Prospective Analysis of Prostate-Specific Markers in Pelvic Lymph Nodes of Patients With High-Risk Prostate Cancer

Anna C. Ferrari, Nelson N. Stone, Jason N. Eyler, Min Gao, John Mandeli, Pamela Unger, Robert E. Gallagher, Richard Stock*

Background: Pathologic evidence of pelvic lymph node involvement is obtained in 12%–20% of patients with localized prostate cancer that exhibits high-risk features (defined on the basis of tumor size, serum prostate-specific antigen [PSA] level, or Gleason score). The rate of systemic failure (i.e., relapse) in patients with this type of prostate cancer and no pathologic evidence of regional lymph node involvement is 55%–92% within 5 years of definitive local therapy. Since reverse transcription–polymerase chain reaction (RT–PCR) methods are likely to be more sensitive than routine pathologic examination in detecting metastatic tumor cells, we compared the ability of the two approaches to detect prostate cells in the pelvic lymph nodes of patients with localized, high-risk disease. Methods: Fifty-eight lymph node specimens isolated from 33 patients before definitive local therapy were examined. Expression of PSA and prostate-specific membrane antigen (PSM) messenger RNAs in the specimens was assessed by means of nested RT–PCR. Results: Pathologic examination identified tumor cells in the lymph nodes of four (12%) of the 33 patients, and PSA and/or PSM expression was positive in specimens from 27 (82%) of the patients (two-sided \(P<.0001\)). The four patients with positive pathologic findings also had positive RT–PCR results. Among the 29 patients with no pathologic evidence of lymph node involvement, 23 (79%) tested positive by means of RT–PCR. In these 23 patients, PSM expression was detected more frequently than PSA expression; however, in two patients, only PSA expression was detected. Conclusions: Expression of prostate-specific markers in the pelvic lymph nodes of patients with localized, high-risk prostate cancer may indicate the presence of metastatic tumor cells. Such cells may be responsible for the high rate of systemic failure seen in these patients. Additional studies are required to determine the prognostic relevance of our findings. [J Natl Cancer Inst 1997;89:1498–1504]

Patients with prostate cancer who present with high-risk features may harbor metastases in the pelvic lymph nodes (LN). Since the extent of lymph node involvement has been shown to correlate with progression and death, these patients will not be cured by local interventions alone (1–4). While the presenting prostate-specific antigen (PSA) level, clinical stage, and Gleason score may be predictive of lymph node involvement (5,6), pathologic assessment of the pelvic lymph nodes before local intervention is required to determine the presence or absence of metastatic extension. This evaluation is performed on frozen sections at the time of laparotomy for radical prostatectomy or on fixed lymph node tissues removed by laparoscopic lymph node dissection before definitive radiation therapy.

In spite of efforts to pathologically stage the disease in patients with prostate cancer, the frequency of detection of lymph node metastasis by routine histologic analysis is not concordant with the frequency of disease recurrence after definitive local therapy (7–11). The difference is most outstanding in the group of patients with localized prostate cancer and high-risk features (12,13), including a T stage of greater than or equal to 2B [TNM classification system (14)], a serum PSA value of greater than or equal to 20 ng/mL, or a Gleason score (15) of greater than or equal to 7. While the incidence of N1–2 disease (i.e., metastasis to regional lymph nodes) for this group of patients ranges from 12% to 20% by routine pathologic examination and while 16% of occult lymph node metastases can be detected in patients with stage T3, N0 disease by means of immunohistochemical analysis with antibodies to PSA or to epithelial-specific intermediate filament cytokeratins (10,11), the rate of failure with rising PSA levels in patients with disease classified as N0 ranges from 55% to 92% within 5 years of local treatment (9,16–19). These discrepancies suggest that metastatic tumor cells in pelvic lymph nodes can escape detection by microscopic procedures and that patients may be subjected to noncurative therapies and their attendant morbidities when treatment is directed to local control...
alone. Therefore, an improved method of detecting patients with lymph node metastasis needs to be identified.

Expression of the PSA (20) and prostate-specific membrane antigen (PSM) (21,22) genes is restricted almost exclusively to prostate epithelial cells. However, these two antigens are expressed heterogeneously by such cells. Whereas PSA expression is stimulated by androgens (23) and the protein is highly expressed by differentiated prostate epithelial cells (24) and tumors (25–28), PSM expression is suppressed by androgens and PSM protein is more highly expressed in undifferentiated prostate epithelial cells and tumors (21,29). Thus, the utilization of these two specific markers has broadened the phenotypic spectrum of prostate cancer metastases that are detectable by molecular methods.

