR, places the burden on investigators to demonstrate the clinical and biological significance of these cells.

hK2 is an androgen-regulated protein that has an ~80% amino acid sequence identity with PSA (23) and is expressed almost exclusively in the prostatic epithelial cells. Immunohistochemical studies have shown an incremental increase in hK2 expression from benign epithelium, to prostatic intraepithelial neoplasia, to prostate cancer (24). Furthermore, hK2 expression, in contrast with PSA (24, 25), is directly associated with the Gleason grade of the primary tumor with foci of prostate cancer metastatic to the lymph nodes demonstrating the highest level of expression (26). Finally, circulating levels of hK2 have been shown to enhance the biochemical detection of prostate cancer when combined with free and total PSA (27, 28). These properties of hK2 suggest that it may represent a better RT-PCR target for the detection of circulating, more biologically aggressive prostate cancer cells, which in turn may better correlate with both occult micrometastatic disease and the risk of disease progression.

We designed a novel, highly sensitive, and specific RT-PCR assay for the detection of prostate cancer cells expressing hK2 mRNA. First, we assessed the performance characteristics of this assay on peripheral blood specimens obtained from men without prostate cancer as well as from patients with documented metastatic disease. Subse-
from each specimen was used for the synthesis of the first strand of cDNA. In

associated with a splice variant of the hK2 gene (29) reported previously. 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, Wash-

Patient Selection and Sample Acquisition. Two hundred and twenty-eight consecutive patients undergoing radical retropubic prostatectomy for clinically localized prostate cancer (cT1–2) at The Methodist Hospital, Houston, TX, between November 9, 1994, and November 2, 1995, were evaluated. Institu-
tional Review Board approved informed consent for the collection of clinical data as well as serum and prostatic tissue samples were obtained for all patients. Peripheral blood specimens were collected into Vacutainer CPT 8-ml tubes containing 1 ml of 0.1M sodium citrate anticoagulant (Becton Dickinson, Franklin Lakes, NJ), typically preoperatively in the morning on the day of surgery; and at least 4 weeks after prostate biopsy or other urological proce-
dure (e.g., cystoscopy). No patient was treated with either neoadjuvant hor-
monal or radiation therapy before radical prostatectomy. Serum PSA was measured by the Hybritech Tandem-R assay (Hybritech, Inc., San Diego, CA). Clinical stage was assigned by the surgeon according to the 1992 Tumor-
Node-Metastasis system. The mean patient age in this study was 61.9 ± 7.6 years (median, 62.9 years; range 37.7–75.5 years).

Control Patients. Controls included patients with documented metastatic prostate cancer and healthy male subjects without prostate cancer. Blood specimens from seven patients with untreated, bone scan-proven metastatic prostate cancer were used. The negative control group comprised 14 subjects attending the Baylor Prostate Center’s prostate cancer screening program who had a normal DRE and a serum PSA level of <0.5 ng/ml (mean 0.38 ± 0.09 ng/ml). These patients are unlikely to have clinically detectable prostate cancer or to develop it over the subsequent 10 years (30).

Pathological Examination. All prostate biopsy and 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, and frozen sections of the lymph nodes were microscopically examined for the presence of micrometastases. The radical prostatectomy specimens were processed by whole-mount technique, and pathological stage was assigned as described previously (31). The location of each tumor focus was assessed, and the presence of extraprostatic extension, seminal vesicle involvement, and lymph node metastases was recorded. For the purposes of analysis, pathological stage was categorized into four mutually exclusive groups as follows: (a) organ-confined; (b) extracapsular extension; (c) seminal vesicle involvement; and (d) lymph node involvement. The Glea-
son grading system was used to assign primary and secondary cancer grades.

