

Circulating Tumor Cell Analysis in Patients with Progressive Castration-Resistant Prostate Cancer

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Abstract Purpose: To better direct targeted therapies to the patients with tumors that express the target, there is an urgent need for blood-based assays that provide expression information on a consistent basis in real time with minimal patient discomfort. We aimed to use immunomagnetic-capture technology to isolate and analyze circulating tumor cells (CTC) from small volumes of peripheral blood of patients with advanced prostate cancer.

Experimental Design: Blood was collected from 63 patients with metastatic prostate cancer. CTCs were isolated by the CellSearch system, which uses antibodies to epithelial cell adhesion marker and immunomagnetic capture. CTCs were defined as nucleated cells positive for cytokeratins and negative for CD45. Captured cells were analyzed by immunofluorescence, Papanicolaou staining, and fluorescence *in situ* hybridization.

Results: Most patients (65%) had 5 or more CTCs per 7.5 mL blood sample. Cell counts were consistent between laboratories ($c = 0.99$) and did not change significantly over 72 or 96 h of storage before processing ($c = 0.99$). Their identity as prostate cancer cells was confirmed by conventional cytologic analysis. Molecular profiling, including analysis of epidermal growth factor receptor (EGFR) expression, chromosome ploidy, and androgen receptor (*AR*) gene amplification, was possible for all prostate cancer patients with ≥ 5 CTCs.

Conclusions: The analysis of cancer-related alterations at the DNA and protein level from CTCs is feasible in a hospital-based clinical laboratory. The alterations observed in EGFR and *AR* suggest that the methodology may have a role in clinical decision making.

Assays to detect circulating tumor cells (CTC) in the peripheral blood have been used clinically to provide prognostic information and to test for minimal residual disease. These assays also have the potential to guide treatment selection based on the molecular profile of the tumor before therapy. Such guidance is particularly important in prostate cancer management because the factors contributing to tumor cell

growth and survival change over time as a result of both the intrinsic biology of the tumor and the specific therapies under which it had progressed (1). Our ability to profile recurrent metastatic prostate cancers is limited because a repeat biopsy is not part of the routine management of the disease, and when it is attempted, the frequency that tumor material is obtained is low (2). Consequently, treatment decisions are often made without current knowledge of whether the tumor expresses the putative targets of the drugs under consideration. Because targeted therapies will only be useful for the subset of patients in whom the target is present, we urgently need blood-based assays to provide this information on a consistent basis with minimal patient discomfort.

A variety of techniques for isolation and characterization of CTCs have been studied, each with specific advantages and limitations. Reverse transcription-PCR assays are sensitive and highly specific when the expression of the target mRNAs is limited to malignant tumor cells (3–7). They are also useful to detect residual disease in the setting of minimal tumor burden, in particular for prostate cancer patients whose level of prostate-specific antigen (PSA) is undetectable (8, 9). Flow cytometry has been used to detect and to authenticate the cells as CTCs (10), but it does not allow visual confirmation of morphology or the discrimination of changes at the subcellular level, such as DNA copy number.

Antibodies to the epithelial cell adhesion molecule (EpCAM) allow circulating epithelial-derived tumor cells to be isolated

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Table 1. Baseline patient characteristics

Characteristic	Number (%) or median (range)
Patients	63
Age (y)	72.5 (52-87)
PSA (ng/mL)	181 (6-12,147)
Initial treatment	
Radical prostatectomy, <i>n</i> (%)	23 (37)
Radiation to prostate, <i>n</i> (%)	14 (22)
No primary treatment, <i>n</i> (%)	26 (41)
Systemic treatment	
Hormonal therapy, <i>n</i> (%)	63 (100)
Chemotherapy, <i>n</i> (%)	42 (67)
Radiation to prostate bed, <i>n</i> (%)	7 (12)
Sites of metastasis	
Bone only, <i>n</i> (%)	41 (65)
Soft tissue only, <i>n</i> (%)	8 (13)
Bone and soft tissue, <i>n</i> (%)	14 (22)

