Circulating Prostate Tumor Cells Detected by Reverse Transcription–PCR in Men with Localized or Castration-Refractory Prostate Cancer: Concordance with CellSearch Assay and Association with Bone Metastases and with Survival

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BACKGROUND: Reverse transcription–PCR (RT-PCR) assays have been used for analysis of circulating tumor cells (CTCs), but their clinical value has yet to be established. We assessed men with localized prostate cancer or castration-refractory prostate cancer (CRPC) for CTCs via real-time RT-PCR assays for KLK3 [kallikrein-related peptidase 3; i.e., prostate-specific antigen (PSA)] and KLK2 mRNAs. We also assessed the association of CTCs with disease characteristics and survival.

METHODS: KLK3, KLK2, and PSCA (prostate stem cell antigen) mRNAs were measured by calibrated, quantitative real-time RT-PCR assays in blood samples from 180 localized-disease patients, 76 metastatic CRPC patients, and 19 healthy volunteers. CRPC samples were also tested for CTCs by an immunomagnetic separation system (CellSearch™; Veridex) approved for clinical use.

RESULTS: All healthy volunteers were negative for KLK mRNAs. Results of tests for KLK3 or KLK2 mRNAs were positive (≥80 mRNAs/mL blood) in 37 patients (49%) with CRPC but in only 15 patients (8%) with localized cancer. RT-PCR and CellSearch CTC results were strongly concordant (80%–85%) and correlated (Kendall τ, 0.60–0.68). Among CRPC patients, KLK mRNAs and CellSearch CTCs were closely associated with clinical evidence of bone metastases and with survival but were only modestly correlated with serum PSA concentrations. PSCA mRNA was detected in only 7 CRPC patients (10%) and was associated with a positive KLK mRNA status.

CONCLUSIONS: Real-time RT-PCR assays of KLK mRNAs are highly concordant with CellSearch CTC results in patients with CRPC. KLK2/3-expressing CTCs are common in men with CRPC and bone metastases but are rare in patients with metastases diagnosed only in soft tissues and patients with localized cancer.

For patients with prostate cancer, there is need for improved predictive markers to facilitate treatment selection and to monitor the effects of treatment. This need is particularly acute for patients with metastatic and/or castration-refractory disease. In these patients, the measured concentration of prostate-specific antigen (PSA) is only loosely associated with survival, bone scans offer only limited information on changes in the disease, and biopsy has poor sensitivity and is not clinically practical on a repeated basis. In addition, there is a need for improved preoperative staging modalities for patients with localized prostate cancer.

These problems have created interest in circulating tumor cells (CTCs) as a potential marker. The numerous techniques that have been used to detect CTCs can be categorized as techniques for detecting tissue- or disease-specific gene expression, such as reverse...
transcription–PCR (RT-PCR), and techniques for detecting CTCs as intact cells, such as flow cytometry and immunomagnetic capture. The one assay approved for clinical use in the US is CellSearch semiautomated immunomagnetic capture and detection (Veridex) (1). It has been hypothesized that a more sensitive method for CTC detection may be real-time RT-PCR of tissue-specific transcripts. End-point RT-PCR for molecular staging of prostate cancer was investigated with high expectations in the early 1990s (2, 3). The hope was that CTC detection might provide a more accurate way to preoperatively predict pathologic stage and the risk of disease recurrence. After initially showing promise, however, CTC-detection methods subsequently have produced often discrepant results, and most reports have failed to show clinical value (4 –11). Furthermore, the proportions of positive samples in different disease stages remain unclear. Frequencies of blood samples positive for prostate-specific RNAs have ranged from 0% to 81% in clinically localized disease and from 31% to 100% in metastatic disease (12, 13). Widespread inconsistencies in results may stem largely from differences in preanalytical sample processing, differences in analytical methods, poor standardization, and qualitative or semiquantitative detection of end-point RT-PCR products.

We have developed sensitive, highly reproducible, and fully standardized real-time quantitative RT-PCR assays for KLK3 (kallikrein-related peptidase 3; i.e., PSA) and KLK2 (human kallikrein 2) (14 –16). In this study, we compared this method to CellSearch for patients with castration-resistant prostate cancer (CRPC), and investigated the association of CTCs with disease characteristics and survival. We also investigated the frequency of KLK3- or KLK2-expressing CTCs in men with clinically localized prostate cancer. Finally, we describe a new real-time quantitative RT-PCR assay for the detection of PSCA (prostate stem cell antigen) mRNA. The PSCA gene is overexpressed in prostate cancer metastases (17), and our objective was to assess the frequency of PSCA-expressing CTCs in patients with CRPC.