The high specificity of PSA and PSM gene expression has made it possible to identify metastatic prostate epithelial cells among nonprostate cells. Using reverse transcription–polymerase chain reaction (RT–PCR) methods, several laboratories have been able to identify one circulating prostate cell in 10 million peripheral blood mononuclear cells of patients with localized and metastatic prostate cancer (29–40). More recently, metastases of prostate cells to the bone marrow (41,42) and lymph nodes (43–46) of prostate cells to the bone marrow (41,42) and lymph nodes (43–46) have also been detected in localized disease, suggesting that prostate cancer cells migrate early in the course of the disease and perhaps are responsible for later systemic failure. To investigate this possibility further, we collected fresh pelvic lymph node tissue by laparoscopic lymph node dissection from 33 patients with high-risk, localized prostate cancer before definitive therapy. We then evaluated the incidence of metastatic prostate cells as judged by the detection—via RT–PCR—of PSA and PSM messenger RNA (mRNA) markers and compared it with the detection of metastasis by pathologic examination of the same specimens.

**Patients, Materials, and Methods**

**Disease Staging**

The tumors in 33 patients with clinically localized, high-risk prostate cancer who had negative bone scans and computerized tomographic scans of the pelvis (T3–T2B, PSA level >20 ng/mL, or Gleason score >7) were clinically and pathologically staged by one urologist (N. N. Stone). Pathologic staging included transrectal, ultrasound-guided seminal vesicle biopsies (three core biopsy specimens from each seminal vesicle) performed 1–2 months before laparoscopic lymph node dissection. Sixty-one lymph node tissue specimens (average of five lymph nodes per specimen) were obtained from 28 patients who had bilateral obturator and hypogastric lymph node areas sampled and five patients who had unilateral lymph node sampling. Patients gave written informed consent, and the study was approved by an institutional review board. Patients with N1 disease received hormone therapy alone, and patients with N0 disease received radiation therapy at least 1 month after pathologic lymph node staging.

**Tissue Processing and RNA Extraction**

Lymph node tissue was removed by laparoscopic lymph node dissection and washed with a 0.9% solution of sodium chloride. Each entire specimen was embedded in Tissue Tek O.C.T. compound (Miles Laboratories, Elkhart, IN) and frozen in a cryostat. A 4-µm-thick section was cut, stained with hematoxylin–eosin, and examined by one pathologist (P. Unger). Next, six to 10 frozen sections were cut from each tissue block. The sections were dropped into 1 mL of a 1:100 dilution of 1× RT buffer, 0.2 mΜ each standard dNTP (Boehringer Mannheim, Mannheim, Germany), 0.1 pmol 5′ and 3′ primers. A 1.25 U AmpliTaq Gold polymerase (The Perkin-Elmer Corp.), and two drops of MgCl₂ (The Perkin-Elmer Corp., Norwalk, CT), 0.2 mΜ each of the standard dNTP and 0.5 mΜ each of the standard dNTPs (dNTPs). The solution was subsequently incubated at 25 °C for 5 minutes. Superscript II Moloney murine leukemia virus reverse transcriptase (200 U) was then added, and the mixture was incubated at 25 °C for an additional 10 minutes. After this incubation, the mixture was incubated at 42 °C for 50 minutes to terminate the reaction, we heated the mixture at 70 °C for 15 minutes and then cooled it on ice for at least 5 minutes. Residual RNA was destroyed by incubation at 37 °C for 20 minutes in the presence of 2 U ribonuclease H.

Under generic PCR conditions, 0.2–1 µg RNA-equivalent of test complementary DNA (cDNA) was mixed in a 50-µL reaction volume with 1× PCR buffer, 1.5 mM MgCl₂ (The Perkin-Elmer Corp., Norwalk, CT), 0.2 mΜ each standard dNTP (Boehringer Mannheim, Mannheim, Germany), 0.1 pmol 5′ and 3′ primers. A 2.5 U AmpliTaq Gold polymerase (The Perkin-Elmer Corp.), and two drops of mineral oil (Sigma Chemical Co., St. Louis, MO). The mixture was then subjected to temperature cycling in an aluminum block (MJ Research, Inc., Waterdown, MA) that was programmed as follows: 95 °C for 10 minutes (step 1); one cycle at 95 °C for 50 seconds, 57 °C for 2 minutes, and 72 °C for 2 minutes (step 2); 29 cycles at 95 °C for 30 seconds, 57 °C for 1 minute, and 72 °C for 1 minute (step 3); and one cycle at 95 °C for 30 seconds, 57 °C for 1 minute, and 72 °C for 10 minutes (step 4). The PCR products were identified on the basis of size after agarose gel (FMC Bioproducts, Rockland, ME) electrophoresis and staining with ethidium bromide.

