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Comparison of Immunohistochemistry with Reverse Transcription-PCR for the Detection of Micrometastatic Prostate Cancer in Lymph Nodes[1]

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ABSTRACT

The objective is to compare the performance of immunohistochemistry (IHC) with that of reverse transcription (RT)-PCR for detecting clinically significant micrometastases in histopathologically normal archival pelvic lymph nodes (PLN) removed at radical prostatectomy from men with locally advanced nonmetastatic prostate cancer. We stained 1864 fixed, paraffin-embedded PLNs from 199 $pT_3N_0M_0$ prostate cancer patients for prostate-specific antigen (PSA) and cytokeratin. We also assessed human glandular kallikrein (hK2) expression in a subset of 164 patients. In addition, all PLN specimens were assayed for hK2 mRNA using a previously described RT-PCR assay. PSA and cytokeratin were expressed in the same 13 of 199 (7%) cases; hK2 was expressed in 3 of 164 (2%) cases. PSA/cytokeratin and hK2 expression were associated with cancer involvement of seminal vesicles, higher Gleason sum, and a positive RT-PCR-hK2 assay result. In standard postoperative multivariable models, IHC-PSA/IHC-Cytokeratin or IHC-hK2 was associated with prostate cancer progression, development of distant metastases, and prostate cancer-specific survival. However, when RT-PCR-hK2 assay result was added to the models, it was the sole predictor of clinical outcomes. Although IHC-PSA/IHC-Cytokeratin and IHC-hK2 were more specific for identifying patients who would suffer biochemical progression and develop metastases and who would ultimately die of prostate cancer, RT-PCR-hK2 was more sensitive and accurate. Although IHC for PSA, cytokeratin, and hK2 appear to be more specific methods for detecting biologically and clinically significant prostate cancer micrometastases in histopathologically normal PLN, RT-PCR-hK2 appears to be a more sensitive method that maintained a reasonable specificity. In pT_3N_0 patients, a positive RT-PCR-hK2 assay result when performed on PLN was the strongest predictor of clinical outcomes after radical prostatectomy.

INTRODUCTION

Patients with locally advanced prostate cancer, in the form of EPE[3] and/or SVI, are at increased risk for disease progression after radical prostatectomy (1–5). In these patients, disease progression is often because of early dissemination of microscopic metastatic disease that remains undetectable by standard staging modalities and histopathological analysis (6). Of all of the adverse pathologic features demonstrated by clinically localized prostate cancer, the presence of histopathologically evident prostate cancer metastases to pelvic lymph nodes is the strongest predictor of future disease progression to distant metastases, regardless of apparent success in eradicating local disease (3, 5, 7, 8). Conventional staging modalities such as imaging techniques and histopathological examination of tissue specimens (*e.g.*, pelvic lymph nodes), however, play a limited role in staging these patients because of their poor performance in detecting early, low-volume prostate cancer metastases (9–12). Identification of patients with clinically relevant occult metastatic involvement of the lymph node would be helpful for selecting patients best suited for clinical trials of early systemic intervention (13, 14), for sparing men who have undergone prostatectomy from the morbidity associated with ineffective local adjuvant or salvage radiation therapy (15, 16), and in predicting patient outcome.

The main problems with current histopathological lymph node evaluation are sampling error and poor sensitivity for detecting individual tumor cells or small tumor foci. It has been calculated that a pathologist has only a 1% chance of detecting a micrometastatic focus that is the diameter of three tumor cells (17). Previous studies have shown that immunohistochemical staining for PSA or cytokeratins of surgically removed archival paraffin-embedded lymph nodes has a higher sensitivity for detection of micrometastatic cancer cells than routine H&E microscopy (18–20). When compared with histology, flow cytometry, and IHC, RT-PCR has the highest sensitivity for detecting small numbers of cells (21). In various cancers, including prostate cancer, RT-PCR has been shown to be superior to standard histological and immunohistochemical approaches in sensitivity and specificity for detecting cells in regional lymph nodes (22–24). Although the molecular and histological techniques used in these studies identified disseminated prostatic cells, the biological and clinical significance of these cells has remained uncertain. Foci of metastatic prostate cancer detectable by conventional modalities (*e.g.*, histology, bone scan, or computed tomography scan) are almost always associated with clinically significant disease, whereas molecular and histological techniques that can identify very small numbers of cells, place the burden on investigators to demonstrate the clinical relevance of these cells.

We have previously developed a highly sensitive and specific splice variant-specific RT-PCR assay for hK2 and demonstrated that it is associated with an increased risk of metastasis to pelvic lymph nodes (25) and of overall and aggressive disease progression (26) when performed on peripheral blood of patients with clinically localized prostate cancer before radical prostatectomy. Given the high rate of RNA degradation in archival paraffin-embedded tissue, we modified our RT-PCR assay for hK2 mRNA by designing a primer set that amplifies a smaller region of hK2 within the span of our original primer set and demonstrated that RT-PCR-hK2 in pelvic lymph nodes of pT_3N_0 patients is associated with an increased risk for prostate cancer progression after primary and salvage local therapy, with the development of clinically detectable distant prostate cancer metastases and with prostate cancer-specific risk of mortality (27). In this study, we sought to assess the power of immunohistochemical staining for PSA, hK2, and/or cytokeratin to detect biologically and clinically significant occult micrometastates in archival, cancer-free pelvic lymph nodes from 199 consecutive patients with locally ad-

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[3] The abbreviations used are: EPE, extraprostatic extension; SVI, seminal vesicle involvement; PSA, prostate-specific antigen; hK2, human glandular kallikrein; RT-PCR, reverse transcription-PCR; IHC, immunohistochemistry.

vanced prostate cancer (pT_3N_0). In addition, we wanted to compare the performance characteristics of immunostaining for PSA, cytokeratin, and hK2 to that of our RT-PCR-hK2 assay for prediction of prostate cancer outcomes.