RNA Preparation. Mononuclear cells were separated from peripheral blood specimens by centrifugation at room temperature for 20 min at 1500 g, and the buffy coat fraction, containing nucleated cells, was recovered using sterile transfer pipettes. The cells were washed once in PBS, lysed with an acid-pH guanidine lysis buffer, and stored at −80°C until processing. Initially RNA was extracted using the acid phenol-chloroform method. Later, RNA was extracted using a modified protocol, which replaced the initial ethanol precipi-
tation by application to Qiagen-RNeasy spin columns (Qiagen Inc.). RNA was ethanol-precipitated and stored at −80°C in 70% ethanol and 85 mM NaOAC (pH 4.2) until assayed.

RT Reaction and cDNA Synthesis. An aliquot containing 1 μg of RNA from each specimen was used for the synthesis of the first strand of cDNA. In

each of the reactions, a 50-μl solution containing 3 μM random hexamers, 25

mm Tris-HCl, 37.5 mM KCl, 1.5 mM MgCl2, 10 mm dithiorthreitol, 0.25 mM each
deoxyribonucleotide triphosphate, 40 units of RNAsin RNase inhibitor (Promega, Madison, WI), and 200 units of SuperScript II RNase Transcriptase (Life Technologies, Inc., Rockville, MD) were processed as described previously (32). Briefly, the annealing mixture was incubated at room temperature for 15 min then incubated in a thermocycler (Perkin-Elmer, Foster City, CA) at 37°C for 75 min. The reverse transcriptase enzymes were inactivated by heating the solution to 95°C for 15 s.

Oligonucleotide Primers. Two sets of PCR primers were designed spanning intron 4 and including a significant portion of the 3’ untranslated region of the hK2 gene with maximum nonhomology to both hK1 and hK3 (PSA) at their 3’ ends (Fig. 1). After preliminary studies, the primer set calculated to yield a 643-bp PCR product was selected for additional analysis. The 5’ end and the 3’ end of our primer were located at position 507 in exon 4 (5’ flanking sequence from 507 to 526), and at position 1150 in the 3’ untranslated region (3’ flanking sequence from 1150 to 1131), respectively. PSA cDNA and our primer for hK2 cDNA differ at six positions within the sequence of the 5’ primer as well as the 3’ primer.

PCR. PCR was performed using Taq polymerase (Promega). The PCR reaction conditions were essentially the same as those reported previously (8) but included 2 μM d11-dUTP and 0.4 μM hK2 primers. The PCR mixture was preheated to 80°C before 10-μl reverse transcription products were added and cycling begun. The PCR was performed for 35 cycles consisting of the following steps: (a) denaturation at 94°C for 1 min; (b) annealing at 66°C for 1 min; and (c) extension at 72°C for 2 min. Then, 12 μl of each PCR product was loaded on the 2% NuSieve agarose gel (FMC BioProducts, Rockland, ME) in TAE buffer and then stained with ethidium bromide. After electrophoresis, PCR products were transferred onto a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN) with pressure blotter (8 Hg) for 2 h (Bio-Rad-Laboratories, Hercules, CA). The membrane then was probed using the Genius System (Boehringer Mannheim). Both gel analysis and a second PCR reaction amplifying the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene were used to assess mRNA integrity. Internal negative control reactions for the RT-PCR were performed using all of the reagents as for the experimental samples, but with lymphoblast RNA in each of the assays. None of the assays exhibited a signal from the internal negative control. Internal positive control reactions for the RT-PCR were performed using hK2 cDNA. RT-PCR results were scored in a blinded fashion to patient clinical and pathological data.

Determination of Specificity and Limit of Detection of RT-PCR Assay for hK2 Message. Preliminary studies using cDNA templates for hK2 and hK3 were performed to determine assay specificity to the target species, hK2. The detection limit of our protocol was tested in two ways. First, we det-

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electrophoresis. Amplification of the cloned hK2 cDNA yielded a 653-bp fragment, which differs from the amplified fragment from human RNA.

