Circulating Tumor Cells in Patients with Castration-Resistant Prostate Cancer Baseline Values and Correlation with Prognostic Factors

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Abstract

Purpose: **Circulating tumor cells (CTC) have been recently accepted by the Food and Drug Administration of the United States as a prognostic tool in advanced prostate cancer. However, a number of questions remain about the use of the test. The optimal clinical cut-off has never been determined. Also, the predictive value of CTCs in the setting of low-burden advanced prostate cancer has not been evaluated. Herein we describe our experience with the CellSearch method of CTC enumeration.**
Experimental Design: **CTCs enumerated from 100 patients with castration-resistant prostate cancer were correlated with clinicopathologic characteristics and conventional biomarkers, such as prostate-specific antigen and lactate dehydrogenase. Patients received ongoing medical oncologic follow-up for up to 26 months, and overall survival status was documented.**
Results: **Forty-nine of the patients (49%) were alive at the end of the study. CTC counts correlate well with overall survival (P < 0.001) but are also tightly interrelated to other biomarkers. Threshold analysis identified 4 CTC/7.5 cc (compared with the approved value of 5) as an optimal cut-off value with respect to correlation with survival outcomes as well as predictive of metastatic disease. Univariate analysis confirmed a tight interrelationship between cut-off CTC values and biomarkers. Multivariate analysis with bootstrap sampling validation identified lactate dehydrogenase (P = 0.002) and CTCs (P = 0.001) as independently prognostically significant.**
Conclusions: **Baseline CTC values provide important prognostic information and specific prediction of metastatic disease. Their presence correlates with classic biomarkers.** (Cancer Epidemiol Biomarkers Prev 2009;18(6):1904–13)

Introduction

Prostate cancer is the most common nondermatologic malignancy in men and the second leading cause of cancer-related death in the United States, with an incidence of 186,320 new cases and 28,660 deaths due to carcinoma of the prostate in 2008 (1). Most patients succumbing to prostate cancer have castration-resistant prostate cancer (CRPC). Halabi et al. and members of the Cancer and Leukemia Group B have extensively analyzed a variety of prognostic factors in >1,200 patients with CRPC entered prospectively into clinical trials. These include pain interference scores, performance status, age, prostate-specific antigen (PSA), lactate dehydrogenase (LDH), and Gleason score (2). These factors can be combined into nomograms that are clinically useful but have not yet been validated prospectively. This lack of a prospectively validated surrogate end point for survival has hampered the testing of new therapies. Exhaustive retrospective analysis has shown the prototypical biomarker, PSA, has limited utility as a survival surrogate in CRPC (3). The utility of radiographic response as a clinical end point is extremely limited as well. Bone scans are notoriously unreliable because the delayed kinetics of bone remodeling after cancer therapy has been largely supplanted by PSA (4). Measurable disease in CRPC is present in <25% of patients and thus limits the usefulness of radiographs in monitoring clinical response (5).

Recently, circulating tumor cells (CTC) have been proposed as a new prognostic and predictive factor in CRPC. The significance of CTCs in prostate and other cancer patients was first reported by Moreno et al. with the use of PCR to detect PSA-positive cells in the circulation of patients undergoing radical prostatectomy (6). Subsequent investigators reported that the reverse transcription-PCR detection of cells in the circulation of patients undergoing a radical prostatectomy (7) or those with metastatic prostate cancer (8) portended a worse prognosis. In 2003, the Cancer and Leukemia Group B reported on a large prospective trial showing that patients with circulating PSA-positive tumor cells detected by reverse transcription-PCR had a median survival of 11 months, compared with 21 months for those with no detectable cells (P = 0.001; ref. 9). The technique was not used in other trials and, thus, its

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prognostic importance could not be prospectively validated. The technique was then refined to detect CTCs by the use of antibodies against epithelial cell surface antigens, such as EpCAM. By linking such antibodies to ferrous particles, the cells could be separated and detected with the use of high-strength magnetic fields and detection devices. This CTC detection system was commercialized and standardized by Immunicon/Veridex, LLC.

With the use of that device and technique, investigators reported that large numbers of cells could be detected in the circulation of patients with lung, breast, colon, and prostate cancer (10). In breast cancer patients, Cristofanelli et al. then showed that ≥5 CTC/7.5 cc of blood was a poor prognostic finding (11, 12). Budd et al. reported that a decline in circulating breast cancer cells 4 weeks after initiation of systemic therapy correlated with a subsequent radiographic response, (13) suggesting that CTCs represented an early surrogate for predicting response. CTCs are present in approximately one third of all patients with colorectal carcinoma (14) and correlate with disease stage, with the majority (60.7 %) of stage IV colorectal carcinoma patients presenting with detectable CTCs (15). In January 2004, the Food and Drug Administration approved the devise for monitoring of breast cancer and, in November 2007, for monitoring colon cancer therapy (16).

In metastatic prostate cancer, Schaffer et al. reported that 65% of CRPC patients had >5 CTC/7.5 cc, with a median of 16 CTC/7.5 cc of blood, (17) and reported in preliminary fashion that the CTCs could be probed for androgen reception and epidermal growth factor receptor overexpression. These findings suggested that the circulating cells could also be used to sample the biology of the cancer.