Materials and Methods

PARTICIPANTS, SAMPLE COLLECTION, AND CellSearch ANALYSIS

The study enrolled 80 patients treated at Memorial Sloan-Kettering Cancer Center (MSKCC) for metastatic CRPC with castrate concentrations of testosterone (< 1.74 nmol/L). Radionuclide bone scans were reviewed for the presence or absence of metastatic bone disease, and computed tomography and/or MRI scans were evaluated for lymph node, liver, or lung soft tissue disease, or for epidural and prostatic/pelvic masses. Four patients without evidence of metastatic disease or castrate concentrations of testosterone were excluded. As controls, the study enrolled 19 healthy volunteers: 12 men younger than 40 years without prostate cancer and 7 women.

We enrolled 3 groups of patients with localized disease. The first consisted of 42 patients who had undergone radical prostatectomy (RP) at MSKCC at least 6 months before sample collection (median, 24 months; interquartile range, 6 – 80 months). Eleven patients in this group (26%) had pathologic stage pT3a, 4 patients (10%) had stage pT3b disease, and 27 patients had pT2 cancer with positive surgical margins (3 patients, 7%), capsular invasion (15 patients, 36%), or no adverse pathologic features (9 patients, 21%). The second group included 87 patients who were scheduled to undergo RP for clinically localized prostate cancer and whose RP sample subsequently showed at least one unfavorable feature, defined as seminal vesicle invasion, extracapsular extension or capsular invasion, positive surgical margin, or lymph node involvement. Thirty-two patients in the second group (37%) had pathologic stage pT3a, 9 patients (10%) had pT3b disease, and 2 patients (2%) were in stage pT4. The remaining patients had pT2 cancer with lymph node involvement (1 patient, 1%), positive surgical margin (7 patients, 8%), or capsular invasion (36 patients, 41%). The third group consisted of 51 patients with prostate cancer diagnosed at University Hospital of Hamburg (UKE), Germany, and who were scheduled to undergo either RP for clinically localized cancer or radiation therapy of the prostate. Six (23%) of the 26 pre-RP patients had a pathologic stage of pT3a, and 6 (23%) had pT3b disease. Fourteen patients had pT2 cancer, 2 (8%) with positive surgical margins and 12 (46%) with no adverse pathologic features. Biochemical recurrence was defined as having at least one serum PSA value > 0.4 ng/mL.

Peripheral blood (2.5 mL) was collected in PAXgene Blood RNA tubes (PreAnalytiX). Samples were incubated at room temperature for 24 h and stored at −20 °C and −80 °C until RNA isolation. For CRPC patients, a second sample of 7.5 mL was collected at the same visit in a CellSave tube (Veridex) and processed for CTC counts by CellSearch immunomagnetic selection as previously described (18). All samples were collected under institutional review board–approved protocols with informed consent.
RNA was isolated with the PAXgene Blood RNA kit (PreAnalytiX) according to the manufacturer’s instructions, including the optional DNase digestion. An internal calibrator RNA, m3PSA (see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue4) was added in predetermined amounts to each patient sample at the beginning of the RNA isolation (step 5 in the PAXgene RNA-isolation protocol) to produce 10,000 molecules/µL of the reverse-transcription reaction. The internal calibrator reflects any variation arising from the different steps of the RT-PCR protocol from RNA isolation to signal detection. RNA was quantified with the RiboGreen RNA-quantitation reagent (Invitrogen), immediately divided into 2 aliquots, and reverse-transcribed with High-Capacity cDNA Archive Kit (Applied Biosystems). RNA priming and reverse transcription are detailed in the online Data Supplement. In brief, an aliquot of RNA was reverse-transcribed with a mixture of sequence-specific RNA primers and anchored oligo(dT)12 primers to detect mRNA and internal calibrator RNA, and a second aliquot was reverse-transcribed with a mixture of random primers and anchored oligo(dT)12 primers for the detection of PSCA expression.