MATERIALS AND METHODS

Patient Selection and Sample Acquisition. We evaluated formalin-fixed, paraffin-embedded, histopathologically uninvolved pelvic lymph nodes from 217 consecutive pathologic stage pT_3N_0 patients who underwent radical prostatectomy for the treatment of clinically localized prostate cancer by a single surgeon (P. T. S.) at The Methodist Hospital (Houston, TX) between December 1983 and November 1996. All patients underwent an extended lymph nodes dissection, which included level I, II, and III lymph nodes as routine (28). Patients were identified from the database of the Baylor SPORE in prostate cancer and selected on the basis of pathologically identified locally advanced prostate cancer and negative lymph nodes (stage pT_3N_0). Usable RNA was isolated from lymphadenectomy specimens from 199 patients. RNA integrity was defined by successful amplification of fragments of *glyceraldehyde-3-phosphate dehydrogenase*-positive controls of appropriate size. Institutional Review Board-approved informed consent for the collection of clinical data, as well as serum and tissue samples, was obtained from all patients. No patient was treated with either neoadjuvant hormonal or radiation therapy before radical prostatectomy or adjuvant radiation therapy before an elevation of PSA levels. The mean patient age in this study was 62.7 ± 6.8 years (median, 63.4 years; range, 49.6–75.1 years).

Pathologic Examination. All lymph node and radical prostatectomy specimens were examined at our institution by a pathologist who was blinded to IHC and RT-PCR-hK2 results and to clinical outcome. The radical prostatectomy specimens were processed by whole-mount technique, and pathological parameters were evaluated as described previously (29). Total tumor volume was computed by computerized planimetry from the whole-mount sections (30). One hundred fifty patients had EPE only and 49 had seminal vesicle invasion (specifically, cancer within the muscular coat of the seminal vesicle, not simply tumor in the fat adjacent to the seminal vesicle (2). The level of EPE, with respect to the stroma of the prostate, prostatic capsule, and periprostatic soft tissue, was classified as described previously (31). Seventy-eight patients (39%) had focal EPE (tumor outside the prostate to a depth of less than one high-power field on no more than two separate sections) and 121 (61%) had established EPE (any amount of extraprostatic tumor more than focal EPE). Standard pelvic lymph node dissection was performed in each patient, with a total of 1846 pelvic lymph nodes removed at the time of radical prostatectomy (mean, 9.6 ± 4.2 lymph nodes/patient). Frozen sections of all lymph nodes were performed 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.

Postoperative Follow-Up. Each patient was scheduled to have a digital rectal examination and serum PSA evaluation every 3 months for the first postoperative year, semiannually from the second through the fifth year, and annually thereafter. Biochemical progression was defined as a sustained elevation, on two or more occasions, of PSA $>$ 0.2 ng/ml. The date of progression was assigned to the date of the first value $>$ 0.2 ng/ml. A staging evaluation, including bone scan, ProstaScint scan, or PSA doubling time calculation, was performed carried out for 63 of 68 patients who had PSA progression before the administration of salvage therapy. For patients who had biochemical progression, postprogression serum PSA doubling time was calculated using the formula: $DT = \log(2) \times T / [\log(\text{final PSA}) - \log(\text{initial PSA})]$ (32), where DT is the serum PSA doubling time, T is the time interval between the initial and final PSA level, final PSA is the presalvage therapy PSA level, and initial PSA is the PSA level noted at the time of the postoperative biochemical progression. All patients had at least three PSA measurements available postprogression. The natural logarithm was used in all logarithmic transformations. Survival data were obtained from the cancer registry at The Methodist Hospital and the patients' medical records. Death certificates were retrieved on all dead patients from the archived death certificates and reviewed for cause of death. Attribution of cause of death on the death certificate is in two parts. Part I lists death caused by (*a*) immediate cause of death (final disease or condition resulting in death) or by (*b*) underlying cause of death, and part II lists other significant conditions contributing to death but not resulting in the underlying cause given in part I. Information abstracted from each death certificate included the date of death and whether prostate cancer was noted in part I or II. For this study, to reduce bias in attribution of cause of death, only men who had prostate cancer listed in part I of the death certificate were considered to have died of prostate cancer.

Salvage Radiation Therapy. Of the 68 patients who had cancer progression, 33 were treated with salvage radiation therapy. Twenty-one of these 33 patients (64%) were treated with external beam therapy at The Methodist Hospital, and the remainder were treated at other institutions. Radiation therapy was limited to the prostatic fossa in 32 patients (97%), and 1 patient received pelvic radiation with an additional boost to the prostatic fossa. Radiation was delivered with 10–23 MV photons. The four-fields technique (anteroposterior/posteroanterior and opposing laterals) with customized field sizes was used. Total radiation therapy dose ranged from 60 to 75.5 Gy (median, 66 Gy), delivered in daily fractions of 1.8–2.0 Gy. After radiation, the patients were followed by physical examination and serum PSA measurements approximately every 3–6 months. Serum PSA measurements of patients who received radiation treatment in other institutions were available through regular follow-up reports. A complete response to salvage radiation therapy was defined as the achievement and maintenance of an undetectable serum PSA level (0.2 ng/ml). Radiation therapy was considered to have failed in a patient if the postradiation serum PSA levels did not fall to, and remained at, an undetectable level.

IHC. The original H&E-stained specimens were examined first for evidence of microscopic foci of prostate cancer. We then performed PSA and cytokeratin staining using serial sections adjacent to the site of the original H&E sections from each archival, formalin-fixed, paraffin-embedded pelvic lymph node removed during radical prostatectomy. Briefly, antigen retrieval was performed by immersing the tissue sections in 0.1 M citrate buffer (pH 6.0) and microwaving at 800 W for 5 min. The primary monoclonal antibody for PSA (ER/PR8, 1/25 dilution; Dako Corp., Carpinteria, CA) and pan-specific mixture antibodies for human cytokeratins (anti-AE1/AE3, 1/50 dilution; Dako Corp.) were incubated for 20 min at room temperature. Secondary antibody (Vector Laboratories) was applied at a dilution of 1:400. Reactivity was visualized with an avidin-biotin complex immunoperoxidase system using diaminobenzidine as the chromagen and Mayer's Hematoxylin as the counterstain (Vector Laboratories). Before lymphadenectomy specimens were analyzed, the PSA and cytokeratin antibodies were tested at various dilutions with prostate cancer specimens to identify the optimal antibody concentration required for reproducible immunohistological staining with minimum background. Prostate tissue and lymph nodes with histological evidence of prostate cancer on H&E served as positive staining controls. Consecutive tumor sections with the primary antibody substituted with a normal mouse IgG were used as negative controls. The controls were included in each series of patient slides stained. All slides were reviewed independently by two investigators (G. W. and S. E.) who were blinded to clinical and pathologic data and reconfirmed by a second set of evaluations by one investigator (S. F. S.) blinded to the interpretations of the first set of evaluations, as well as clinical and pathologic data. Immunostaining results were compared and discrepancies were reviewed. A consensus of all reviewers was required for immunostaining results to be considered positive.