and concentrated from peripheral blood, so that they can be inspected by microscopy (11–13). More recently, a semi-automated system, CellSearch, has been developed using an EpCAM antibody-based immunomagnetic capture and automated staining methodology. The sensitivity, linearity, and reproducibility of the automated CTC selection platform have been described using cultured tumor cells (14), and factors predictive of detection of CTCs in prostate cancer have been reported (15–17). The system and reagents have been Food and Drug Administration–cleared for predicting progression-free survival and overall survival in patients with metastatic breast cancer (18, 19). In a pivotal trial, the detection of >5 CTCs per 7.5 mL of blood at the start of chemotherapy and after each cycle of therapy was associated with shorter progression-free and overall survival in patients with metastatic breast cancer. CTC counts were more predictive of the outcomes than were standard clinical parameters (20, 21). For prostate cancer, preliminary analysis of the correlation of CTC counts with mRNAs for PSA or prostate-specific membrane antigen and available clinical predictors have been encouraging (16).

In this study, we show that CTCs isolated and enumerated from patients with progressive prostate cancer on androgen depletion represent true neoplastic cells that can be profiled at the protein level and for chromosomal changes by fluorescence *in situ* hybridization (FISH). Cell counts remained stable for up to 72 h from the time of phlebotomy. Unique aspects of this study are that it was done in a hospital-based clinical laboratory setting, blood samples were obtained in the context of routine patient management, and protein expression was analyzed by immunocytochemistry using a semi-automated method.

Materials and Methods

Patients. Blood was collected from 63 patients with metastatic prostate cancer and from 17 control subjects without prostate cancer at the Memorial Sloan-Kettering Cancer Center under protocols approved by the Institutional Review Board and with informed consent. All patients in the prostate cancer group had evidence of progression on androgen depletion, as documented by rising PSA despite castrate (<20 ng/dL) levels of testosterone. Blood was collected into CellSave tubes (Immunicon, Huntingdon Valley, PA). In most cases, two samples with at least 7.5 mL of blood were collected at one visit.

Automated immunomagnetic isolation and immunofluorescent staining of CTCs. The methodology for automated immunomagnetic selection of CTCs, based on capture with an anti-EpCAM antibody and immunofluorescent staining and analysis, has been described (14, 18, 20). In short, samples were drawn in tubes containing cell preservatives, maintained at room temperature, incubated with EpCAM antibody-covered ferroparticles at room temperature, and processed on the CellTracks Autoprep (Immunicon). Circulating epithelial cells expressing EpCAM were isolated by a magnetic field without centrifugation. After the supernatant containing unbound cells was removed, the 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 (14). Stained cells were analyzed on a fluorescence microscope using the Cell Track Analyzer II (Immunicon). Automatically selected images were reviewed by the operator for identification and counting of CTCs, which were defined as cytokeratin-positive and 4',6-diamidino-2-phenylindole–positive nucleated cells lacking CD45. Quality control was maintained via standard procedures. The CellSearch System is available from Veridex LLC (Warren, NJ).

To assess the consistency of results between laboratories, two separate tubes were collected at the same phlebotomy from 18 men (14 with prostate cancer and 4 without a cancer diagnosis) and processed blindly at the Memorial Sloan-Kettering Cancer Center Clinical Chemistry Laboratory and the Immunicon Laboratory. To assess whether cell counts change over time, separate samples were obtained and processed within 24 h or at 72 or 96 h after collection from 14 patients and 1 volunteer.