**PCR for PSA and PSM**

Outer and inner primer sets for double-nested PCR of both PSA and PSM nucleotide sequences were synthesized by Bio-Synthesis, Inc., Lewisville, TX. The sequences used for the external PSA 20-mer primers were obtained from GenBank and were selected for the lowest homology with human kallikrein nucleotide sequences by use of the DNA Strider 1.2 program for Macintosh. These primers were previously reported to identify circulating prostate tumor cells in the peripheral blood (31). They were anchored in exon 3 at nucleotides 648–667 (5′-GATGACTCCAGGCCAGCCT-3′) and in exon 5 at nucleotides 1357–1338 (5′-CACGACCCCCCTATCCT-3′), incorporating a cDNA segment of 710 base pairs (bp). The nested PSA 20-mer primers were anchored in exon 4 at nucleotides 860–879 (5′-GATATGTCTCCAGGCGCATGC-3′) and in exon 5 at nucleotides 1315–1296 (5′-GCGACATCCGCCCTTCGAGG-3′), incorporating a cDNA segment of 455 bp. The sequences for the PSM cDNA and the two sets of PSM primers were obtained from the literature (29,38). The external primers were a 23-mer anchored at nucleotides 1368–1390 (5′-CAGATGTCCATTCTTGAGGAGTCC-3′) and a 21-mer anchored at nucleotides 2015–1995 (5′-AACACATCCCTTCTCAGGACC-3′), incorporating a 647-bp cDNA segment. The nested primers were a set of 24-mers anchored at nucleotides 1689–1712 (5′-CCTAACAAAGGAGTCGAAAGGGC-3′) and nucleotides 1923–1899 (5′-ACTGTTGATCAGGGTATGCCGT-3′), incorporating a cDNA segment of 234 bp.

For PCR amplifications of PSA and PSM with outer primers, 1 µg RNA-equivalent of cDNA was added to each reaction mixture and subjected to the temperature-cycling steps described above except that the number of cycles in step 3 was increased to 49. Nested reactions utilized 4 µL of a 1:100 dilution of

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the first-round PCR product, and the number of cycles was decreased to 29 in step 3. Included in each PSA or PSM PCR experiment were two negative control reactions—a reaction with no added cDNA and a reaction containing human female peripheral blood mononuclear cell cDNA. A reaction containing cDNA synthesized from RNA derived from a mixture of one LNCaP cell and 10⁶ peripheral blood mononuclear cells served as a positive control in each experiment.

**Competitive G3PDH/G3PDH MIMIC PCR**

To control for RNA integrity and to determine the significance of a negative RT–PCR assay, we performed a competitive RT–PCR assay involving the product of the housekeeping gene G3PDH (glyceraldehyde-3-phosphate dehydrogenase). A set of 20-mer primers anchored at nucleotides 416–435 (5’-CACAATGCTCCTGCTTCAG-3’) and nucleotides 859–840 (5’-CTCCCGAGCAGCGTTCAC-3’) of the G3PDH cDNA was synthesized to amplify a fragment of 444 bp (47). A G3PDH MIMIC competitor DNA of 289 bp consisted of a fragment of v-erbB DNA flanked by the G3PDH-specific primers (47). An aliquot of each cDNA sample was amplified simultaneously with a known amount of the synthetic G3PDH MIMIC DNA as a specific competitor for amplification of endogenous G3PDH sequences (Fig. 1). The standard for cDNA/RNA integrity was established in a series of reactions with the use of a fixed amount of control cDNA (0.2 μg RNA-equivalent) that was prepared from one LNCaP cell mixed with 10⁶ female peripheral blood mononuclear cells and variable amounts of the MIMIC competitor DNA (from 0.01 to 5 amol). We found that the cDNA equivalent of 0.2 μg of intact control RNA yielded amplification equal to 0.1 amol of G3PDH MIMIC DNA. A PCR reaction containing these ratios of sample cDNA and exogenous MIMIC competitor was used to assess the integrity of all test RNAs (Fig. 1). If no signal from the endogenous G3PDH template was observed in the presence of the MIMIC, the RNA was considered to be degraded and not assessable. On the other hand, if a decreased but visible endogenous G3PDH signal was observed, the degree of degradation was estimated from the G3PDH/G3PDH MIMIC titration curve by means of visual inspection, and a repeat reaction containing an appropriate compensatory amount of cDNA was performed.