Recently, deBono et al. (18) reported on 231 patients in whom a baseline CTC count ≥5 cells/7.5 cc before the start of the first (67% of patients, the majority of whom were receiving docetaxel therapy) or subsequent (33 % of patients) lines of chemotherapy was associated with a significantly shorter median overall survival compared with those with <5 cells (11.5 months versus 21.7 months; P <.0001). The kinetics of response to chemotherapy were rapid; a decline of the CTCs to <5 at 2 to 5 weeks post therapy had a dramatic effect on prognosis, with a median overall survival improved to 20.7 months compared with 9.5 months for those whose CTCs did not decline. The predictive ability of CTCs was shown to be superior to that of PSA. These collective findings led to the approval of CTCs by the Food and Drug Administration in February 2008 for monitoring prostate cancer therapy (18).

Despite these recent advances, a number of questions remain about the use of CTC monitoring in patients with CRPC, such as the relationship to other well-defined prognostic factors (i.e., the Halabi nomogram, its use in the absence of metastatic disease, its role in hormone-sensitive metastatic disease, its cost-effectiveness, as well as the optimal cut-off value, which has been by convention set at 5 cells/7.5 cc, the value used for breast cancer patients; ref. 11). The Nevada Cancer Institute received a Veridex device in January 2007, and we now present the experience with the test in our first 100 patients with CRPC. We report that a baseline CTC count ≥4 cells/7.5 cc blood provides important prognostic information and is a highly specific indicator of metastatic disease yet may be predicted by classic biomarkers, such as PSA, LDH, and alkaline phosphatase.

Materials and Methods

Patients. From January 2007 through August 2008, blood was collected from 100 patients with histologically proven CRPC. All samples were collected and processed at the Nevada Cancer Institute under protocols approved by the Institutional Review Board and with written informed consent. Castration-resistant disease was defined as a documented castrate testosterone within 3 mo with disease progression by PSA working group criteria (19) or radiographic progression while on androgen deprivation therapy with a gonadotropin-releasing hormone agonist or after prior orchiectomy. Patient clinical information was linked to the CTC information with the use of the Mosaiq (Elekta AB) electronic medical record system. The date of diagnosis was defined as the date on the first pathology report showing prostate cancer or the date of radical prostatectomy. The Gleason sum was that recorded on the radical prostatectomy pathology report or on the transrectal ultrasound–guided biopsy (if a radical prostatectomy was not done). The dates of radical prostatectomy and of PSA failure were recorded. The latency period was defined as the number of days between the date of diagnosis and the date of first CTC sampling. The pathologic stage or, if not available, the clinical stage at diagnosis was recorded per American Joint Committee on Cancer 2002 tumor-node-metastasis criteria as stages I to IV. Metastatic disease status was abstracted from clinical data and coded as bone involvement based on bone scan data or as soft tissue involvement based on the presence of lymph node or visceral metastasis (lung, liver, or other) on the most recent computed tomography scan. The number of prior lines of hormonal therapy and chemotherapy was recorded for each patient at the time of the CTC draw. A gonadotropin-releasing hormone agonist and orchiectomy were by convention considered a single line of hormonal therapy, with antiandrogen or estrogen therapy considered additional lines. Chemotherapy was defined as a standard cytotoxic therapy and did not include newer targeted agents.

CellSearch CTC Assay and Biomarker Collection. A single 7.5 mL tube of blood was collected into CellSave tubes (Immunicon) at each visit for automated immunomagnetic isolation and immunofluorescent staining of CTCs. The methodology for automated immunomagnetic selection of CTCs, based on capture with an anti–epithelial cell adhesion molecule antibody and immunofluorescent staining and analysis, has been previously described (14). In short, samples were drawn in tubes containing cell preservatives, maintained at room temperature, incubated with epithelial cell adhesion molecule antibody–covered ferroparticles at room temperature, and processed on the CellTracks AutoPrep (Immunicon) system. Circulating epithelial cells expressing epithelial cell adhesion molecules were isolated by a magnetic field without centrifugation. After the supernatant containing unbound cells was removed, the

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enriched samples were processed for fluorescent staining. Nucleic acids were stained with 4′,6-diamidino-2-phenylindole, and epithelial cells were stained with anti–cytokeratin-phycoerythrin. Leukocytes were excluded with an allophycocyanin-conjugated anti-CD45 antibody as previously described (12, 14). Stained cells were analyzed on a fluorescence microscope with the use of the CellTrack Analyzer II (Immunicon). Automatically selected images were reviewed by the operator (B.G.) for identification and counting of CTCs, which were defined as cytokeratin-positive and 4′,6-diamidino-2-phenylindole–positive nucleated cells lacking CD45. All findings were reviewed and confirmed by the pathologists (Y.M. and L.F.). Quality control was maintained via standard procedures. Patients also had routine clinical prostate cancer biomarker monitoring consisting of PSA, testosterone, LDH, prostatic acid phosphatase, total acid phosphatase, alkaline phosphatase, and complete blood counts (hemoglobin, platelet count, and WBC) generally drawn simultaneously or within 48 h of the CTC draw.

Study Design. After the baseline draw, patients were followed through the end of the study period, or until death, during which time they received either standard therapies: hormonal therapies or chemotherapy, or treatment on clinical trials. The biomarkers, PSA, LDH, prostatic acid phosphatase, total acid phosphatase, alkaline phosphatase, hemoglobin, platelet count, and WBC counts, were evaluated as continuous variables. At the end of the study period, survival outcome was correlated with baseline CTC results. Overall survival was defined as the date of first CTC sample to the date of death. Locked databases containing the biomarker and clinical data were reviewed independently by the first author (O.G.) and the biostatistician (J.S.).