We also performed hK2 (Hybritech mouse monoclonal antibody G586, clone 4M4745; Beckman Coulter, Inc. San Diego, CA) staining using serial sections from the same paraffin-embedded blocks as for PSA and cytokeratin staining. HK2 immunostaining was done using the automated Ventana system (NexES; Ventana Medical Systems, Inc., Tucson, Arizona) that uses an indirect avidin-biotin-peroxidase technique with diaminobenzidine as the chromogen for the antigen localization. Antigen retrieval was performed using citrate buffer (pH 6) in a pressure cooker for 10 min. Before lymphadenectomy specimens were analyzed, the hK2 antibody was tested at various dilutions (2–10 μg/ml) on primary prostate cancers with different Gleason scores and metastatic prostate cancer specimens to identify the optimal antibody concentration required for reproducible immunohistological staining with minimum background, especially in macrophages, because they are commonly observed in lymph node sinuses. The ideal concentration was found to be 6 μg/ml. As a negative control, a nonreactive, IgG1 mouse monoclonal antibody (MOPC

21) was substituted for the primary antibody on consecutive tumor sections. Prostate tissue and known positive lymph node specimens were used as a positive control. All slides were reviewed by one investigator (M. P. R.) without knowledge of staining status of the other markers or of pathologic data.

RNA Preparation, Oligonucleotide Primers, Reverse Transcription Reaction, cDNA Synthesis, PCR, and Assay Interpretation and Scoring. We have previously described the RT-PCR-hK2 assay in peripheral blood (25) and lymph node tissue (26). Briefly, after sections for immunohistochemical staining were obtained, the remainder of each formalin-fixed, paraffin-embedded lymph node specimen was cut into ~50-μm ribbons. We had already developed a primer set for amplification of hK2 (spanning intron 4′ and including a significant portion of the 3′ untranslated region of the hKLK2 gene; Ref. 25). It differentially amplifies the native hk2 transcript, which encodes for the full-length hK2 protein [607 bp (33)], and an alternate spliced transcript (644 bp), which contains an additional 37 nucleotides downstream from the native splice donor site in intron 4 (34). Given the high rate of RNA degradation in archival paraffin-embedded tissue, we designed our primer set for this study to amplify a smaller fragment within the sequence amplified by our previously described primer set. The upstream primer anchored, respectively, in exon 4, nt 563–582: 5′-ATGTGTGCTAGAGCTTACTC-3′ and the downstream primer anchored in exon 5, nt 648–667: 5′-AAGTGGACCCCCA-GAATCAC-3′. The primer was calculated to yield two distinct amplified DNA fragments: the alternate spliced transcript of ~142 bp and the native hk2 transcript of ~105 bp. Reverse transcription was performed as previously described but contained 1.25 μg of RNA (25). The PCR conditions were similar to those previously reported (25), except for cycling, which was performed for 33 cycles with an annealing temperature of 63°C for 1 min, an extension at 72°C for 2 min, and a final extension step at 72°C for 7 min. After 12 μl of each PCR product was loaded on the 2% NuSieve agarose gel (FMC BioProducts, Rockland, ME) in TAE buffer, ethidium bromide staining was performed and followed by gel documentation. After electrophoresis, PCR products were transferred onto a charged nylon membrane (Boehringer Mannheim, Indianapolis, IN), which 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 but without added 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 100 and 1000 copies of hK2 cDNA, as well as at least two sets of formalin-fixed, paraffin-embedded prostate tissue. We chose to score RT-PCR results solely for detection of the native hK2 fragment, which we have previously found to be associated with a more biologically aggressive prostate cancer phenotype (25, 26). The test results were categorized as positive if a clear 105-bp product was visible either after ethidium bromide staining of the gel before blotting or if a signal was clearly evident after probing; as equivocal if no 105-bp product band was evident on ethidium bromide staining and a barely discernable signal was present on the probed membrane; and as negative if no hK2 product was seen either during gel documentation or after membrane probing, but a band was present in each of the positive controls, indicating that the RNA was able to be reverse transcribed and subsequently amplified. To provide internal consistency to the application of these admittedly subjective criteria, all RT-PCR assay results were scored by the same investigators, and the investigators were blinded to clinical and pathologic data and to results from immunohistochemical staining.

Statistical Analysis. The Fisher exact test and the χ^2 test were used to evaluate the association of IHC-Cytokeratin, IHC-PSA, and IHC-hK2 results with clinical and pathologic characteristics. Differences in variables with a continuous distribution across ranked categories were assessed using either Mann-Whitney *U* test or Kruskal-Wallis nonparametric ANOVA. Multivariable survival analysis was performed with the Cox proportional hazards regression model. Preoperative PSA level had a skewed distribution and, therefore, was modeled with a log transformation in the Cox models. Statistical significance in this study was set as $P < 0.05$. All reported *P*s are two-sided. All analyses were performed with the SPSS statistical package (SPSS version 10.0 for Windows).

RESULTS

Detection of Lymph Node Micrometastases Using IHC-PSA/IHC-Cytokeratin and IHC-hK2 Staining Results and RT-PCR-hK2. Occult microscopic foci of prostate carcinoma were found by IHC, RT-PCR, reexamination of the original H&E specimens, and recuts of the specimens. IHC analysis for PSA revealed PSA expressing disseminated cells in lymph nodes from 13 of the 199 patients (7%). All positive cases by IHC-PSA also showed staining for cytokeratin. The cytokeratin stain was darker and easier to interpret. In all 13 cases, only one lymph node was involved. In 3 of 13 cases (23%), the prostate cancer cells that had been missed by the original examination were detected on reexamination of the original H&E slide, guided by the immunohistochemical results. Prostate cancer cells were seen on a recut H&E slide in 8 of 13 cases (62%) but were not identified, even in retrospect, on the original H&E slide. In 2 of 13 cases (13%), the micrometastasis was not seen on the original or recut H&E slide but was seen on IHC-PSA and IHC-Cytokeratin slides. We also performed hK2 staining on pelvic lymph nodes from 164 patients for whom serial sections adjacent to the site of the original H&E sections from the original tissue blocks were available. Three of 164 patients (2%) exhibited hK2 staining (Fig. 1). One patient had four lymph node sections showing a few cells in the lymph node cortex. The second patient had hK2-staining micrometastatic emboli in a vessel contiguous to a lymph node, and the third patient had a micrometastasis-exhibiting, nonlymphoid cell morphology in one lymph node. In all three cases, slides were also IHC-PSA/IHC-Cytokeratin positive. In the 3 cases, no micrometastasis was detected on the original H&E slides. RT-PCR-hK2 assay results were scored as negative in 80 patients (40%), as equivocal in 79 patients (40%), and as positive in 40 patients (20%).