**Statistical Methods**

Statistical comparisons were made by use of McNemar’s two-sample test for binomial proportions for paired data from patients (48). The reported P value is two-sided.

**Results**

**Sensitivity and Specificity of PSA and PSM RT–PCR Assays**

The sensitivity of the PSA and PSM RT–PCR assays was established by cell dilution experiments in which human LNCaP cells were mixed with human female peripheral blood mononuclear cells at ratios of 1:10⁴ to 1:10⁷. Our nested RT–PCR assay increased the sensitivity of prostate cell detection from 1:10⁶ cells to 1:10⁷ cells. When RNA integrity was high, as indicated by adequate amplification of cDNA from endogenous G3PDH RNA in the presence of a fixed amount of G3PDH/G3PDH MIMIC DNA competitor, one LNCaP cell admixed with 10⁷ peripheral blood mononuclear cells (1:10⁷) could be detected (Fig. 1). The specificity of the amplified PSA and PSM products was determined on the basis of PCR product size and homology (Southern blot transfer and hybridization with a specific oligomer probe) or, in some instances, by PCR product sequence analysis (data not shown).

**RT–PCR Analysis of Pelvic Lymph Node RNAs**

RNAs were extracted from 61 lymph node tissue specimens (average of five lymph nodes per specimen) from 33 patients. As a first step, G3PDH/G3PDH MIMIC competitive assays were performed to evaluate RNA integrity (see ‘‘Patients, Materials, and Methods’’ section). On this basis, RNAs from three samples were excluded because of complete degradation. PSA and PSM RT–PCR assays were then performed on the remaining 58 RNA samples (still representing 33 patients). In Fig. 2, eight representative RT–PCR results from six patients are shown. Partial RNA degradation, as indicated by a decreased endogenous G3PDH signal relative to the G3PDH/G3PDH MIMIC signal, was observed in lanes 5, 6, 7, 9, and 10 (panel B). Consequently, a compensatory amount of cDNA was utilized to repeat these assays, and the final results for the G3PDH/G3PDH MIMIC assay are shown in the corresponding lanes of panel C. The final nested PCR results for PSA and PSM are shown in panel A. This illustrative gel analysis shows a full spectrum of possible PSA/PSM RT–PCR results: both mRNAs being present (lane 3, left lymph nodes of patient 29), PSM mRNA alone being present (lanes 4 and 6, left lymph nodes of patients 21 and 31, respectively), PSA mRNA alone being present (lane 5, right lymph nodes of patient 30), and neither mRNA being present (lanes 7, 8, 9, and 10, from right and left lymph nodes of patients 32 and 33, respectively).

By this form of analysis, 41 (71%) of 58 lymph node RNAs analyzed were RT–PCR positive: Seven (12%) of 58 RNAs were positive for PSA only, 18 (31%) of 58 RNAs were positive for PSM only, and 16 (28%) of 58 RNAs were positive for both. Seventeen (29%) of the 58 RNAs, corresponding to specimens
Fig. 2. Reverse transcription–polymerase chain reaction (RT–PCR) analysis of prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSM) messenger RNA in eight representative pelvic lymph node RNAs from six patients with high-risk prostate cancer. A) Nested PSA and PSM RT–PCR products as follows: lane 1, restriction endonuclease Hae III-digested φ×174 DNA size markers; lane 2, the amplification products from female peripheral blood mononuclear cell DNA; lane 3, left lymph node from patient 29; lanes 4 and 6, left lymph nodes from patients 21 and 31; lane 5, right lymph nodes from patient 30; and lanes 7, 8, 9, and 10, left and right lymph nodes from patients 32 and 33, respectively. B) First G3PDH (i.e., glyceraldehyde-3-phosphate dehydrogenase) G3PDH MIMIC DNA competitive assay results. C) Results of the second G3PDH/G3PDH MIMIC DNA competitive assay after increasing the amount of complementary DNA for those samples showing partial messenger RNA degradation (lanes 5–10 in panel B). See text for more details.

from 15 patients, were RT–PCR negative for both markers (see Tables 1 and 2 and see below).