Statistical Design. CTCs were correlated with all continuous variables by determining the Spearman rank correlation coefficients. For categorical variables, the rank transformation of CTCs was analyzed with the use of a one-factor ANOVA. The statistical significance of the categorical variables was tested with the ANOVA F-test. Pairwise comparisons between subgroups were tested with the Tukey-Kramer test, which controls the nominal type I error rate.

Overall survival was analyzed with the use of Kaplan-Meier techniques to estimate survival rates and generate the survival curve. Univariate Logistic regression analysis was conducted to assess the individual prognostic importance of the baseline biomarkers. Significant ($P < 0.05$) individual prognostic factors were included in a Cox multiple regression analysis, and backward elimination was used to identify independent prognostic factors. The criterion for staying in the model was $P < 0.05$. Log_{10} transformations of biomarkers with highly skewed distributions were included in the analyses. To assess the robustness of the final model, 2,000 bootstrap samples were randomly selected from the full dataset (20, 21). Random sampling was done with replacement, and each sample contained 100 observations. For each sample, the final model selection as described above was determined. The frequency and percentage out of 2,000 samples for inclusion in the final model were calculated for each biomarker.

Threshold analysis to determine optimal CTC cut-offs was done for both metastatic disease status (any versus none) and survival. The optimal cut-off was defined as the lowest CTC value maximizing the sum of sensitivity and specificity. A univariate Cox regression analysis was used to correlate biomarkers with the presence of any and 4 CTCs, corresponding to the metastatic disease and survival cut-offs (see Results for details). For survival prediction, the CTC cut-off analysis was replicated 2,000 times with the use of bootstrap resampling as described above. For each CTC value, the frequency it was the optimal cut-point was calculated. Unless otherwise noted, all references to statistical significance correspond to an $\alpha = 0.05$ significance level.

Results

Patient Characteristics and Correlation of Biomarkers with CTC Counts. From January 2007 through August 2008, 100 patients with CRPC consented to an analysis of baseline CTCs and biomarkers, and were followed through the end of the study period with respect to survival outcome. The patient demographics at the time of study entry are presented in Table 1A. The median age was 71 years, and the median time from diagnosis to study entry, or latency period, was nearly six years. Approximately 50% of patients presented with metastatic disease at original diagnosis. Whereas the majority of the patients were chemotherapy naïve (58%), 20% had two or more lines of chemotherapy. Metastatic disease was present in 84%, whereas 16% were CRPC but had no radiographic evidence of metastatic disease. The clinicopathologic characteristics at the time of prostate cancer diagnosis was documented (Table 1B). Forty-seven percent of the patients had a Gleason sum of ≥8. The baseline biomarkers were tabulated in Table 1C (serologic) and Table 1D (blood counts). The median baseline PSA was 23.6 ng/mL (range, 0-4,469).

Spearman rank correlation coefficients for CTCs were calculated for all continuous variables in Table 2A. Alkaline phosphatase, PSA, LDH, total acid phosphatase, and prostatic acid phosphatase all correlated positively with CTCs. Negatively correlating variables included hemoglobin ($P < 0.0001$), WBC count, age at baseline draw ($P = 0.02$), and latency period ($P = 0.003$). PSA at diagnosis, and baseline albumin, glucose, platelets, and bone-specific alkaline phosphatase failed to correlate with CTCs. All variables with statistical significance were analyzed with scatter plots (Fig. 1). For LDH, alkaline phosphatase, and prostatic acid phosphatase, a biphasic relationship with CTCs was observed, becoming elevated at a certain CTC threshold. Other variables, such as total acid phosphatase and hemoglobin, were linearly related to the CTC log.

Next, we did ANOVA on the categorical variables (Table 2B). Although there was a trend for increasing median CTC count both for total and high primary tumor Gleason score, this was not statistically significant. Stage at diagnosis and the type of definitive therapy had no correlation with baseline CTC number. The number of lines of chemotherapy (Fig. 2A) and the presence of metastatic disease proved to correlate most closely with CTC counts (Fig. 2B). Race correlated with differences in CTCs, with Hispanic patients having higher median CTC

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counts than Asian patients, but this difference was based on small numbers of patients.

We further assessed whether radiographically detectable site of disease correlated with baseline CTC number (Fig. 2C). There was a trend towards higher median CTC counts in the presence of bony metastatic disease when compared with soft tissue–only disease. Threshold analysis of each integral CTC cut-off was done (data

Table 1.