Association of Lymph Node IHC-PSA/IHC-Cytokeratin and IHC-hK2 Staining Results with Clinical and Pathologic Characteristics. Association of IHC-PSA/IHC-Cytokeratin and IHC-hK2 results with pathologic characteristics and RT-PCR-hK2 assay results are shown in Table 1. IHC-PSA/IHC-Cytokeratin and IHC-hK2 expression were associated with prostate cancer involvement of the seminal vesicles ($P = 0.019$ and $P = 0.015$, respectively), higher Gleason sum ($P = 0.019$ and $P = 0.033$, respectively), and RT-PCR-hK2 assay results ($P = 0.038$ and $P = 0.004$, respectively). All 3 patients who had IHC-hK2-expressing cells in their lymph nodes had a positive RT-PCR-hK2 assay result. There was no difference in preoperative serum PSA levels, age, and tumor volume between patients with a positive or negative IHC-PSA/IHC-Cytokeratin ($P = 0.123$, $P = 0.743$, and $P = 0.141$, respectively) or IHC-hK2 staining ($P = 0.178$, $P = 0.842$, and $P = 0.141$, respectively).

Association of Lymph Node IHC-PSA/IHC-Cytokeratin and IHC-hK2 Staining Results with Biochemical Progression. Overall, 68 of 199 patients (34%) demonstrated prostate cancer progression as evidenced by PSA elevation after radical prostatectomy. The median follow-up period for nonprogressing patients was 99.3 months (range, 50.3–189.3 months). Thirty-five patients had at least 10 years of progression-free follow-up. The actuarial 6- and 12-year probabilities of PSA progression after surgery for all patients were 33 and 35%, respectively. IHC-PSA/IHC-Cytokeratin ($P = 0.013$) was associated with biochemical progression when adjusted for the effects of surgical margin status ($P = 0.019$), SVI ($P = 0.002$), prostatectomy Gleason sum (overall $P = 0.033$, Gleason sum 5 and 6 *versus* 7, $P = 0.027$, and 7 *versus* 8–10, $P = 0.019$), preoperative PSA ($P = 0.028$), and level of prostatic capsular invasion ($P = 0.136$). Likewise, IHC-hK2 ($P = 0.004$) was associated with biochemical progression when adjusted for the effects of surgical margin status ($P = 0.001$), SVI ($P = 0.007$), prostatectomy Gleason sum (overall $P = 0.044$, Gleason