Distribution of Pathologic Staging and RT–PCR Results in Lymph Nodes and Patients

Pathologic findings and RT–PCR assay results for the 58 lymph node specimens are shown in Table 1. Four lymph node specimens were pathologically positive, corresponding to 7% of the lymph nodes and four (12%) of the 33 patients. These same lymph nodes and patients were positive for both PSA and PSM. Thus, in this small sample, RT–PCR showed 100% concordance.

Table 1. Distribution of pathologic diagnosis and reverse transcription–polymerase chain reaction (RT–PCR) assay results at the level of the pelvic lymph node specimen and the patient*

<table>
<thead>
<tr>
<th>Analysis level</th>
<th>Pathology</th>
<th>RT–PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node specimens</td>
<td>POS (N1–2)</td>
<td>No.</td>
</tr>
<tr>
<td></td>
<td>NEG (N0)</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>58</td>
</tr>
<tr>
<td>Patients</td>
<td>POS (N1–2)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>NEG (N0)</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>33</td>
</tr>
</tbody>
</table>

*Pathologic diagnosis of lymph node involvement is either positive (POS) or negative (NEG). N0 and N1–2 indicate the extent of involvement [TNM classification system (14)].
†The four pathologically positive (N1–2) lymph nodes were RT–PCR positive for prostate-specific antigen and prostate-specific membrane antigen.

Table 2. Distribution of reverse transcription–polymerase chain reaction (RT–PCR)-positive assays for prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSM) messenger RNA expression in pathologically negative lymph node specimens and in patients with disease classified as N0 (negative LNs)*

<table>
<thead>
<tr>
<th>Category</th>
<th>PSA only (%)</th>
<th>PSM only (%)</th>
<th>PSA + PSM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>49</td>
<td>19 (39)</td>
<td>30 (61)</td>
</tr>
<tr>
<td>Lymph node specimens</td>
<td>37 (7)</td>
<td>18 (49)</td>
<td>12 (32)</td>
</tr>
</tbody>
</table>

*TNM classification system (14).

with pathologically established lymph node metastasis. Among the 54 pathologically negative lymph nodes, 37 (69%) were RT–PCR positive for either PSA, PSM, or both. Seventeen (31%) were RT–PCR negative for both markers. The RT–PCR-positive lymph nodes were found in 23 (79%) of 29 patients with pathologically negative (N0) disease. Only six (21%) of 29 patients with N0 disease remained RT–PCR negative. Overall, the difference in the rate of detection of metastatic prostate cancer cells by pathologic analysis (12%) versus RT–PCR (82%) was statistically significant (P<0.0001).

Distribution of PSA and PSM RT–PCR Assays Among Pathologically Negative Lymph Node Samples and Patients With N0 Disease

Because of the critical importance of RT–PCR results in the pathologically negative lymph nodes of patients with N0 disease, we examined the distribution of PSA and PSM assay results in these groups. From this analysis, we hoped to gain some insight into the frequency of false-positive assays and to determine the contribution of individual markers to the detection of metastatic prostate cancer cells. There were 108 RT–PCR assays performed on the 54 pathologically negative lymph node specimens (one PSA and one PSM assay performed on each specimen); 59 (55%) assays were negative and 49 (45%) assays were positive. The 49 RT–PCR-positive assays (Table 2) that occurred in 37 pathologically negative lymph node specimens were more frequently positive for PSM (81%: 49% + 32%, in Table 2) than for PSA (51%: 19% + 32%, in Table 2). Overall, the 23 corresponding patients with N0 disease were also more frequently RT–PCR positive for PSM (92%: 35% + 57%, in Table 2) than for PSA (66%: 9% + 57%, in Table 2), and over half of them expressed both markers.

In spite of the higher frequency of patients with PSM RT–PCR-positive lymph nodes, two of the 23 patients with N0 disease were identified by PSA RT–PCR alone, and they would have been missed if only PSM RT–PCR testing had been performed.