Flow cytometry. After completion of the CellSearch assay, stained, fixed cells were transferred from the magnetic cartridge into TruCount Absolute Count tubes containing fluorescent counting beads (BD Biosciences, San Jose, CA) and analyzed on either a FACSCalibur or FACSCanto flow cytometer (BD Biosciences) equipped with 488-nm argon ion and 635-nm red diode lasers. Data acquisition and analysis was done using either the CellQuest 3.3 or FACSDiva 4.1.1 (BD Biosciences) programs, until ~30,000 beads were collected in each sample tube. Multiparameter analysis was done on list mode data. The criteria for delineating the CTCs were based on nucleated cells staining with cytokeratin-phycoerythrin antibody and the absence of staining with CD45-allophycocyanin.

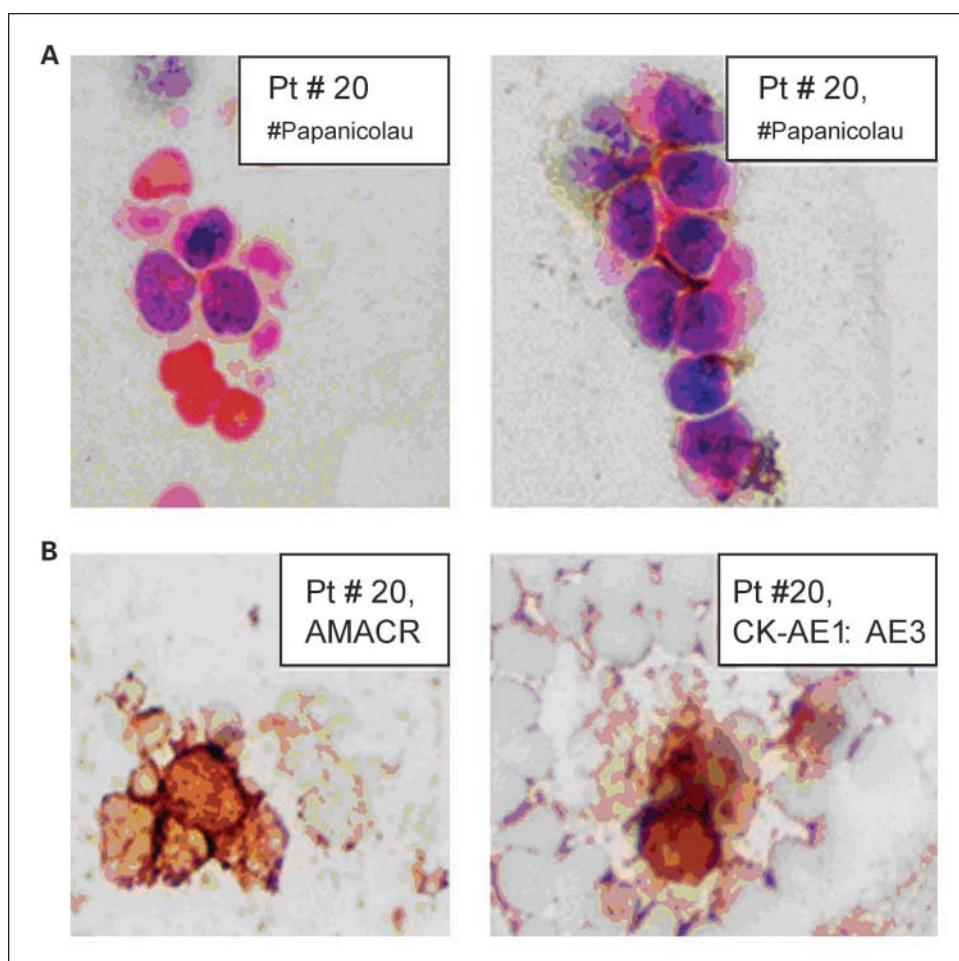
Slide preparation and immunocytochemistry. Duplicate enriched samples were processed for both automated cell counting, which was also later used for FISH studies, and for standard cytology. Following automated immunomagnetic selection using the CellSearch profile kit

Table 2. Circulating tumor cell number in patients with progressive metastatic castration-resistant disease

CTC number	n.a.	0-2	3-4	5-10	11-25	26-50	51-100	101-200	≥201
No. patients (%)	4 (6)	9 (14)	10 (16)	7 (11)	8 (13)	7 (11)	7 (11)	5 (8)	6 (10)

NOTE: n.a., not available because of uninterpretable data.