CALIBRATION CURVE
As an external calibration curve, we used calibrators containing varying amounts of KLK3 and KLK2 mRNA (2.5 to 10^4 mRNA copies/µL of the reverse-transcription reaction, corresponding to 160 to 6.4 × 10^5 copies/mL of blood) and a fixed amount of m3PSA RNA (10,000 RNA molecules/µL of reverse-transcription reaction). Calibrator RNAs were diluted into 100 ng/µL tRNA (Escherichia coli MRE 600 tRNA; Roche Applied Science) in sterile water. The external calibrators were analyzed along with all patient samples. In vitro production of RNA calibrators and data analysis have been described (19–21).

REAL-TIME RT-PCR METHODOLOGY
The real-time quantitative RT-PCR methodology for KLK3 and KLK2 mRNA has been described in detail (14–16, 19). In this study, 2.5 µL cDNA, representing 39 µL of blood, was used as template in 10-µL amplification reactions. All samples were run in duplicate. The mean within-assay variation (maximum difference between duplicate reactions) was <0.5 threshold cycles for the internal calibrator mRNA and <1 threshold cycle for KLK3 and KLK2 mRNAs.

RT-PCR ASSAY FOR PSCA mRNA
An internally calibrated real-time quantitative RT-PCR assay was developed for PSCA mRNA. The primer and probe sequences were designed to span the exon splice junctions; oligonucleotide sequences are shown in Table 1 in the online Data Supplement. The reporter probe (Thermo Fisher Scientific) had a 5’ aminolinker and a 3’ phosphate; it was terbium-labeled at the 5’ end and purified as described (16). The quencher probe was modified with a 3’ Dabcyl quencher moiety (Thermo Fisher Scientific). PCR reactions were run in duplicate with 5 µL of cDNA as template in 25-µL reactions, with reaction compositions and thermal cycling as described for KLK3 mRNA (15). Data were analyzed as previously described (22). The PSCA mRNA assay was performed on 71 of the 76 samples from CRPC patients.

STATISTICAL METHODS
A positive RT-PCR result was defined as ≥80 copies of target RNA per milliliter of blood in both PCR replicates, with at least 20% recovery of the internal calibrator. A positive CellSearch result was defined as ≥5 CTCs per 7.5 mL of blood sample.

We analyzed the relationship between KLK mRNA and CellSearch CTC results in 2 ways. First, we calculated the concordance: the proportion of patients categorized as either both positive or both negative in KLK mRNA and CellSearch assays. Second, we considered the KLK mRNA and CTC results as continuous variables and calculated the Kendall τ to estimate correlation.

We tested the association of a positive RT-PCR result to clinical characteristics of the CRPC patients: presence or absence of biochemical progression at the time of research blood draw, location of metastasis, and overall survival. The probability of survival after the time of blood draw was estimated with Kaplan–Meier methods. Univariate associations of PSA, log CTC count, log KLK2 mRNA, and log KLK3 mRNA were evaluated with Cox proportional hazards regression models. Predictive accuracy is given by the concordance probability estimate (CPE). The CPE measures the level of concordance between the survival time and the prognostic index as determined by the Cox model. The CPE ranges between 0.5 and 1.0, with 1.0 representing perfect concordance between the prognostic index and survival time, and 0.5 representing absence of a relationship between prognostic index and survival time [for further information on the CPE, see (23)]. Owing to the limited follow-up, we were unable to compare actual recurrence outcomes for the patients with localized disease. We instead used the postoperative nomogram probability as a surrogate for recurrence outcomes. The postoperative nomogram probability (24) was computed for all patients with locally advanced disease who underwent RP (42 patients with postoperative blood samples and 87 patients with...
preoperative blood samples from MSKCC; 26 patients with preoperative blood samples from UKE), and the Mann–Whitney $U$-test was used to compare probabilities for patients positive for KLK3 or KLK2 mRNA with those for patients negative for both KLK3 and KLK2 mRNAs.

Results

CHARACTERISTICS OF THE CRPC PATIENTS
The clinical characteristics of the 76 CRPC patients included in the study are detailed in Table 2 in the online Data Supplement. At the time of research blood draw, 60 (79%) of the patients had increasing PSA concentrations under androgen-depletion therapy. Metastases were limited to soft tissue in 9 patients (12%), limited to bone in 26 patients (34%), and in both soft tissue and bone in the remaining 41 patients (54%). The median serum PSA concentration at the time of research blood draw was 111 ng/mL (interquartile range, 31–433 ng/mL).