Availability of Unilateral Versus Bilateral Lymph Node Samples and Detection of RT–PCR-Positive Patients

The availability of lymph node tissues from bilateral dissections affected the outcome of the RT–PCR analysis. The patients with N0 disease who had a bilateral dissection were more likely to be classified as RT–PCR positive, 19 (90%) of 21, than the patients with unilateral lymph node specimens, four (50%) of
eight (Table 3). In patients with N1–2 disease, the contralateral, histologically normal lymph nodes were RT–PCR positive 50% of the time (two of four).

**Comparison of RT–PCR Results With Prostate Tumor Size, Gleason Score, and Serum PSA Value**

Table 4 shows the distribution of patients according to primary tumor stage, lymph node pathologic diagnosis, and PSA/PSM RT–PCR assay outcome. The distribution of frequencies of positive RT–PCR assays ranged from 50% to 100%. Patients with stage T2A primary tumors had the lowest frequency of RT–PCR-positive lymph nodes (50%), but, notably, there were more unilateral lymph node biopsy specimens in this subgroup (three of six). Overall, there was no indication of any association between tumor stage (Table 4), Gleason score (not shown), or serum PSA levels (not shown) and the RT–PCR results, and the number of patients was too small to permit statistical associations.

**Discussion**

This study indicates that RT–PCR analysis can detect molecular evidence of PSA and PSM mRNA expression in 69% of pathologically normal pelvic lymph node specimens from patients with high-risk prostate cancer. This finding suggests that these lymph nodes frequently contain occult metastatic prostate cancer cells and that the incidence of lymph node metastasis estimated from clinical nomograms and routine pathologic staging [12%–20% (5,6,12,13)] and immunohistochemical staging [16% (10,11)] is likely underestimated. This finding also suggests that the 5-year failure rate (55%–92%) following definitive therapy in patients with high-risk disease and pathologically normal lymph nodes (9,16–19) may be related to some of these patients to the presence of these metastatic prostate cancer cells.

Supporting evidence for the potential clinical relevance of these metastatic prostate cancer cells is provided by a retrospective PSA RT–PCR analysis of RNAs from archival, fixed, pathologically negative lymph node tissues. Among 36 patients with prostate cancer treated with radical prostatectomy, 16 (44%) were RT–PCR positive, and 14 (88%) of these 16 patients had recurrences within 5 years, whereas only six (30%) of 20 RT–PCR-negative patients had recurrences (45). This association between RT–PCR-positive lymph nodes and clinical outcome suggests that the molecularly detected prostate cancer cells in the archival lymph node tissues represented established micrometastatic foci of tumor cells responsible for the systemic spread and failure experienced by the patients. In contrast, it remains unclear whether circulating prostate cells detected in the peripheral blood or the bone marrow of patients with clinically localized prostate cancer are able to predict pathologic stage and lead to distant metastasis (31,36).

A number of issues related to molecular staging of pelvic lymph nodes by RT–PCR require critical assessment before it can be extended to clinical practice. These issues include the following: the sensitivity and specificity of RT–PCR to identify prostate cancer cells in the tissues of patients, the number of markers required to identify prostate cancer cells, the number of lymph node samples needed to have confidence in the prediction of metastases for individual patients, and the clinical relevance of these prostate cancer cell metastases for overall prognosis. A potential source of misleading information can arise from false-negative and false-positive results. To address this question, we analyzed the distribution of the RT–PCR assay results among the 58 lymph node tissues examined.

Although the sample size was small, there were no false-negative cases among the four pathologically positive lymph node specimens; i.e., all four were RT–PCR positive for both PSA and PSM. Further evidence against false-negative results is the extremely high sensitivity of the assay to detect prostate cancer cells among nonprostate cells (one prostate cell in 10^7 nonprostate cells). This analysis also suggested that false-positive assays, which can arise from various sources, were not a significant factor influencing our conclusions. Our observation of a greater number of negative rather than positive PSA and PSM RT–PCR assays, i.e., 59 versus 49, strongly favors the interpretation that the finding of higher proportions of RT–PCR-positive lymph nodes and patients is not due to cross-

**Table 3. Effect of unilateral or bilateral lymph node analysis on the distribution of reverse transcription–polymerase chain reaction (RT–PCR) assay results in patients with N0 disease (negative nodes)**

<table>
<thead>
<tr>
<th>Lymph node source</th>
<th>Total No. of patients</th>
<th>RT–PCR results†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>POS (%)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>21</td>
<td>19 (90)</td>
</tr>
<tr>
<td>Unilateral</td>
<td>8</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>23</td>
</tr>
</tbody>
</table>

*TNM classification system (14).
†POS = positive; NEG = negative.