Fig. 1. CTC cytopathologic analysis. Immunomagnetically isolated CTCs stained by the Papanicolau method are shown under high power (*top*). Cells destained, then stained by immunocytochemistry for cytokeratins (CK) or α -methyl CoA-racemase (AMACR), a specific marker of prostate cancer cells (*bottom*).



to isolate and collect cells of interest without the staining procedure, the cell suspension was pipetted onto a bioadhesive-coated glass slide, air-dried, and methanol fixed. Papanicolau staining was done by standard protocols, and stained cells were examined under high power by a cytopathologist (O. Lin). For immunocytochemical analysis, destained alcohol-fixed slides were restained with the anti-cytokeratin monoclonal antibodies AE1/AE3 (Biogenex, San Ramon, CA; 1:800) after

pretreatment with protease and with α -methyl CoA racemase (Zeta, Sierra Madre, CA; 1:50) after heating for antigen retrieval.

Fluorescence in situ hybridization. Before FISH processing, the slides prepared for automated CTC counting were fixed in 95% ethanol, and then air-dried. Probe sets were produced using BAC or PAC clones spanning RP11-94L15 (RP11-479J1 and RP4-808O4) and *ERBB2* (RP11-62N23, RP1194L15, and CTD-3211L18) obtained from

Table 3. FISH analysis of CTC

Patient	CTC count	AR copy number	X cen copy number	Ratio*	ERBB2 copy number	17 cen copy number	Ratio [†]	Ploidy
A	9	1	1	1	2	2	1	Diploid
B	25	3	3	1	3-5	3-5	1.15	Tetraploid
C	27	2-3	2-3	1	3-4	3-4	1	Tetraploid
D	36	1	1	1	2	2	1	Diploid
E	50	Amp	2-3	>10	3-4	3-4	1	Tetraploid
F	90	Amp	1	>10	2	2	1	Diploid
G	114	2	2	1	4	4	1	Tetraploid
		Amp	2	>10	3-4	3-4	1	Tetraploid
H	179	Amp	2-3	>10	3-4	3-4	1	Tetraploid
J	561	Amp	1	>10	2	2	1	Diploid
		Amp	2-3	>10	3-5	3-5	1	Tetraploid

NOTE: Ploidy was inferred from FISH signal counts.

Abbreviations: cen, centromere; Amp, amplification of gene locus.

*Ratio of AR copy number to X centromere copy number.

[†]Ratio of *ERBB2* copy number to 17 centromere copy number.