A. Baseline patient demographics

Variable	*n*	Categories	Values
Age at diagnosis (y)	99	Mean ± SD	64 ± 8
		Median (range)	64 (40-85)
Age at baseline draw (y)	100	Mean ± SD	71 ± 9
		Median (range)	71 (49-91)
Latency period (y)	99	Mean ± SD	6.5 ± 5.2
		Median (range)	5.6 (0.23-21)
Race	100	African American	5 (5%)
		Asian American	4 (4%)
		Hispanic	5 (5%)
		Caucasian	86 (86%)
Definitive therapy	100	Radical prostatectomy	24 (24%)
		Radiation therapy	25 (25%)
		None	49 (49%)
		Unknown	2 (2%)
Lines of hormonal therapy	100	Unknown	2 (2%)
		1	10 (10%)
		2	50 (50%)
		≥3	38 (38%)
		Range	1-5
Lines of chemotherapy	100	0	58 (58%)
		1	22 (22%)
		2	11 (11%)
		≥3	9 (9%)
		Range	0-6
Metastatic disease	100	None	16 (16%)
		Bone only	47 (47%)
		Soft tissue only	12 (12%)
		Bone + soft tissue	25 (25%)

B. Primary tumor characteristics

Variable	*n*	Categories	Values
Gleason sum	100	Unknown	14 (14%)
		≤6	11 (11%)
		7	28 (28%)
		≥8	47 (47%)
AJCC stage at diagnosis	100	Unknown	21 (21%)
		Stage I	0 (0%)
		Stage II	19 (19%)
		Stage III	14 (14%)
		Stage IV	46 (46%)
PSA at diagnosis (ng/mL)	78	Mean ± SD	299 ± 1216
		Median (range)	17.5 (1.9-9,300)

C. Baseline biomarkers

	Biomarker							
	CTC (per 7.5 cc)	PSA (ng/dL)	LDH (U/L)	AP (IU/L)	Acid phosphatase		Albumin (g/dL)	Glucose (g/dL)
					TAP (U/L)	PAP (ng/mL)		
Median	4	23.6	181	92	7.6	4.1	4.0	103
Range	0-2,572	0-4,469	101-2,240	24-1,196	3.9 to ≥70	0.4 to ≥500	2.9-4.9	72-482
N	100	100	96	98	77	80	97	97

D. Baseline blood counts

Variable (units)	Lineage		
	Leukocytes (10^3 cells/mm^3)	Hemoglobin (g/dL)	Platelets (10^3 cells/mm^3)
Median	6.1	12.0	237
Range	1.7-17.1	7.1-15.4	25-487
N	90	90	90

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Table 2.

A. Spearman coefficients for continuous biomarker–CTC correlation

Biomarker	n	Spearman coefficient	P
AP	98	0.71	<0.001
Bone-specific AP	20	0.35	0.134
PSA	100	0.58	<0.001
LDH	96	0.62	<0.001
PAP	80	0.65	<0.001
TAP	77	0.60	<0.001
Albumin	97	-0.18	0.077
Glucose	97	-0.10	0.344
Hemoglobin	90	-0.51	<0.001
Platelets	90	-0.14	0.185
WBC	90	-0.30	0.004
Age at diagnosis	99	-0.07	0.470
Age at baseline draw	100	-0.23	0.022
Latency	99	-0.30	0.003
PSA at diagnosis	78	-0.09	0.425

B. ANOVA* CTC count with respect to categorical variables

Variable	Categories	n	CTC median (range)	P †	Pairwise comparison ‡
Race	African American	5	4 (0-241)	0.034	Asian vs Hispanic (P = 0.020)
	Asian	4	0 (0-2)		
	Hispanic	5	65 (4-2,572)		
	Caucasian	86	4 (0-1,966)		
Stage at diagnosis	II	19	2 (0-924)	0.564	None
	III	14	30 (0-1,966)		
	IV	46	5 (0-2,572)		
	Unknown	21	5 (0-400)		
Definitive therapy	None/unknown	51	5 (0-2,572)	0.405	None
	RT	25	5 (0-924)		
	RP	24	1 (0-1,966)		
Lines of hormonal therapy	1	10	2 (0-2,572)	0.882	None
	2	50	4 (0-1,563)		
	≥3	38	5 (0-1,966)		
Lines of chemotherapy	0	58	3 (0-924)	0.004	0 vs 2 (P = 0.004)
	1	22	1 (0-2,572)		1 vs 2 (P = 0.017)
	≥2	20	123 (0-1,966)		
Total Gleason	Unknown	14	3 (0-132)	0.249	None
	≤6	11	0 (0-1,220)		
	7	28	5 (0-1,966)		
	≥8	47	8 (0-2,572)		
High Gleason	Unknown	18	4 (0-175)	0.244	None
	2-3	11	0 (0-1,220)		
	4	34	4 (0-1,966)		
	5	37	15 (0-2,572)		
Metastatic disease	None	16	0 (0-3)	0.001	None vs bone (P = 0.001)
	Soft tissue	12	2 (0-924)		None vs both (P = 0.003)
	Bone	47	17 (0-1,966)		
	Both	25	12 (0-2,572)		

*ANOVA conducted on rank-transform of CTC count.
† P value for ANOVA F-test.
‡ P value for Tukey-Kramer pairwise comparisons.

not shown), revealing an optimum CTC count of 4 that maximized the sum of sensitivity (61%) and specificity (100%).

Correlation of Biomarkers with Survival Outcomes and the Presence of CTCs. At the end of study follow-up and among the 100 patients included in the survival analysis, 49 (49%) patients were alive and 51 (51%) patients were deceased. The median duration of follow-up was 303 days (range, 21-766 days). The Kaplan-Meier estimate of the median survival time was 461 days (Fig. 3A). We subsequently analyzed biomarkers with respect to end-of-study survival status. In the univariate analysis (Table 3A), CTCs, alkaline phosphatase, PSA, LDH, prostatic acid phosphatase, total acid phosphatase, albumin, and hemoglobin proved to be the most tightly linked variables, with univariate P values all <0.003. Notably, Gleason sum and stage had nonsignificant P values. In the multivariate analysis, CTCs (hazard ratio, 1.76) and LDH (hazard ratio, 8.0) were retained as the only significant factors in the final model; all other biomarkers, including PSA, were eliminated. Results from the bootstrap validation (Table 3B) indicate CTCs (69%) and LDH (73%) were included in the final model most frequently among the biomarkers. Moreover, for all but one instance when CTCs or LDH were included in

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the final model, they were associated with increased risk of death. All other biomarkers were included in the final model in <42% of the bootstrap samples.