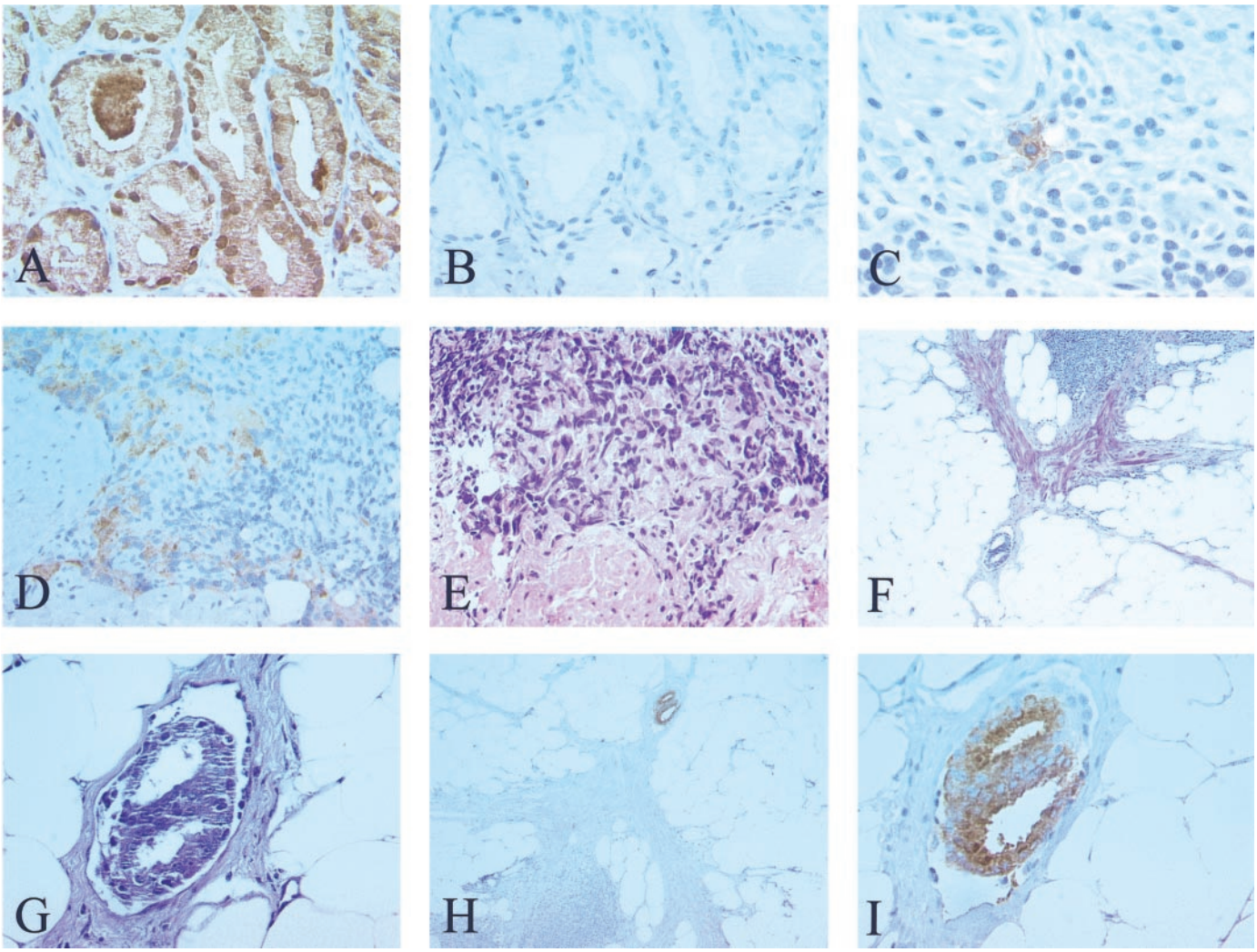

Fig. 1. *A*, a representative area of a positive control for hK2 staining, ×50. *B*, negative control of sample for hK2 staining, MOPC21, ×50. *C*, single cell expressing hK2 in lymph node, ×100. *D*, several cells expressing hK2 in the cortex of lymph node, ×50. *E*, H&E section of *D* shows crush artifacts where cancer cells are impossible to identify, ×50. *F*, micrometastasis in a vessel contiguous to a normal lymph node, H&E, ×10. *G*, emboli consist of tumor cells forming two glands in a vessel contiguous to a normal lymph node, H&E, ×50. *H*, same micrometastasis as in *F*, in a vessel contiguous to lymph node, hK2, ×10. *I*, emboli consist of cells expressing hK2, hK2, ×50.

sum 5 and 6 *versus* 7, $P = 0.046$, and 7 *versus* 8–10, $P = 0.034$), preoperative PSA ($P = 0.162$), and level of prostatic capsular invasion ($P = 0.125$). In a model that included both IHC-PSA/IHC-Cytokeratin and IHC-hK2 in addition to standard postoperative features, only surgical margin status ($P = 0.001$) and SVI ($P = 0.006$) were associated with biochemical progression. When RT-PCR-hK2 assay status was added to any model, surgical margin status (Ps $<$ 0.001) and RT-PCR-hK2 (Ps $\leq$ 0.006) were the only predictors of biochemical progression (*e.g.,* Table 2).

Association of Lymph Node IHC-PSA/IHC-Cytokeratin and IHC-hK2 Staining Results with Response to Salvage Radiation Therapy. Eight patients did not undergo additional treatment after disease progression; 22 underwent hormonal therapy as primary treatment of prostate cancer progression, and 33 underwent local salvage radiation therapy. In patients undergoing salvage therapy, PSA doubling time between radical prostatectomy failure and initiation of salvage therapy was shorter in patients with a positive IHC-PSA/IHC-Cytokeratin staining result (median, 9.5 months; range, 3.4–23.6 months) than in those with a negative result (median, 21.3 months; range, 3.2–45.3 months; $P = 0.043$). Similarly, patients with a positive IHC-hK2 staining result had a shorter PSA doubling time between radical prostatectomy failure and start of salvage therapy than those with a negative staining result (median, 7.3 months; range, 3.7–23.6 months *versus* median, 13.6 months; range, 3.2–45.3 years).

The median follow-up of patients who had a favorable response to radiation was 69.3 months (range, 23.9–102.6 months). IHC-PSA/IHC-Cytokeratin was associated with biochemical failure after salvage radiation therapy ($P = 0.037$) in a multivariable preradiation model that adjusted for the effects of preradiation serum PSA level ($P = 0.102$) and PSA doubling time between radical prostatectomy failure and salvage radiation therapy start ($P = 0.047$). In a model that included IHC-hK2 instead of IHC-PSA/IHC-Cytokeratin, only PSA doubling time ($P = 0.035$) was a predictor of biochemical failure after salvage radiation therapy. When RT-PCR-hK2 assay status was added to any of the models, it was the sole predictor of biochemical progression after salvage radiation therapy (Ps $\leq$ 0.048).

Association of Lymph Node IHC-PSA/IHC-Cytokeratin and IHC-hK2 Staining Results with Development of Clinically Evident Distant Prostate Cancer Metastases. Thirty patients developed bone metastases as evidenced by imaging studies. The median follow-up period for patients who did not develop metastases was 101.8 months (range, 42.5–189.3 months). There were 53 patients with at least 10 years of metastasis-free follow-up. IHC-PSA/IHC-Cytokeratin ($P = 0.016$) was the only predictor of distant prostate

Table 1 *Association of PSA, cytokeratin, and hK2 IHC staining performed on histopathological negative pelvic lymph nodes with pathological characteristics and lymph node RT-PCR-hK2 assay results from 199 consecutive patients with locally advanced prostate cancer (stage pT_3N_0)*

	IHC-PSA/IHC-Cytokeratin			IHC-hK2		
	No. of patients (%)	Positive	*P*	No. of patients (%)	Positive	*P*
Total	199	13 (7)		164	3 (2)	
Seminal vesicle involvement (%)						
Negative	150 (75)	6 (4)		123 (75)	0	
Positive	49 (25)	7 (14)	0.019	41 (25)	3 (7)	0.015
Capsular invasion (%)						
Focal	78 (39)	3 (4)		64 (39)	1 (2)	
Established	121 (61)	10 (8)	0.256	100 (61)	2 (2)	1.000
Surgical margin status (%)						
Negative	162 (81)	11 (7)		133 (81)	3 (2)	
Positive	37 (19)	2 (5)	1.000	31 (19)	0	1.000
RP[a] Gleason sum (%)						
5–6	63 (32)	1 (2)		51 (31)	0	
7	107 (54)	7 (7)		89 (54)	1 (1)	
8–10	29 (15)	5 (17)	0.019	24 (15)	2 (8)	0.033
RT-PCR-hK2 result (%)						
Negative	80 (40)	7 (9)		62 (38)	0	
Equivocal	79 (40)	1 (1)		67 (41)	0	
Positive	40 (20)	5 (13)	0.038	35 (21)	3 (9)	0.004
IHC-PSA/IHC-Cytokeratin (%)						
Negative	—	—		155 (93)	0	
Positive	—	—	—	9 (7)	3 (33)	<.001

[a] RP, radical prostatectomy.

cancer metastases in a multivariable postoperative model that adjusted for the effects of preoperative PSA ($P = 0.115$), SVI ($P = 0.277$), level of prostatic capsular invasion ($P = 0.405$), surgical margin status ($P = 0.585$), and prostatectomy Gleason sum (overall $P = 0.304$, Gleason sum 5 and 6 *versus* 7, $P = 0.199$, and 7 *versus* 8–10, $P = 0.164$). Similarly, IHC-hK2 ($P < 0.001$) was the sole predictor of distant prostate cancer metastases in a model that adjusted for the effects of preoperative PSA ($P = 0.114$), SVI ($P = 0.190$), level of prostatic capsular invasion ($P = 0.235$), and surgical margin status ($P = 0.736$) and prostatectomy Gleason sum (overall $P = 0.275$, Gleason sum 5 and 6 *versus* 7, $P = 0.202$, and 7 *versus* 8–10, $P = 0.261$). However, in a model that included standard pathologic parameters, IHC-PSA/IHC-Cytokeratin, and IHC-hK2, and RT-PCR-hK2, staining results were the sole predictors of distant metastases ($P < 0.001$, Table 2).