**Nucleotide Sequencing.** The amplified 680-bp and 643-bp fragments of hK2 cDNA, referred to as hK2-U and hK2-L amplified from LNCaP RNA, respectively, were excised from a 2% agarose gel and purified with Bio101 Geneclean Kit (ISC BioExpress, Kaysville, UT). The purified DNA was directly cloned using the TA Cloning Kit (Invitrogen Co., San Diego, CA). The cloned product was then sequenced using a 373 DNA Sequencer (Perkin-Elmer). The sequencing reactions and analysis were performed using the manufacturer’s recommendations.

**Statistical Analysis.** The Pearson $\chi^2$ test was used to evaluate the association between hK2 RT-PCR results and pathological stage. Univariate and multivariate logistic regression analyses were used for the prediction of final pathological lymph node status. Variables in the model included typical predictors (e.g., preoperative serum PSA, preoperative biopsy Gleason score, and clinical stage) in addition to RT-PCR-hK2 results. Preoperative biopsy Gleason score and the natural logarithm of serum PSA level were examined as continuous variables. For the statistical analysis, RT-PCR-hK2 results were examined as hK2-U (positive or negative) alone, as hK2-L (positive or negative) alone, as combined hK2-U and hK2-L, as well as either hK2-U or hK2-L. A $P \leq 0.05$ was considered statistically significant. All analyses were performed with SAS software (version 6.12).

**RESULTS**

**Assay Performance.** To determine the specificity of our primer set for hK2, PCR assays on samples of increasing copy numbers of PSA cDNAs (from 1 to $10^6$) were run and compared with a control sample of 100 copies of hK2 cDNA (Fig. 2). The PCR assay amplified hK2 cDNA appropriately, but not PSA cDNA, demonstrating the absence of cross-reactivity with PSA using this assay. To determine the detection limit of the assay, we performed serial dilutions of the plasmid hK2 cDNA, yielding 0 to 100 copies/reaction. Our PCR assay reliably detected as low as five copies of the plasmid containing hK2 cDNA. However, lower copy numbers occasionally yielded positive results (Fig. 3 and data not shown). Serial dilution of LNCaP cells into immortalized human lymphoblasts at ratios of $1:10^4$ to $1:10^9$ demonstrated that the lower limit of detection for this assay was at least one LNCaP cell diluted in $10^9$ lymphoblasts (Fig. 4).

**Detection of hK2 Messages.** Whereas amplification of hK2 cDNA yielded the expected 680-bp product, when performed on RNA isolated from LNCaP cells, two distinct amplified DNA fragments of approximately 680 bp (referred to as hK2-U) and 643 bp (referred to as hK2-L) were identified (Fig. 4). DNA sequencing analysis demonstrated that the larger fragment, hK2-U, corresponded to the amplified product of a previously reported expressed, but not translated, splice variant in the splice donor site of intron IV in the hGK-1 gene (33). The smaller fragment, hK2-L, was found to correspond to the amplified product of the native hK2 mRNA. This fragment has the
same sequence as positions 507 to 1150 of hK2 cDNA (GenBank accession no. S39329).

Detection of hK2 mRNA in Normal Control Subjects and Metastatic Prostate Cancer Patients. The specificity of the assay was tested on the peripheral blood specimens of 14 healthy control subjects. Five of these specimens (35.7%) tested positive for hK2-U and two (14.3%) tested positive for hK2-L. We also performed RT-PCR assays on peripheral samples from seven patients with metastatic disease. Six (85.7%) of seven specimens tested positive, five for both hK2-U and hK2-L (71%), and one for hK2-U only (Fig. 5).