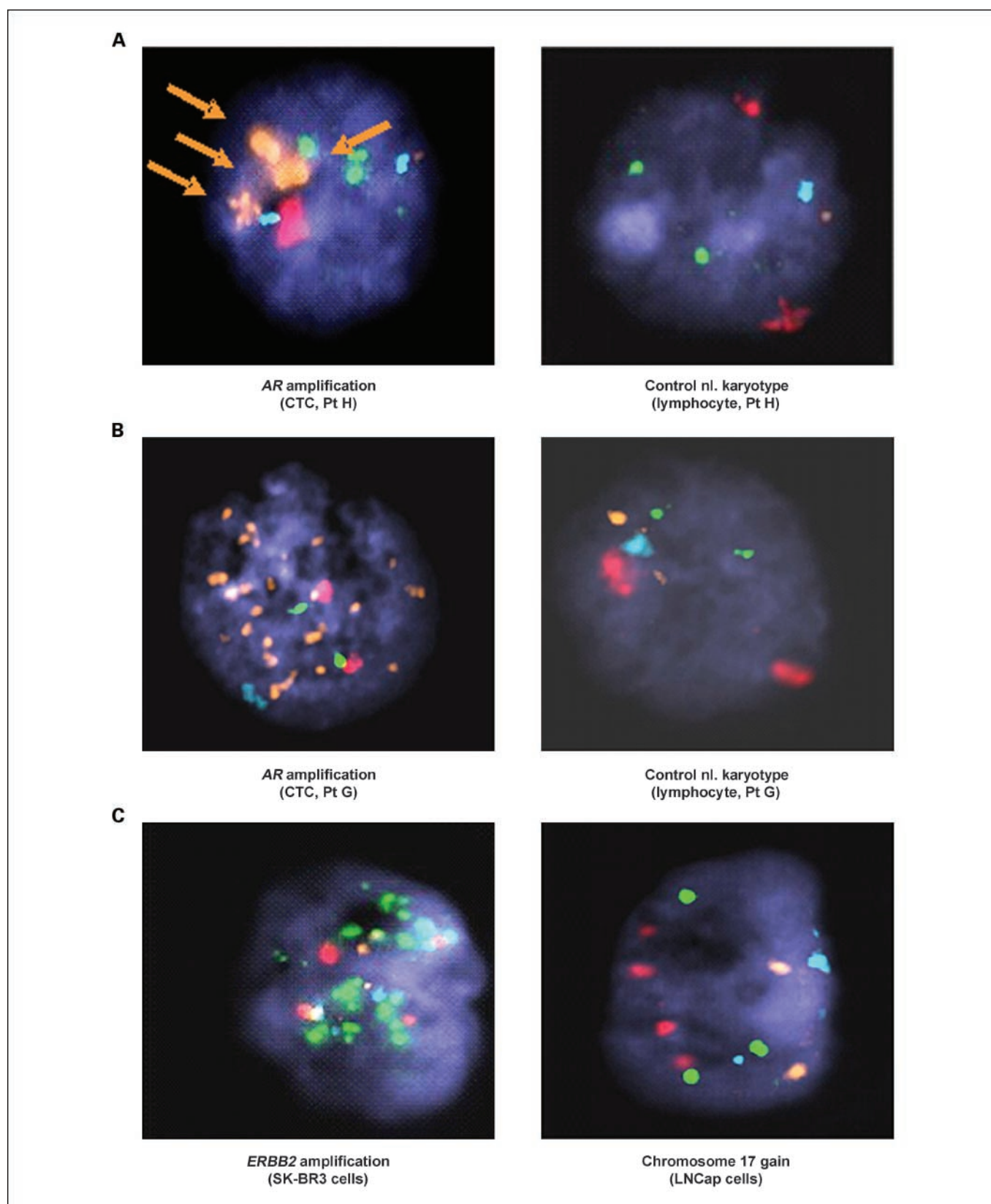
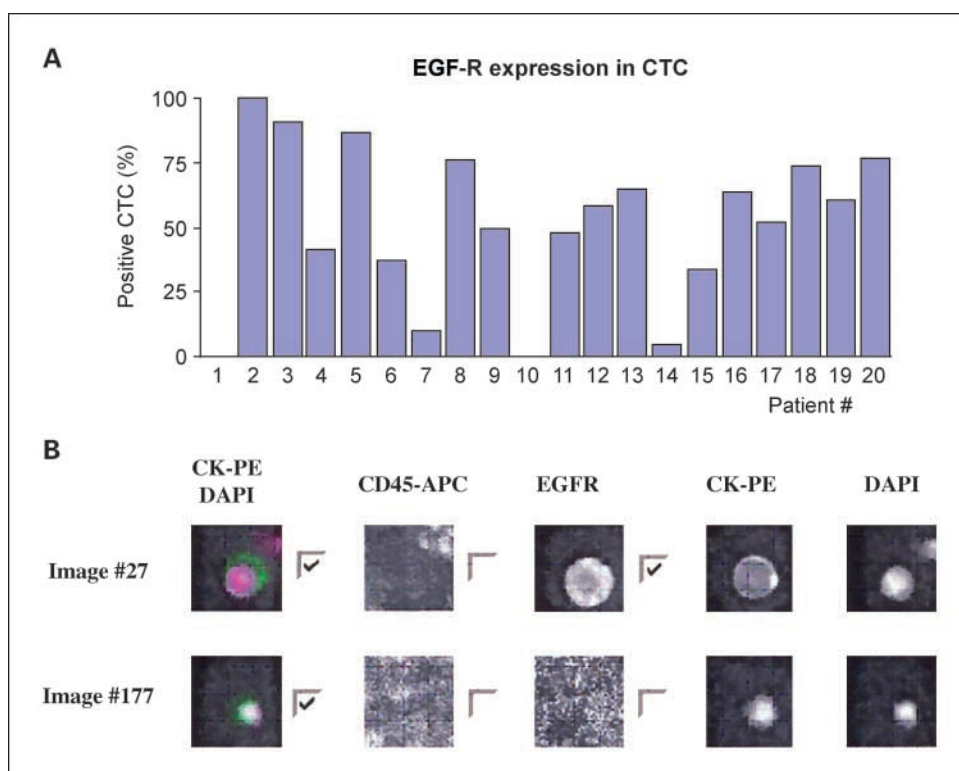