Association of Lymph Node IHC-PSA/IHC-Cytokeratin and IHC-hK2 Staining Results with Patient Survival. Survival data were available for all 199 patients. Thirty-eight of 199 patients (19%) were dead at the time of analysis. Of the 38 patients, 18 died of metastatic prostate cancer and 20 died of other causes. The median follow-up was 110.1 months (range, 42.6–191.8 months) after prostatectomy. There were 73 patients with at least 10 years follow-up. IHC-PSA/IHC-Cytokeratin ($P = 0.003$) was the sole predictor of prostate-specific survival after prostatectomy in a postoperative multivariable model that adjusted for the effects of surgical margin status ($P = 0.809$), SVI ($P = 0.087$), level of prostatic capsular invasion ($P = 0.986$), prostatectomy Gleason sum (overall $P = 0.380$, Gleason sum 5 and 6 *versus* 7, $P = 0.311$, and 7 *versus* 8–10, $P = 0.388$), and preoperative PSA ($P = 0.251$). In model that included IHC-hK2 instead of IHC-PSA/IHC-Cytokeratin, IHC-hK2 was the sole predictor of prostate-specific survival after prostatectomy ($P = 0.001$). However, when RT-PCR-hK2 result was added to any model, it was the sole predictor of prostate cancer death (Ps ≤ 0.002; *e.g.*, Table 2).

Clinical Performance of Lymph Node IHC-PSA/IHC-Cytokeratin, IHC-hK2, and RT-PCR-hK2 Assay for Prediction of Prostate Cancer Outcomes. Table 3 shows the sensitivity, specificity, and positive and negative predictive value of IHC-PSA/IHC-Cytokeratin, IHC-hK2, and RT-PCR-hK2 for predicting biochemical progression in patients who had at least 5 years of follow-up after radical prostatectomy and for predicting development of clinically evident metastases and death from prostate cancer in patients who had 10 years of follow-up. IHC-PSA/IHC-Cytokeratin and IHC-hK2 had a very low sensitivity but high specificity for all three end points. RT-PCR-hK2 had a markedly higher sensitivity and accuracy but a slightly lower specificity than IHC-PSA/IHC-Cytokeratin and IHC-

Table 2 *Multivariable cox regression analyses of postoperative features for the prediction of biochemical progression, development of clinically evident distant prostate cancer metastases, prostate cancer-specific mortality in 199 consecutive patients with locally advanced prostate cancer and histopathologically negative lymph nodes (stage pT_3N_0)*

	Biochemical progression			Development of clinically evident distant metastases			Prostate cancer-specific mortality		
		Hazard ratio			Hazard ratio			Hazard ratio	
	P	Estimate	95% CI[a]	*P*	Estimate	95% CI	*P*	Estimate	95% CI
Preoperative PSA levels[b]	0.252	1.434	0.997–2.064	0.116	1.972	0.834–4.663	0.199	2.117	0.832–5.387
Surgical margin status	<.001	3.340	1.754–6.359	0.352	1.615	0.588–4.436	0.158	2.481	0.872–7.059
EPE	0.243	1.420	0.656–3.074	0.459	1.573	0.474–5.220	0.646	1.116	0.352–3.538
SVI	0.076	2.077	0.976–4.420	0.478	1.376	0.569–3.328	0.567	1.204	0.353–4.107
RP Gleason sum[c]	0.086	1.998	0.957–4.173	0.064	2.254	0.987–5.147	0.164	2.239	0.763–6.570
IHC-PSA/IHC-Cytokeratin	0.063	2.352	0.876–6.316	0.080	2.156	0.475–9.786	0.095	2.655	0.685–10.291
IHC-hK2	0.175	1.841	0.404–8.392	0.733	1.250	0.240–6.510	0.967	1.044	0.139–7.841
RT-PCR-hK2	0.006	2.776	1.177–6.548	<.001	2.885	1.585–5.252	0.002	3.195	1.520–6.716

[a] CI, confidence interval; RP, radical prostatectomy.
[b] Preoperative PSA levels were logarithmically transformed.
[c] RP Gleason sum was categorized as grade 2–6 *versus* grade 7 *versus* grade 8–10.

Table 3 *Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of PSA, cytokeratin, and hK2 IHC and RT-PCR-hK2 assay results for biochemical progression at 5 years, development of clinically evident metastases at 10 years, and death of prostate cancer at 10 years in 199 consecutive patients with locally advanced prostate cancer and histopathologically negative lymph nodes (stage pT_3N_0)*

		Biochemical progression at 5 years				
	No. of patients	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
IHC-PSA/IHC-Cytokeratin	196	11.8	96.9	66.7	67.4	67.3
IHC-hK2	164	4.8	100	100	62.7	63.4
RT-PCR-hK2[a]	196	33.8	87.5	60.0	71.3	68.9
		Distant metastases at 10 years				
	No. of patients	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
IHC-PSA/IHC-Cytokeratin	83	16.7	90.4	50	65.3	63.4
IHC-hK2	70	7	97.5	66.7	59.1	59.4
RT-PCR-hK2[a]	83	60.0	82.7	66.7	78.2	74.4
		Prostate cancer-specific survival at 10 years				
	No. of patients	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
IHC-PSA/IHC-Cytokeratin	91	27.8	93.1	50	83.8	79.6
IHC-hK2	76	11.8	98.3	66.7	79.2	78.7
RT-PCR-hK2[a]	91	77.8	82.2	53.8	90.8	84.2

[a] RT-PCR-hK2 was analyzed as a dichotomous variable (equivocal or negative assay results *versus* positive assay result).

hK2 for all three end points. These performances were independent of the time period (2–12 years).