CONCORDANCE AND CORRELATION OF CellSearch CTC COUNTS WITH KLK AND PSCA mRNAs
The KLK3 mRNA status was positive in 27 (36%) of the 76 patients with CRPC, and the KLK2 mRNA status was positive in 32 patients (42%). Results for one or both KLK mRNAs were positive in 37 patients (49%). In contrast, results for both KLKs were negative in all 19 healthy volunteers. The CellSearch CTC status was positive in 31 (48%) of the 65 CRPC patients with evaluable results. The median CTC count was 4 cells per 7.5 mL of blood (interquartile range, 1–31 cells). Thirty-one patients (48%) had $\geq$5 CTCs, and 24 (37%) had $\geq$10 CTCs. A positive status for PSCA mRNA occurred at much lower frequency (10%, 7 of 71 patients tested). Six of the 7 patients positive for PSCA mRNA were also positive for one or both KLK mRNAs.

Table 1 summarizes the concordance between the KLK mRNA and CellSearch CTC data. The status for either KLK3 or KLK2 mRNA was concordant with CellSearch CTC counts in 82% of the CRPC patients. Of the 34 patients with a negative CTC status ($\leq$5 cells), 7 (21%) had detectable KLK3 or KLK2 mRNA. To assess the level of confidence that KLK mRNA is a marker of CTCs, we computed the CI that a CTC-positive patient was also positive for KLK mRNA (i.e., sensitivity of KLK mRNA for detecting CTCs). The sensitivity of both KLK3 and KLK2 mRNA was 74% (95% CI, 55%–88%), and that of either KLK3 or KLK2 mRNA was 84% (95% CI, 66%–95%). Because CTCs are given per 7.5 mL of blood and 5 CTCs therefore correspond to only 0.67 cells/mL, we also evaluated the level of confidence that KLK mRNA was a marker of 2 CTCs/mL (i.e., $\geq$15 CTCs per 7.5 mL blood). The sensitivities were 82% (18 of 22 patients; 95% CI, 60%–95%) for KLK3 mRNA, 91% (20 of 22 patients; 95% CI, 71%–99%) for KLK2 mRNA, and 95% (21 of 22 patients; 95% CI, 77%–100%) when either KLK3 or KLK2 mRNA was considered. We note that this sensitivity was achieved with a much smaller volume of blood than is used in the CellSearch assay (39 mL of blood per PCR reaction). These results are also consistent with prior data showing that the KLK assays are sufficiently sensitive to detect $<2$ LNCaP cells per milliliter of blood in a background of $10^7$ nucleated cells (or 160 mRNA copies/mL of blood) (15).

<table>
<thead>
<tr>
<th>Patients, n</th>
<th>Concordance</th>
<th>Kendall $\tau$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CellSearch CTCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLK3 RNA</td>
<td>85%</td>
<td>0.68</td>
</tr>
<tr>
<td>Negative</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>KLK2 RNA</td>
<td>80%</td>
<td>0.60</td>
</tr>
<tr>
<td>Negative</td>
<td>29</td>
<td>8</td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>KLK3 and KLK2</td>
<td>82%</td>
<td>NT*</td>
</tr>
<tr>
<td>Negative</td>
<td>27</td>
<td>5</td>
</tr>
<tr>
<td>Either KLK positive</td>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td>KLK2 RNA</td>
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</tr>
<tr>
<td>KLK3 RNA</td>
<td>80%</td>
<td>0.66</td>
</tr>
<tr>
<td>Negative</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

* NT, not tested.

ASSOCIATION WITH DISEASE CHARACTERISTICS
A positive status for KLK3 and KLK2 mRNAs was found only in patients who had clinical evidence of bone metastases; one or both KLK mRNAs were positive in 13 patients (50%) with bone metastases alone, in
24 patients (59%) with bone and soft tissue metastases, and in none of the patients with soft tissue metastases alone (Fig. 2; see Table 3 in the online Data Supplement). Similarly, a positive CellSearch result was more frequent in patients with bone metastases. PSA concentration in serum (measured within 3 days of collecting blood for CTC analyses) was modestly correlated with KLK mRNA copy number and with CellSearch CTC count. Kendall $\tau$ coefficients of correlation were 0.43 for serum PSA and KLK3 mRNA, 0.35 for serum PSA and KLK2 mRNA, and 0.42 for serum PSA and CTC counts (all $P < 0.001$).