Relation of hK2 RT-PCR Results to Clinical and Pathological Features. RT-PCR-hK2 was then performed on peripheral blood specimens obtained preoperatively from 228 consecutive patients who underwent radical prostatectomy for the treatment of localized prostate cancer (example, Fig. 6). We evaluated the relationship between pathological stage and the results of the RT-PCR-hK2 assay, scored separately for amplification of either hK2-L, hK2-U, or combinations of both fragments. There was no correlation between pathological stage and RT-PCR-hK2 results of any of the examined fragments, although there was a trend of association between a positive RT-PCR-hK2-L result and a more advanced pathological stage that approached statistical significance \((P = 0.056; \text{Table 1})\). RT-PCR-hK2-L (OR, 3.95; 95% CI, 1.96–8.49; \(P = 0.028\)) and the combination of RT-PCR of hK2-L or hK2-U (OR, 4.24; 95% CI, 1.90–8.11; \(P = 0.025\)) were found to be significant predictors of final lymph node status (Table 2). Multivariate logistic regression analysis was performed for the prediction of final pathological lymph node status. In a model that included preoperative PSA, clinical stage, and biopsy Gleason score, along with RT-PCR-hK2-L, preoperative serum PSA level and clinical stage failed to predict lymph node status, whereas preoperative biopsy Gleason score (OR, 3.28; 95% CI, 1.31–6.23; \(P = 0.0112\)) and RT-PCR-hK2-L (OR, 4.95; 95% CI, 1.20–9.39; \(P = 0.0269\)) were found to predict the presence of pelvic lymph node metastases (Table 3).

DISCUSSION

We have developed a highly sensitive and specific RT-PCR assay for hK2 mRNA that was designed to differentially amplify two previously described transcripts of the \(hKLK2\) gene. When performed before radical prostatectomy on the peripheral blood of patients with clinically localized prostate cancer, the native hK2-amplified fragment (RT-PCR-hK2-L) was associated with the risk of metastasis to pelvic lymph nodes \((P = 0.028)\).

We designed our assay to provide maximum sensitivity and specificity for hK2 without any cross-reactivity to the closely related kalikreins, hK1 and hK3. Furthermore, we designed our primers to 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 in intron IV (33). This larger, alternatively spliced mRNA is predicted to encode a truncated version of the hK2 protein, although no such species of hK2 protein has been identified \(in vivo\). Recently, three additional species of hK2 transcripts have been described, including two 3.0 kb hK2 transcripts corresponding to the two known 1.5 kb major isoforms, but each with an additional 1.5 kb of 3′ untranslated region attributable to
transcription through the first polyadenylation signal to a second signal located downstream. A third form, with a deletion of 13 nucleotides between exons 3 and 4 of the hKLK2 transcript that would result in a truncated hK2 protein missing 67 amino acids at the COOH-terminus of the protein, was also identified (34). This second predicted truncated hK2 protein has also not been identified in vivo. Because of the overlapping position of our 5′ primer with the 13-bp deletion in intron 3, our assay would not be predicted to amplify this third species of hK2 mRNA. However, our primers were predicted to amplify a 643-bp fragment from both the native 1.5 kb and the related 3.0-Kb transcript with the longer 3′ UTR. Similarly, our primers were predicted to amplify the larger 680-bp fragment from both the 1.5 kb, and the related 3.0-kb, alternate intron IV splice variants.

By differentially amplifying these splice variants, we were able to associate a more biologically aggressive phenotype with the amplified native hK2-L fragment but not to the fragment encoding a predicted truncated and, presumably, nonfunctional hK2 protein. hK2 encodes a highly active protease that has multiple biological activities and that may contribute functionally to prostate cancer invasion and metastasis. Other active proteases such as uPA and plasmin are known to be involved in cancer invasion and metastasis. hK2 has been shown to activate single-chain uPA to the active a two-chain form of uPA (35, 36), which is highly correlated with prostate cancer metastasis (37). More recently, hK2 has been shown to inactivate the major tissue inhibitor of uPA, plasminogen activator inhibitor-1 (38). Thus, hK2 may influence the progression of prostate cancer by the activation of uPA and by the inactivation of the primary inhibitor of uPA, plasminogen activator inhibitor-1.