DISCUSSION

Lymph node involvement is almost invariably associated with clinical disease progression in patients with prostate cancer. Intriguingly, however, up to one-third of prostate cancer patients whose regional lymph nodes are tumor free by conventional histopathological methods will develop disease progression despite effective local therapy. Although the etiology of disease progression is likely multifactorial, the findings suggest that conventional methods of assessing the lymph nodes may fail to detect a significant proportion of clinically important metastatic foci. Using immunohistochemical staining of lymph nodes from $pT_3N_0M_0$ prostate cancer patients, we detected PSA-/cytokeratin- and hK2-expressing prostatic cells that were missed by routine histopathological examination. However, careful reexamination of the original H&E-stained lymph node sections revealed prostatic cells in 23% of misdiagnosed patients, and examination of recuts adjacent to the original sections revealed prostatic cells in an additional 62% of the misdiagnosed patients. Immunohistochemical staining for PSA/cytokeratin and hK2 were associated with established features of biologically aggressive prostate cancer, including seminal vesicle invasion and higher Gleason sum. In addition, IHC-PSA/IHC-Cytokeratin and IHC-hK2 were associated with the results of a novel RT-PCR assay for hK2 mRNA performed on the same lymph nodes. Furthermore, after adjustment for the effects of standard pathologic features, results showed that patients with a positive IHC-PSA/IHC-Cytokeratin or IHC-hK2 were at increased risk of suffering biochemical progression after radical prostatectomy, of developing clinically apparent distant metastases, and most importantly, of dying of prostate cancer. However, when RT-PCR-hK2 was added to the models, it was the sole marker associated with prostate cancer outcomes. Although RT-PCR-hK2 had a higher sensitivity and accuracy, immunohistochemical analysis had a higher specificity for prostate cancer progression, metastasis, and mortality.

We found that immunohistochemical staining detects prostate cancer cells in normal regional pelvic lymph nodes of 2% (IHC-hK2) to 7% (IHC-PSA/IHC-Cytokeratin) of pT_3 patients, understaged by conventional H&E assessment. Previous studies have reported that immunostaining for PSA and cytokeratins yields 3–16% occult metastases in routine histopathologically metastasis-free lymph nodes removed at radical prostatectomy from men with locally advanced disease (18, 20, 35–37). Current lymph node evaluation involves microscopic examination of H&E-stained tissue sections and suffers from two major limitations: (*a*) single tumor cell or small foci of cells are easily missed; and (*b*) because only one or two tissue sections are studied, the greater part of each node is left unexamined. Serial sectioning can overcome the issue of sampling error. Besides the technique used, the incidence of occult metastases depends on the skill and commitment of the pathologist. For example, we, like Potter *et al.* (20), discovered a significant number of small deposits of originally unnoticed tumor on reexamination of the original slides or examination of slides showing areas adjacent to the those appearing in the original slides. Although microscopic involvement of a single lymph node confers an unfavorable outcome (8, 38), the biological and clinical significance of submicroscopic disease in lymph nodes detected by IHC remains uncertain. Not all disseminated cancerous cells are able to avoid apoptotic stimuli, bypass the immune response, adapt to the new microenvironment, induce neovascularization and angiogenesis, and proliferate quickly into large metastatic deposits. The ultimate fate of cancer cells in the lymph nodes likely depends on individual phenotype of the cells as well as their interaction with the microenvironment. In addition to the number and volume of the metastases, active proliferation of the micrometastatic cells is also associated with early clinical disease progression (38–41).

We found that IHC-PSA/IHC-Cytokeratin or IHC-hK2 was independently associated with prostate cancer progression, development of overt metastases, and most importantly, cancer-specific death in a large cohort of consecutive patients with pathologic locally advanced prostate cancer but without lymph node metastases detectable by routine methods (pT_3N_0). In agreement with Freeman *et al.* (18), we found that IHC-PSA/IHC-Cytokeratin staining of routine histopathological normal pelvic lymph nodes removed at radical prostatectomy was associated with invasion of the seminal vesicles and higher Gleason sum. Similarly, expression of hK2 was associated with SVI and higher Gleason sum. Although these associations are important, the association with clinically important end points such as clinical disease progression in patients treated effectively for clinically localized disease would be more informative to physicians faced with managing these patients (42, 43). Potter *et al.* (20) found that 50% of the $pT_{3b}N_0$ patients who had lymph node micrometastases detected by IHC-PSA and/or IHC-Cytokeratin suffered either biochemical or clinical disease progression. Immunohistochemical assay has several advantages, including applicability in all cases and easy adaptability to

current pathologic analysis. However, IHC may be limited by variation in assay results according to the antibodies, subjectivity of interpretation, and differences in specimen handling (formalin pH, fixatives temperatures, and durations of fixation) and technical procedure (*e.g.,* antibody dilution and procedure reagents). In addition, step-sectioned immunohistochemical evaluation of lymph nodes would be too costly and time consuming for routine analysis, limiting its usefulness to evaluation of malignancies with sentinel lymph nodes.

RT-PCR for hK2 was the strongest predictor of biochemical progression, development of clinically overt metastases, and disease-specific survival in multivariable models that adjusted for the effects of IHC-PSA/IHC-Cytokeratin, IHC-hK2, and standard pathologic features. RT-PCR overcomes the problem of sampling error because larger amounts of tissue can be analyzed and identifies more positive lymph nodes than IHC (44–49). Indeed, we analyzed ribbons from all lymph nodes from each single patient in one assay and detected a 3–10-fold higher rate of positive results than found with IHC. In addition, RT-PCR involves less operator variability and establishes the presence or absence of specific molecular abnormalities at the RNA level. In agreement with every study, of which we are aware, that compared IHC and RT-PCR for the detection of occult metastases (44–50), we found that RT-PCR was consistently more sensitive and accurate than immunostaining for identifying patients who are likely to suffer clinical disease progression and eventually death of prostate cancer. However, IHC was more specific than RT-PCR for detecting biologically and clinically significant subclinical metastases. An advantage of IHC over RT-PCR is that it allows an additional cytomorphological evaluation of suspicious cells useful for targeted therapy, as well as an assignment of the findings to histotopographical regions when performed on tissue sections. False positive RT-PCR-hK2 assay results are, at least in part, attributable to the phenomenon of ectopic gene, which results in very low background levels of expression of most genes in all tissue types (51, 52). In addition, the detection of specific molecules in draining lymph nodes may not necessarily indicate the presence of intact prostate cancer cells in the nodes. Although we used a qualitative, gel-based RT-PCR method that was associated with prostate cancer outcomes, it is now becoming apparent that this simple positive/negative method may not be the most reliable sign of micrometastases. Novel quantitative RT-PCR assays have been shown to improve test specificity while maintaining the same sensitivity as gel-based single marker RT-PCR assays (49). In addition, quantitative RT-PCR assays are objective, standardized, faster, simpler, reproducible, and would allow quantification of the extent of lymph node involvement. On the other hand, no universal tumor marker for biologically aggressive prostate cancer has been identified to date. Therefore, multimarker RT-PCR assays may eliminate some of the inherent problems associated with single-marker techniques such as tumor heterogeneity, clonal selection, and variable expression of individual genes.