A positive status for KLK mRNAs and for CTC count also varied with the number of systemic therapies administered to the patients. The frequency of patients positive for either KLK mRNA was higher in patients after failure of treatment with multiple chemotherapeutic regimens (61%, 11 of 18 patients) compared with patients with first-line chemotherapy (45%, 9 of 20 patients) or only hormonal therapy (42%, 16 of 38 patients). Interestingly, this effect was even more pronounced for CellSearch CTC results, which were positive for 10 (71%) of 14 patients with failure of multiple regimens, 10 (53%) of 19 patients who received first-line chemotherapy, and 11 (34%) of 32 patients who received only hormonal therapy.

ASSOCIATION WITH SURVIVAL

Of the 76 CRPC patients, 34 died during follow-up, with a median time to death of 17 months. The median follow-up for survivors was 14 months. Kaplan–Meier survival estimates show that survival times were...
shorter for patients who tested positive for \( KLK \) mRNA (Fig. 3). The corresponding plot constructed for the 60 patients with evidence of disease progression at the time of blood draw was essentially identical (data not shown). A univariate analysis indicated that \( KLK2 \) mRNA, \( KLK3 \) mRNA, CTC count, and serum PSA concentration were all highly associated with survival (all \( P < 0.001 \); Table 2). We constructed several multivariable models with these variables (Table 2) and included only patients with available PSA measurements and CTC counts (\( n = 60 \)) to facilitate comparisons between models. The predictive accuracy of serum PSA alone as assessed by the CPE was 0.728. The inclusion of \( KLK3 \) mRNA produced similar results (0.726), but the CPE increased to 0.749 and 0.765 with the inclusion of \( KLK2 \) mRNA or CellSearch CTC counts, respectively. The full model (PSA + CTCs + \( KLK2 + KLK3 \) had a CPE of 0.759, which was similar that of the model that included PSA and CTC counts only.

**KLK mRNAs IN LOCALIZED DISEASE**

Either or both \( KLK3 \) and \( KLK2 \) mRNAs were detected in only a small proportion of the patients with localized disease. Tables 4 and 5 in the online Data Supplement summarize patient characteristics according to \( KLK \) mRNA status. Of the patients treated at MSKCC, one or both \( KLK \) mRNAs were detected in 6 (14%) of the 42 patients with samples collected after RP and in 6 (7%) of the 87 patients with samples collected before RP. Results were similar for the 51 pretreatment samples

![Fig. 3. Kaplan–Meier survival probability according to KLK mRNA status for the 76 patients with CRPC.](image)

**Table 2. Univariate analysis of associations with survival by Cox proportional hazards regression and predictive accuracy of univariate and multivariable models predicting survival.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients, n</th>
<th>Hazard ratio</th>
<th>95% Cl</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log PSA</td>
<td>69</td>
<td>1.67</td>
<td>1.30–2.13</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Log CTC</td>
<td>65</td>
<td>1.29</td>
<td>1.16–1.45</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Log ( KLK2 ) mRNA</td>
<td>76</td>
<td>1.16</td>
<td>1.09–1.25</td>
<td>0.0007</td>
</tr>
<tr>
<td>Log ( KLK3 ) mRNA</td>
<td>76</td>
<td>1.18</td>
<td>1.10–1.26</td>
<td>&lt;0.0005</td>
</tr>
</tbody>
</table>

**Model**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients, n</th>
<th>Hazard ratio</th>
<th>95% Cl</th>
<th>CPE (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>69</td>
<td>0.728 (0.042)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( KLK2 ) mRNA</td>
<td>69</td>
<td>0.670 (0.032)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( KLK3 ) mRNA</td>
<td>69</td>
<td>0.688 (0.028)</td>
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</tr>
<tr>
<td>PSA + ( KLK2 ) mRNA</td>
<td>76</td>
<td>0.749 (0.036)</td>
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<tr>
<td>PSA + ( KLK3 ) mRNA</td>
<td>76</td>
<td>0.726 (0.041)</td>
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<tr>
<td>PSA + ( KLK2 ) mRNA + ( KLK3 ) mRNA</td>
<td>76</td>
<td>0.741 (0.034)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTC</td>
<td>76</td>
<td>0.718 (0.029)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA + CTC</td>
<td>76</td>
<td>0.765 (0.033)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA + CTC + ( KLK2 ) mRNA + ( KLK3 ) mRNA</td>
<td>76</td>
<td>0.759 (0.036)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Calculated for the subset of 60 CRPC patients with PSA and CTC data to facilitate comparisons between models.
from UKE; only 3 patients (6%) were positive for KLK mRNA.