Fig. 2. CTC FISH analysis. *A*, *AR* amplification in a CTC from patient H compared with the normal FISH signal in a leukocyte from the same sample. Orange *AR* signals are present in clusters of multiple copies, whereas the reference X centromere probe (*aqua*) is present in two copies. Additional signals for *ERBB2* (green) and 17 centromere (red) suggest a near-tetraploid karyotype. *B*, *AR* amplification in patient G is seen as dispersed orange signals with other probes showing normal copy number in this cell. *C*, in FISH controls, *ERBB2* amplification and chromosome 17 gain is present in SK-BR-3 breast cancer cells, whereas LNCaP prostate cancer cell shows copy numbers consistent with a near-tetraploid chromosome content.

Fig. 3. Quantitation of EGFR expression in CTCs by the automated immunofluorescent assay. The percentage of CTCs positive for EGFR was analyzed in samples with ≥ 5 CTCs (*top*). In the bottom, image 27 shows an individual CTC that stained positive for EGFR expression. Image 177 shows a CTC from the same patient with no EGFR staining. These images are representative for patient 20, and both cells showed positive cytokeratin staining and no CD45 staining, whereas 4',6-diamidino-2-phenylindole showed intact nuclei.



Invitrogen (Carlsbad, CA) or from a cancer clone set obtained from the Sanger Institute as part of their 1-Mb clone set (22), together with human centromere-specific repetitive clones for chromosomes X and 17. DNA was labeled by nick translation to assemble a four-color probe set as follows: AR with SpectrumOrange-dUTP, *ERBB2* with SpectrumGreen-dUTP, and 17 centromere with SpectrumRed-dUTP (Vysis, Abbott Molecular, Inc., Des Plaines, IL), and X centromere with DEAC-dUTP (Perkin-Elmer, Life and Analytical Sciences, Waltham, MA). The hybridization mix was applied to pepsin-treated slides, codenatured at 80°C for 3 min, and then incubated overnight on a HYBrite-automated hybridization station (Vysis, Abbott Molecular, Inc.). Unbound probe was removed by standard procedures. After 4',6-diamidino-2-phenylindole staining, the slides were mounted in antifade solution (Vectashield, Vector, Burlingame, CA) and examined using a Zeiss Axioplan 2 epifluorescence microscope controlled by Isis imaging software (MetaSystems GmbH, Altlusheim, Germany).

Statistical methods. The concordance correlation coefficient, c , was computed to assess the reproducibility of the assay. If the Immunicon and Memorial Sloan-Kettering Cancer Center assays are plotted against each other, then the concordance correlation coefficient measures the variation of the points around a 45° line through the origin. c was also computed for the cell counts obtained on samples obtained at the same time and processed within 24 h, or 72 and 96 h (23). Kendall's τ statistic was used to estimate the association between the number of cytokeratin-positive, CD45-negative CTCs found by immunomagnetic isolation and by flow cytometry (24).