Patients with organ-confined, extracapsular disease and seminal vesicle invasion had similar, low rates of positive results (13–24%), in contrast to patients with lymph node involvement, 55% of whom had a positive result. Interestingly, overall 7-year, progression-free survival rates after radical prostatectomy in patients with organ-confined, extracapsular disease and seminal vesicle invasion, in whom the surgeon was able to achieve a negative surgical margin, are approximately 95%, 80%, and 50%, respectively, indicating that the majority of patients with specimen-confined prostate cancer adequately treated for their local disease will not fail distantly (39–41). Conversely, most, if not all patients, with lymph node involvement will eventually fail local therapy with distant metastases regardless of the success of eradicating local disease (42–44). One reason for the lack of association found between hK2 and pathological stage, despite the association between hK2 and LN+, could be that the ordinal logistic regression analysis of pathological stage makes additional assumptions about the relationship between hK2 and each level of pathological stage. In particular, it assumes that the effects of hK2 on each pathological stage are equal among adjacent pathological stages.

In studies published previously, Corey et al. (45) used primers that amplified a fragment spanning exons 2 to 4 of hK2, and Kawakami et al. (46) used primers that amplified a fragment spanning exons 3 to 4; neither assay was thus designed to differentially amplify these alternate mRNA species, and neither found any association between RT-PCR-hK2 status and pathological stage. Although we also did not find an association between RT-PCR-hK2 status and extracapsular disease or seminal vesicle involvement, RT-PCR for hK2-L, but not hK2-U, was a significant predictor of the final pathological lymph node status in a multivariate analysis that included clinical parameters such as PSA, Gleason score, and clinical stage. Neither Corey et al. (45) nor Kawakami et al. (46) detected an association between RT-PCR-hK2 positivity and lymph node metastasis, although Corey et al. (45) included only one patient with lymph node metastasis in their study.

Our assay was highly specific for hK2 without any detectable cross-reactivity for PSA. In addition, our assay was highly sensitive, as demonstrated by a reliable detection of five copies of hK2 cDNA and at least one LNCaP cell in 10⁵ cultured lymphoblasts. Corey et al. (45) reported results using an assay that could detect 10 copies of hK2 cDNA and one LNCaP cell diluted in 10⁷ peripheral blood mononuclear cells. Kawakami et al. (46) used an assay that only detected one LNCaP cell in 10⁷ lymphocytes. These assays were thus 100- to 1000-fold less sensitive than our assay. We evaluated our RT-PCR assay on peripheral blood specimens obtained from healthy male subjects, patients with prostate cancer, and patients with untreated metastatic prostate cancer, and scored specifically for both hK2-U and hK2-L. Our assay was more sensitive (71% positive for hK2-L) for detecting hK2-expressing cells in patients with metastatic disease than the assays used in previous studies by Corey et al. (31%; Ref. 45) and Kawakami et al. (41%; Ref. 46).

Fourteen percent of the control specimens obtained from men at low risk for harboring prostate cancer tested positive for hK2-L, whereas 36% tested positive for hK2-U. In this same group of patients, 7% demonstrated a positive result with a highly sensitive RT-PCR for PSA (data not shown), which has been reported previ-
ously to detect at least one LNCaP cell diluted in 10^6 lymphoblasts (47). The single control patient (1/14) who tested positive for RT-PCR-PSA was found to be negative for RT-PCR for hK2-L and hK2-U. Whereas the low rate of false positives for PSA and hK2-L were similar, the false positive rate for hK2-U was higher. However, RT-PCR-hK2-U was not a predictor of lymph node status in patients with clinically localized prostate cancer, questioning the biological relevance of this marker. The consistent results with positive and negative controls in each assayed cohort, and the low number of positive RT-PCR results for PSA, which are consistent with results from other groups, argue against a technical problem in our RT-PCR assay. More likely, our assay detected basal levels of hK2 expression, because of the high sensitivity of our PCR primer. Previously, other groups have reported a loss of diagnostic specificity of their assay cause of the high sensitivity of our PCR primer. Presently, other groups have reported a loss of diagnostic specificity of their assay because of overly sensitive PCR primers (49, 50).

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