Threshold analysis of sensitivity and specificity for CTCs with respect to survival status for each cut-off revealed an optimal CTC count of 4 cells/7.5 cc, with a sensitivity of 70% and a specificity of 69% (Fig. 3B). The median survival time for patients with at least 4 CTC (Fig. 3A) was 8.4 months versus 15.1 months for all 100 patients. Survival in patients with <4 CTC was significantly better, and the median could not be estimated due to high censoring. The hazard ratio for ≥4 CTC was 3.65 ($P < 0.001$). Results from 2,000 bootstrap samples showed 4 cells/7.5 cc was the most common optimal cut-point; however, the percentage was only 23%. A cut-off of 2 and 5 CTC were both optimal in ~20% of the bootstrap samples. These results suggest that although a baseline CTC count ≥4 is most predictive of poor survival, lower cut-points retain significant predictive value.

To assess potential interrelationships between CTCs and other biomarkers, we next conducted univariate logistic regression analysis to predict any and ≥4 CTC (the cut-off for both metastatic disease and survival). A number of variables correlated with the CTC cut-offs in a univariate analysis, including PSA, LDH, alkaline phosphatase, total acid phosphatase, prostatic acid phosphatase, hemoglobin, and WBC.

Discussion

This analysis of our first 100 CRPC patients reveals that a CTC count ≥4 cells/7.5 cc was a powerful univariate predictor of survival ($P < 0.001$) and that a CTC count ≥4 was 100% specific for the presence of radiographically detectable metastatic disease. This analysis of baseline CTC number and biomarkers assessed overall survival as a primary end point, showing that independent multivariate predictors of survival include PSA and LDH, not CTCs, highlighting the prognostic value of classic biomarkers (9). As this analysis evaluated only baseline values, it was not intended to be longitudinal.

CTCs for the most part do not correlate well with the clinicopathologic characteristics of the primary tumor.

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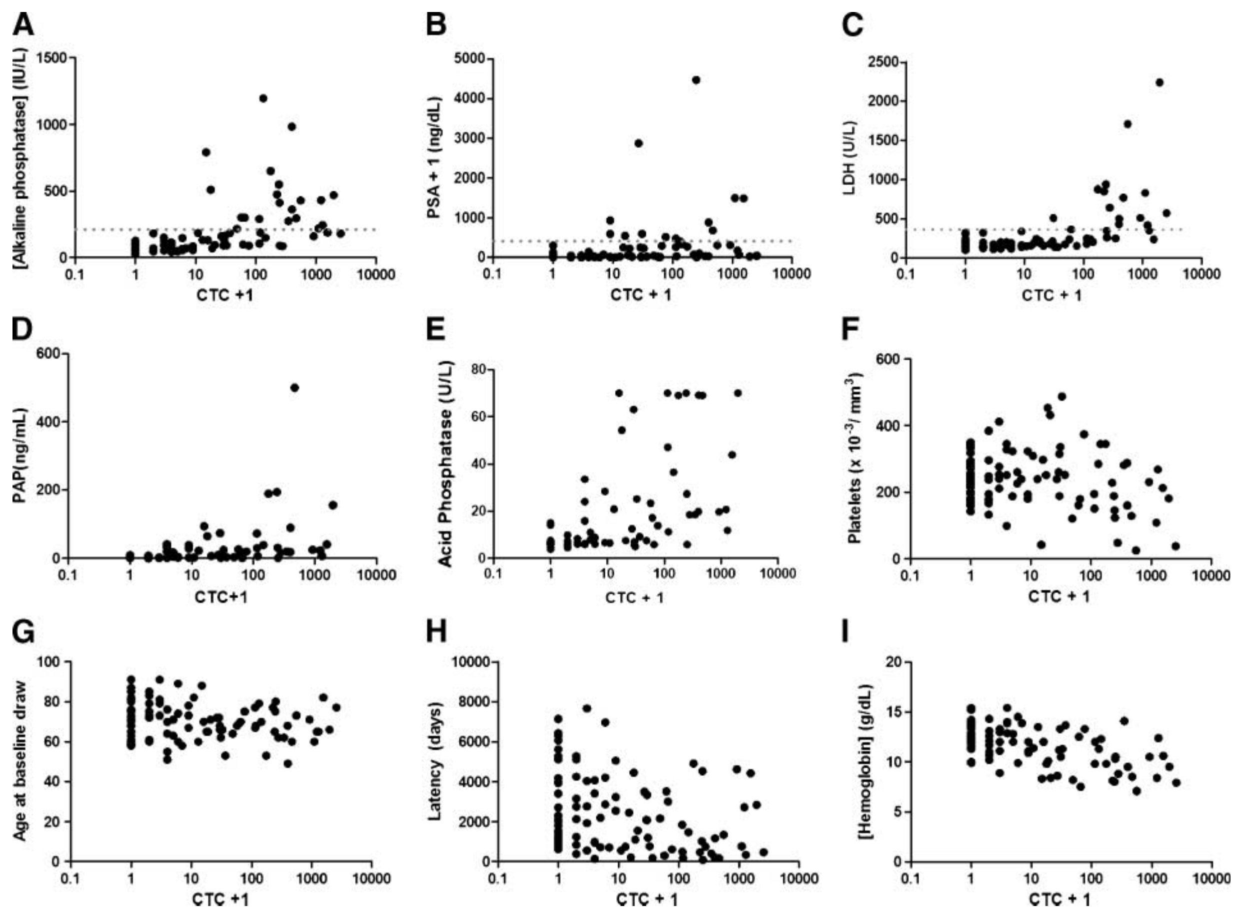