The accuracy of RT-PCR-hK2 was lowest for prediction of biochemical progression, increased for prediction of development of distant metastases, and was highest for prediction of prostate cancer-specific death. The rate of false negative RT-PCR-hK2 assay results decreased markedly from 66% for prediction of biochemical progression at 5 years after surgery, to 40% for prediction of development of metastases at 10 years after surgery, and to 22% for prediction of death of prostate cancer at 10-year follow-up. The false positive rate increased only slightly from 12 to 17 and 18%, respectively. Biochemical progression may result from local failure related to residual disease present after radical prostatectomy, to occult metastatic nodal or distant metastatic disease present at the time of surgery, or to some combination of these. These forms of recurrent disease have variable progression rates with regard to metastases and eventual death. Pound *et al.* (43) reported that many patients who experience disease progression after radical prostatectomy will not develop clinically evident metastases, nor will they die of prostate cancer. The lower accuracy of RT-PCR-hK2 for biochemical progression after surgery may be because of a lack of association of RT-PCR-hK2 with local and/or biologically more indolent disease.

The low sensitivity of the molecular and immunohistochemical assays for clinical outcomes in prostate cancer patients suggests that other mechanisms of disease dissemination, via the peripheral blood and bone marrow, which bypass local lymph nodes, most likely, also play an important part in disease progression (53–55). In this study, all patients who had a positive immunostaining and/or RT-PCR assay result and who ultimately developed distant metastases had large tumor deposits. This suggests that lymph node micrometastases do not only reflect lymphogeneous spread but may also signal the early phase of hematogeneous systemic tumor spread. Morphologically, lymph nodes contain not only lymphatic channels but also blood vessels, and the prostate cancer cells detected in lymph nodes might reflect peripheral blood metastases rather than lymphatic metastases. Kollermann *et al.* (56) found that the rate of seeding to lymph nodes is lower than the rate of seeding to bone marrow. However, because some patients had isolated carcinoma cells in the lymph nodes only, they concluded that in some patients at least, initial tumor cell dissemination occurs via the lymphatic vessels. We have previously shown that preoperative peripheral blood RT-PCR-hK2 and postoperative peripheral blood RT-PCR/PSA assays are independently associated with overall and aggressive disease progression after radical prostatectomy (26).[4] This study included 39 consecutive patients who were part of these studies and therefore had preoperative RT-PCR/PSA and RT-PCR-hK2 and postoperative RT-PCR/PSA performed on peripheral blood specimens. In all 39 patients and in patients who experienced disease progression in the first 5 years after radical prostatectomy, results from peripheral blood and lymph node assays were concordant in $<30\%$ of cases (data not shown). These findings suggest that the primary tumor can shed tumor cells by different routes and that each of these routes can be sufficient for clinical progression of prostate cancer. On the other hand, 40% of the patients who developed distant metastases and 100% of the patients who died of prostate cancer within 7 years of follow-up had positive results for both lymph node and peripheral blood assays.(data not shown) On the basis of these findings, we hypothesize that patients who have detectable disseminated cells in the bloodstream and in the nodal tissue have more biologically aggressive prostate cancer, putting them at the highest risk for early metastasis and death from prostate cancer.

Several limitations in this study should be considered. The current population was restricted to patients who underwent radical prostatectomy and bilateral pelvic lymphadenectomy by a single highly experienced surgeon (P. T. S.). In our present series of patients with locally advanced disease, the positive margin rate was only 19%. Positive margin rates after radical prostatectomy are strong predictors of disease progression in patients with pT_3 prostate cancer (57, 58). In addition, 47 of 199 patients underwent radical prostatectomy before institution of routine assessment of postoperative PSA levels at our department (1989). Furthermore, the sample size and the follow-up interval of this study were limited. Some variables that were inconclusive because of limited statistical power may attain statistical significance if the sample size or the length of follow-up is increased. Finally, the lymph node sampling technique may have failed to pick

[4] S. F. Shariat, M. W. Kattan, W. Song, D. Bernard, E. Gottenger, T. M. Wheeler, and K. M. Slawin. Early postoperative peripheral blood RT-PCR assay for prostate-specific antigen is associated with prostate cancer progression in patients undergoing radical prostatectomy, submitted for publication.

up metastases in nodes not resected. Although the topic of the extent of lymph node sampling remains controversial, there is recent evidence that an extended pelvic lymphadenectomy is associated with a high rate of lymph node metastases outside of the fields of standard lymphadenectomy (28, 59). Bader *et al.* (28), for example, reported that 40% of pT_3 patients have positive lymph nodes on extended lymph node dissection (median of 21 lymph nodes excised/patient). In this study, a standard pelvic lymph node dissection was performed in each patient with a mean of 9.6 lymph nodes/patient removed at the time of radical prostatectomy.

Although still falling short of perfect performance, RT-PCR and IHC upstaged some patients misdiagnosed as having cancer-free lymph nodes by routine H&E examination. Immunohistochemical staining for PSA, cytokeratin, or hK2 detected with high specificity clinically and biologically significant cancer cells in histopathological normal lymph nodes of pT_3 prostate cancer patients. However, RT-PCR-hK2 detected subclinical lymph node deposits with higher sensitivity and accuracy and was therefore more strongly associated with prostate cancer progression, metastasis, and survival. Although the methodologies used in this study are too cumbersome to influence clinical management at this point, it might serve as a model. Ultra-staging of prostate cancer on routine histopathological normal pelvic lymph nodes may improve both clinical decision making regarding adjuvant therapy and the comparability of patient populations. In addition to detecting earlier metastatic disease and thereby improving prognosis and monitoring of disease, molecular and histological technologies may help tailor therapy by evaluating individual patterns of cancer gene expression (60–62). Furthermore, ultra-staging may give new insights into lymphatic drainage patterns and provide a new tool for the investigation of the biology of metastasis.

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