KLK mRNA status showed no apparent association with unfavorable localized disease features (see Tables 4 and 5 in the online Data Supplement). There was no significant difference in the postoperative nomogram probability of local recurrence between patients positive for KLK3 or KLK2 mRNA and patients negative for both mRNAs (P = 0.3 for MSKCC preoperative; P = 0.6 for MSKCC postoperative; and P = 0.6 for UKE preoperative). Furthermore, there was no association between a positive status for KLK mRNAs and the time from the last biopsy or other prostate manipulation (such as transurethral prostatectomy) to research blood draw (data not shown).

Discussion

RT-PCR has been used extensively as a means of detecting circulating prostate tumor cells, but it has yet to become accepted, in part because of discrepant results. Our approach has been to use internally and externally calibrated quantitative real-time RT-PCR, extensively optimized assay conditions, and sample-collection procedures that help to preserve the true RNA profile. With this method, we have shown high correlation and concordance between results obtained with our real-time RT-PCR assays and those obtained with an independent assay for CTCs (CellSearch) in patients with metastatic CRPC. Real-time RT-PCR assays were able to detect KLK3 or KLK2 mRNAs in ≥95% of samples that had 15 or more CTCs per 7.5 mL of blood according to the CellSearch assay. CellSearch has been approved by the US Food and Drug Administration for predicting progression-free and overall survival in metastatic breast cancer (1, 25–27) and has recently been approved for use in advanced prostate cancer. Cells isolated via CellSearch technology from patients with progressive CRPC have molecular features of malignant prostate epithelial cells (18). The high concordance between KLK mRNA and CellSearch CTC results suggests that both methods target the same cell population and that real-time RT-PCR assays targeting KLK3 and KLK2 mRNAs reliably detect CTCs in the majority of men with metastatic prostate cancer.

The proportion of patients with metastatic CRPC positive for KLK3 or KLK2 mRNA was approximately 50%. In contrast, few patients were positive for another androgen receptor–responsive gene, PSCA, although a PSCA-positive status was highly associated with a positive status for KLK mRNAs.

Although the overall correlation between KLK and CTC results is high, the scatter plots suggest the possibility of a distinct subpopulation of patients who shed CTCs with high KLK mRNA copy numbers that escape detection by the CellSearch assay. Approximately 20% of patients with <5 CTCs per 7.5 mL of blood had detectable KLK3 or KLK2 mRNA. Two of these patients had 0 CTCs, 2 had 1 CTC, and 3 had 2–4 CTCs per 7.5 mL of blood. No healthy volunteers had a KLK mRNA signal, implying that these transcripts are limited to CTCs and that the detection of such prostate-specific transcripts in blood is indicative of tumor cell dissemination. In addition, very few patients with localized prostate cancer were positive for KLK3 or KLK2 mRNA. Hence, samples that are positive for KLK mRNA but negative by the CellSearch assay cannot be explained by a compromised specificity; instead, we hypothesize that these CTCs were too few or lacked cell surface markers for detection by the CellSearch assay. Conversely, some CRPC patients shed CTCs with very little or no detectable KLK mRNAs. This finding may reflect therapeutic repression of androgen receptor function. From the scatter plots it is evident that the numbers of KLK2 and KLK3 mRNAs per CTC vary among CRPC patients. These numbers, which are derived from combining RT-PCR data with CellSearch CTC data, might prove informative with respect to androgen receptor function, which in turn may relate to critical disease characteristics, treatment options, and outcome. Determining the clinical value of this information will require further studies of KLK mRNA status in CTCs in a large cohort of patients with advanced cancer.