Figure 1. Correlation of continuous variables with CTC counts. Variables with rank correlation P values <0.05 corresponding to positive Spearman coefficients (**A**, alkaline phosphatase; **B**, $\log_{10}$ [PSA+1]; **C**, LDH; **D**, prostatic acid phosphatase; **E**, acid phosphatase) and negative Spearman coefficients (**F**, platelets; **G**, age at baseline draw; **H**, latency; **I**, hemoglobin) are scatterplotted versus log CTC. From the appearance of the plots, a number of the variables exhibit specific cut-offs (*dotted lines*) that correlate with the presence of CTCs (see text for details).

However, we did not have the primary tumor specimen to review and were limited to the original pathology report in most cases. In general, patients with a long clinical latency period are less likely to have an elevated

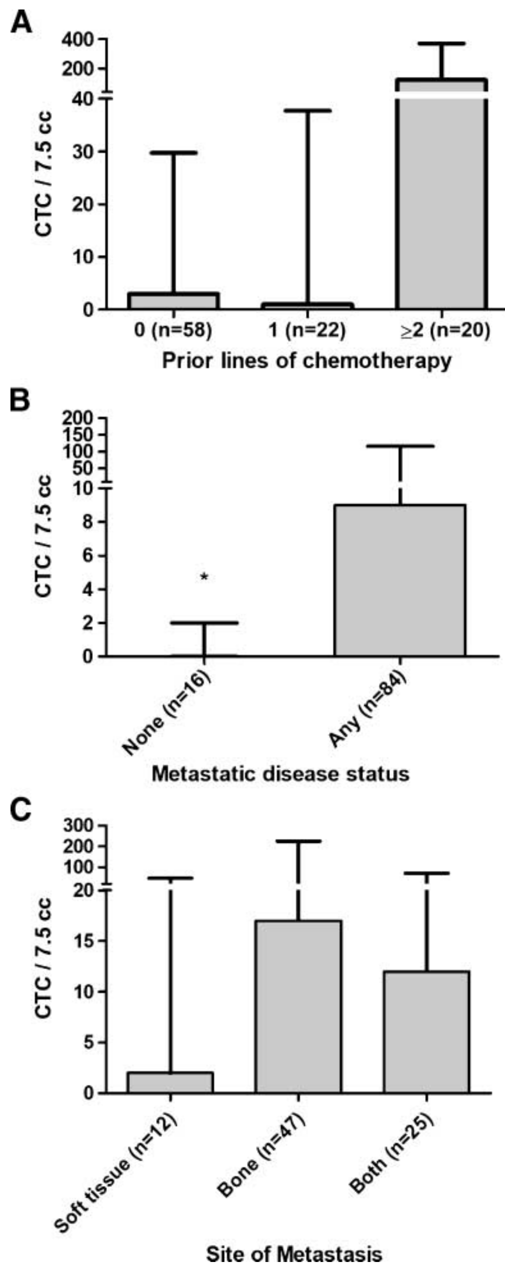

Figure 2. A. Correlation of median CTC counts with lines of chemotherapy. Median values are plotted for 0, 1, and ≥2 lines. By ANOVA, $P = 0.004$. **B.** Correlation of metastatic disease status with median CTC counts. Median CTC counts are correlated with presence and absence of metastatic disease. *, $P = 0.0002$. Error bars, 95% confidence interval. Sensitivity and specificity are optimized with a cut-off of 4, and are 61% and 100%, respectively. Error bars, interquartile data ranges. **C.** Correlation of metastatic disease site with median CTC counts. Median CTC counts are correlated with site of metastatic disease and plotted. Error bars, interquartile data ranges.