**Table 4. Distribution of reverse transcription–polymerase chain reaction (RT–PCR) assay results according to clinical tumor stage**

<table>
<thead>
<tr>
<th>Clinical stage of tumor</th>
<th>Patient No.</th>
<th>Lymph nodes POS,† No.</th>
<th>PSA/PSM NEG, No.</th>
<th>PSA POS, No.</th>
<th>PSM POS, No.</th>
<th>PSA + PSM POS, No.</th>
<th>PSA/PSM POS, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1C</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>T2A</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>T2B</td>
<td>11</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>82</td>
</tr>
<tr>
<td>T2C</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>T3A</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>T3C</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>

*TNM classification system (14). POS = positive; NEG = negative; PSA = prostate-specific antigen; PSM = prostate-specific membrane antigen.
†Positive by pathologic analysis.
contamination of PCR products. In addition, the two negative-control samples, routinely included in our assays, remained free of cross-contamination with PSA or PSM products throughout the experiments. Similarly, the risk of false-positive results due to nonspecific expression of markers by nonprostate cells is unlikely in view of the 17 pathologically negative lymph node specimens that remained negative for both PSA and PSM RT–PCR signals in spite of the high sensitivity of the assays. Furthermore, the possibility of positive results arising from circulating tumor cells traversing the lymphatic circulation or the blood vessels of the pelvic lymph nodes or adjacent tissues was not supported by RT–PCR analysis of RNA from the peripheral blood mononuclear cells of 10 patients in this study. Only one of the 10 patients examined had detectable PSM-positive circulating cells at the time of the staging laparoscopic lymph node dissection (data not shown). This patient was among seven in the group with RT–PCR-positive and pathologically negative pelvic lymph nodes.

This study also illustrates the utility of using two distinct molecular markers to increase the detection of phenotypically heterogeneous tumor cells (20–22). Although there was a higher number of PSM-positive RT–PCR assays than PSA-positive assays in pathologically negative lymph nodes and patients with N0 disease (Table 2), this difference was not statistically significant (P = .11). Two patients, in fact, expressed PSA mRNA exclusively (Table 2). Therefore, both markers contributed independently to the detection of metastatic prostate cancer cells. With a larger cohort of patients, these markers may contribute to the detection of phenotypically separate subgroups with possible differences in outcomes and responses to treatment.

These data also suggest that patients should undergo a bilateral lymph node dissection. The patients with N0 disease in this study who had a unilateral dissection had only a 50% likelihood of a positive RT–PCR assay. This result contrasts with the 90% positive assays among the patients with a bilateral dissection. Thus, while the RT–PCR assays for PSA and PSM are highly sensitive in the detection of micrometastatic prostate cancer cells, adequate tissue specimens are required for accurate evaluation of the lymph node tissues.

In summary, the clinical value of these molecular indicators of metastasis remains to be established over time. Several criteria of our analysis suggest that a positive PSA or PSM RT–PCR assay most likely represents metastatic prostate tumor cells not detectable by light microscopy. These criteria include the sensitivity of our assay, the specificity of the PSA and PSM markers, the absence of false-negative assays, and the lack of evidence for false-positive results. Thus, the detection of PSA and PSM mRNA by the procedures described in this article may provide a more accurate estimate of the true incidence of prostate cancer cell micrometastases to the pelvic lymph nodes than currently utilized pathologic approaches.

Ultimately, the role of RT–PCR in detecting prognostically significant metastases needs to be tested prospectively through the analysis and follow-up of a larger cohort of patients who have different levels of risk of failure. If substantiated by correlation with systemic progression, this approach to molecular analysis of the pelvic lymph nodes may become an additional staging option for the majority of patients with clinically localized prostate cancer who are undergoing definitive therapy. In this way, a subgroup of patients with a higher risk of disease progression may be identified to test prospectively the potential of early systemic intervention to affect the rate of progression. Neoadjuvant/adjuvant hormonal therapy or novel therapeutic strategies, such as biologic response modifiers, tumor vaccines, or gene therapy, could be targeted at this measurable, residual tumor cell population.

References