CTCs were more frequent in patients who had undergone 2 or more chemotherapy regimens than in those with fewer systemic treatments. This effect was stronger for CellSearch CTCs than for KLK mRNAs. This result may possibly be attributable to the tendency of androgen receptor function, and therefore KLK2 and KLK3 expression as well, to diminish in more advanced disease. In patients with only hormonal therapy, KLK mRNA analysis appears to be the more sensitive assay. Higher CTC numbers have previously been reported in patients receiving second-line therapy (28).

CTCs, whether detected by KLK mRNAs or by CellSearch, were very strongly associated with diagnosis of bone metastasis in the CRPC patients. Metastatic disease was confirmed by bone scans and soft tissue imaging, which, although not perfectly accurate, are the standard methods for diagnosing prostate cancer metastases. Notably, none of the patients who had soft tissue metastasis alone had a detectable KLK mRNA signal, although the number of patients was small. A similar trend was observed in a study that used CellSearch technology with a larger patient population (28). This association with the specific site of metastases, along with the weakness of the correlation of KLK
RT-PCR and CellSearch results with the serum PSA concentration, indicates that these assays can provide distinct information that is not related simply to increased tumor burden.

KLK mRNA and CellSearch CTC results were both strongly associated with survival. The shorter survival time for KLK-positive patients also held among the patients whose disease was progressing, suggesting that analysis of KLK mRNAs will have utility in this subset of patients. The accuracy of serum PSA concentration for predicting survival was enhanced with the addition of KLK mRNAs and CellSearch results, and the full model with all these variables had a predictive accuracy of 0.759. This result was similar to that of a model that included only PSA and CellSearch results (0.765) and was similar to a published model that includes PSA, CellSearch results, and albumin (28).

A limitation of the survival analysis is that because of the cohort size, we were unable to test whether KLK mRNA and CellSearch results were associated with survival independent of potential confounding factors. One such factor could be patients’ disease state as reflected by the history of chemotherapy. CTCs were more frequently found in patients who had experienced treatment failure with chemotherapy, a group that might be expected to have shorter survival times than chemotherapy-naive patients. In another study, however, CellSearch CTC results were associated with survival independent of other prognostic factors, including number of prior chemotherapies (29). Testing whether this is also the case for KLK mRNA results will require a larger study.

Our RT-PCR results imply a very low frequency of CTCs in patients with clinically localized disease, even among those with adverse pathologic features. Davis et al. used CellSearch and also found a low CTC frequency in men with early prostate cancer (3 of 97 patients with ≥3 CTCs per 22.5 mL of blood) (30). In that study, however, CTCs at similar frequency were detected in men without prostate cancer, whereas no CTCs were detected in healthy individuals with our RT-PCR methodology. The very low frequency of CTCs in patients with localized disease appears consistent with the close association of CTC counts with skeletal metastases revealed in our RT-PCR assays. This low frequency of CTCs also suggests that large studies with extended follow-up will be required to reliably assess whether the detection of CTCs before treatment is associated with systemic disease (e.g., bone metastases) or more generally with a worse outcome. Because of the limited follow-up time in this study, we were unable to analyze the actual recurrence outcomes of the patients with localized disease.

The Gleason score still provides the gold standard for assessing prostate cancer aggressiveness at diagnosis. Gleason score, however, cannot be easily assessed repeatedly over the course of the disease, and because prostate cancer has a highly variable natural history, current information on the patient’s disease is required for optimal targeting of therapies. Hence, there is need for predictive markers that can be easily assessed repeatedly. This study has shown that KLK mRNA assays and CellSearch CTC assays provide prognostic information and enhance the predictive accuracy of serum PSA alone in patients with CRPC. The sample material for CTC assays is readily obtainable by standard venipuncture, and real-time quantitative RT-PCR is currently one of the most sensitive methods for detecting CTCs. The concordance between RT-PCR and CellSearch methods, albeit in a small patient population, provides proof of concept that these approaches may be equally valid for detecting disseminated prostate tumor cells. Moreover, the approaches are complementary: CellSearch enables intact CTCs to be counted and characterized by fluorescence in situ hybridization and immunohistochemistry, and KLK mRNA assays provide sensitive and quantitative detection of CTC-specific gene expression. The pathobiologic mechanisms that contribute to the shedding of these cells remain to be defined, however, and so investigation continues to define patient groups in which CTC detection has the most clinical potential.
Circulating Tumor Cells in Localized and Metastatic Prostate Cancer

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