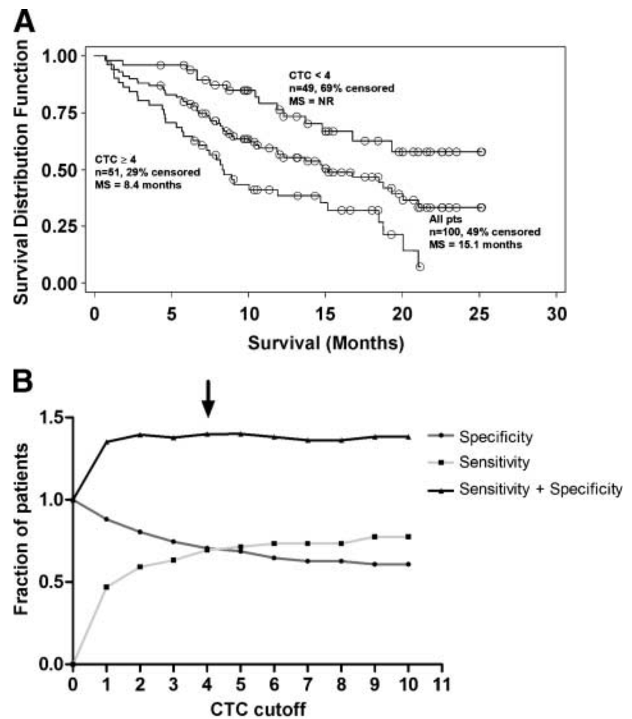

Figure 3. A. Kaplan-Meier survival analysis as a function of CTC category. The median follow-up time is 10.0 mo. The hazard ratio for ≥4 CTC was 3.65 ($P < 0.001$). **B.** Threshold analysis for CTC cut-off determination with the use of a survival end point. Sensitivity, specificity, and the sum of specificity and sensitivity are plotted. The optimal CTC cut-off is 4 cells/7.5 cc, in which sensitivity nearly equals specificity (*arrow*) as confirmed by bootstrap validation.

CTC count, reflective of the indolent nature of the disease. Patients younger than 60 years or those with a higher primary Gleason score are more likely to have an elevated CTC count, reflective of an aggressive disease variant with a shorter latency. Stage at diagnosis was not predictive of CTCs, suggesting that the intrinsic tumor biology, not the disease extent at diagnosis, correlates the presence CTCs. The median latency of 5.6 years represents the time elapsed between initial diagnosis and study entry. Compared with other disease, this is a relatively long natural history and is unique given the transition to castration resistance in the setting of androgen deprivation. These factors may account for the lack of correlation between CTCs and the initial clinicopathology.

That disease extent correlates well with a CTC count ≥4 as well as the lack of surrogacy of radiographic response for survival outcomes raise the possibility that CTC monitoring could reduce the need for routine radiographic monitoring once the presence of metastatic disease has been established radiographically. Alternatively, a CTC count ≥4 in an otherwise asymptomatic patient could replace radiographic imaging, as the presence of metastatic disease is assured. This has also been raised with regard to computed tomography monitoring in breast cancer (13). A prospective trial

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Table 3.

A. Cox regression analysis of overall survival to assess prognostic value of biomarkers

Biomarker	Statistic	Alive (n = 49)	Dead (n = 51)	Univariate*		Multivariate †	
				HR	P	HR	P
CTC ‡	Mean	12	294	2.34 (1.79-3.07)	<0.001	1.76 (1.25-2.47)	0.001
	Median (range)	1 (0-117)	29 (0-2,572)				
AP ‡	Mean	93	239	8.59 (3.92-18.82)	<0.001	—	0.328
	Median (range)	73 (24-300)	132 (36-1,196)				
PSA ‡	Mean	70	342	1.82 (1.31-2.54)	<0.001	—	0.857
	Median (range)	15 (0-593)	51 (0-4,469)				
LDH ‡	Mean	172	387	39.1 (14.0-109.4)	<0.001	8.00 (2.19-29.16)	0.002
	Median (range)	155 (101-512)	239 (113-2,240)				
PAP ‡	Mean	9	44	3.39 (2.09-5.51)	<0.001	—	0.308
	Median (range)	2 (1-74)	18 (0-500)				
TAP ‡	Mean	12	25	6.05 (2.64-13.88)	<0.001	—	0.990
	Median (range)	7 (4-70)	19 (5-70)				
Albumin	Mean	4.1	3.9	0.23 (0.09-0.57)	0.002	—	0.099
	Median (range)	4 (4-5)	4 (3-5)				
Glucose ‡	Mean	118	117	—	0.503	—	NS
	Median (range)	100 (80-482)	105 (72-296)				
Hemoglobin	Mean	12.3	10.9	0.73 (0.63-0.84)	<0.001	—	0.696
	Median (range)	13 (8-15)	11 (7-15)				
Platelets	Mean	252	222	—	0.184	—	NS
	Median (range)	252 (121-453)	220 (25-487)				
WBC ‡	Mean	6.2	6.3	—	0.614	—	NS
	Median (range)	6 (2-11)	6 (2-17)				
Gleason sum	Mean	7.6	8.1	—	0.389	—	NS
	Median (range)	8 (4-10)	8 (6-10)				
Stage	Mean	3.3	3.4	—	0.790	—	NS
	Median (range)	4 (2-4)	4 (2-4)				

B. Bootstrap validation of multiple regression model (2,000 samples)§

	Inclusion in final model	Inclusion (increased risk)	Inclusion (decreased risk)
Biomarker	No. (%)	No. (%)	No. (%)
CTC	1,386 (69.3)	1385 (69.3)	1 (0.1)
AP	552 (27.6)	549 (27.5)	3 (0.2)
PSA	302 (15.1)	151 (7.6)	151 (7.6)
LDH	1,468 (73.4)	1468 (73.4)	0 (0)
PAP	649 (32.5)	596 (29.8)	53 (2.7)
TAP	478 (23.9)	387 (19.4)	91 (4.6)
Albumin	824 (41.2)	2 (0.1)	822 (41.1)
Hemoglobin	272 (13.6)	108 (5.4)	164 (8.2)

*Results from Cox univariate regression analysis to assess individual prognostic value of biomarkers; hazard ratio (95% confidence interval).
† Results from Cox multiple regression analysis to assess independent prognostic value of biomarkers; hazard ratio (95% confidence interval).
‡ Survival analysis conducted on $\log_{10}$ transformation of the biomarker.
§Number values indicate the frequency with which the biomarker was included in the final model. The corresponding percentages are out of 2,000 bootstrap samples.

would be needed to validate CTC use in the various clinical scenarios.

Our two separate threshold analyses were designed to maximize the sensitivity and specificity of two important clinical end points, radiographically detectable metastatic disease and survival. For both scenarios, we obtained values of 4 cells/7.5 cc compared with 5 cells/7.5 cc, which is conventionally used. With regard to detecting metastatic disease, this finding indicates that a high CTC number assures the presence of metastatic disease and, although not proof, supports a causal link between metastases and CTCs. This also implies mechanistically that detectable metastases occur under conditions of elevated CTC counts and not from preexisting micrometastatic disease.

The observation that the cut-off of 5 cells/7.5 cc has been used is based largely on earlier data in breast cancer patients (13) and, although prognostically significant, (18), this was never shown to be the optimal cut-off. Although our survival cut-off differs by only 1 CTC/7.5 cc (4 versus 5), because the cut-offs are so near the median CTC count (4 cells/7.5 cc), this small difference could result in substantial differences in apparent prognoses. Notably, in our analysis, this cut-off seems to afford a greater ability to discriminate between outcomes, with the overall survival for patients with a CTC <4 not yet reached but seeming to be at least 3-fold greater than those with a CTC ≥4, compared with ~2-fold for a CTC cut-off of 5 (18). In addition, the trend with the ongoing follow-up of these patients is that the CTC cut-off is trending downward (data not shown), reflecting decreased survival over time with lower CTC values. Furthermore, the threshold analysis with bootstrapping detected CTC cut-offs as low as 2 as optimal, suggesting

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that the presence of even lower CTC counts may be an ominous finding. A key difference in the current analysis versus the deBono study is that CTC count was analyzed as a continuous variable versus as a categorical cut-point, and this too may account for the greater prognostic significance of this biomarker.

As Danila et al. observed (22), the site of metastatic disease tends to correlate well with median CTC counts, with metastatic disease to bone as opposed to soft tissue correlating with higher CTC counts. In our study, this trend did not reach statistical significance, but our numbers of patients were smaller than Danila et al. There are several possible explanations for this observation: (*a*) the primary production of CTCs is in the osseous compartment (e.g., bone marrow) with resultant hematogenous dissemination via a myelophthisic mechanism, (*b*) there are specific differences in the biology of disease in the soft tissue and bony compartments (i.e., soft tissue disease may be more indolent and therefore less prone to the production of CTCs), and (*c*) lymph nodes and viscera act to trap or filter the CTCs, thereby retaining them in a nonhematogenous compartment.

In the multivariate analysis, CTCs and LDH were independent prognostic factors. However, CTCs and the other biomarkers were strongly correlated. Qualitatively this was apparent by the examination of plots of biomarkers related to CTCs in a biphasic matter. For example, patients with an LDH >325 or ~1.5 times the upper limit of normal (Fig. 1C), or a hemoglobin of <10 (Fig. 1I) were uniformly observed to have elevated CTC numbers, indicating that these variables are specific predictors of CTCs (Fig. 1). We show that CTCs are tightly associated with a number of biomarkers, including PSA, LDH, and alkaline phosphatase, exhibiting a threshold relationship in which, for a given biomarker, exceeding the threshold predicts an elevated CTC count with 100% specificity. For PSA, LDH, and alkaline phosphatase, these values are 300 ng/mL, 325 U/L, and 200 U/L, respectively (Fig. 1A to C, *dotted line*). With the use of these three variables, CTCs can be predicted with 64% sensitivity and 100% specificity. These findings support the notion that the presence of elevated CTCs may be predicted from classic biomarker profiles. Clinically, these observations could be used either as an inexpensive surrogate for CTCs in centers where the test is not available or conversely as justification for CTC enumeration in the same patient as a more robust indicator of the patient's prognosis.

We have observed significant morphologic heterogeneity in a patient's CTCs (data not shown), suggesting that CTC subsets may themselves provide further insights into a given patient's tumor biology and, potentially, additional prognostic information. Given the significant association between metastatic disease and CTC count, we hypothesize that CTCs reflect ongoing metastasis or that a subpopulation of the CTCs are tumor stem cells. In additional to morphologic heterogeneity, a small Asian study showed by fluorescence in situ hybridization analysis that the *TMPRSS2-ERG* translocation has been shown to be expressed in a majority (10 of 15) of CTC samples, without any evidence of active TMPRSS2:ETV1 transcription (23).

A CTC cut-off of 4 cells/7.5 cc has now been statistically validated as predictive of both the presence of metastatic disease and overall survival with the use of threshold analysis, the first such validation ever done in a prostate cancer patient cohort. Further prospective studies are needed to validate its routine use as a prognostic tool. To that end, a large prospective study has been launched within the Southwest Oncology Group (SWOG 0421 phase III trial of docetaxel ± atrasentan) to determine whether CTC values provide independent prognostic and predictive information. Longitudinal analysis in conjunction with other biomarkers will provide additional insight into its predictive value.

Disclosure of Potential Conflicts of Interest

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