Results

Clinical characteristics of the patient population. The clinical characteristics of the 63 patients studied are shown in Table 1. All had progressive metastatic prostate cancer with rising PSA despite castrate levels of testosterone. A total of 37 (59%) had received treatment for localized disease by radical prostatecto-

my (23 cases) or radiation therapy (14 cases) and later developed metastatic disease, whereas 26 (41%) presented with metastatic disease and had not received treatment of the primary tumor. The patterns of metastatic spread included disease in soft tissue only in 8 patients (13%), in bone and soft tissue in 14 (22%), and in bone only in 41 (65%). The median PSA at the time of inclusion in this study was 180.8 ng/mL (range, 6-12,147 ng/mL).

CTC counts. The median CTC count in patients with prostate cancer was 16 cells per 7.5 mL blood sample; counts ranged from 0 to 847. Table 2 shows the distribution of CTC counts. In 4 cases (6%), the isolation and analysis of CTCs produced uninterpretable data. This was most frequently the result of a sample volume that was too small (<7.5 mL suggested by the manufacturer).

Reproducibility in different laboratories and over time. The CTC counts between samples independently processed and reviewed at different laboratories were reproducible ($c = 0.99$), as were the cell counts from duplicate samples processed within 24 h versus 72 or 96 h after collection ($c = 0.99$).

The CTC counts were compared with counts determined by flow cytometry. The rank correlation between the number of cytokeratin-positive, CD45-negative CTCs found by immunomagnetic isolation versus flow cytometry from the same samples was $r = 0.68$. There was, however, a reproducible 40% loss of cells upon transfer from the CellSearch magnetic cartridge to a flow cytometry tube.

Cytopathologic examination of CTCs. Of the samples positive for CTCs, 25 were analyzed by Papanicolaou staining for cytokeratins (AE1/AE3 antibodies) and for α -methyl CoA racemase. All 15 samples containing ≥ 15 CTCs by the CellSearch methodology showed cytologically abnormal cells in the Papanicolaou-stained duplicate sample. In addition, six

out of nine samples with between 2 and 12 CTCs showed abnormal cells by cytology. The abnormal cells isolated from the enriched CTC samples stained positively for cytokeratin AE1/AE3 and α -methyl CoA racemase (Fig. 1). Background staining was present in the immunostained slides due to the presence of the magnetic beads. Nonetheless, the morphologic findings in combination with the pattern of staining allowed the identification of the CTCs.

FISH in CTCs. Samples from the first nine patients with CTC counts >5 were analyzed by FISH using probes for the androgen receptor locus (*AR*), *ERBB2* (*HER2/Neu*), and centromere sequences for chromosomes X and 17, the chromosomes that contain *AR* and *ERBB2*, respectively. The results are presented in Table 3. Marked amplification of the *AR* was observed in five patients, all with CTC counts of 50 or more. For four of these five patients, the other FISH probes showed numbers of signals consistent with tetraploidy, and two patients without *AR* amplification also showed apparent tetraploidy. Examples from two patients with *AR* amplification are shown in Fig. 2. Two of the patients with *AR* amplification had mixed CTC populations. Patient G had a subset of near-tetraploid cells without *AR* amplification, whereas patient J had both diploid and tetraploid cells with multiple copies of *AR*. No abnormality was detected in samples from two patients. None of the samples analyzed had amplification of *ERBB2*.

Analysis of epidermal growth factor receptor protein expression in CTCs. Twenty samples with five or more CTCs were subjected to automated immunofluorescent staining and cell sorting for the epidermal growth factor receptor (EGFR) protein. The percentage of EGFR-positive CTCs relative to total CTCs ranged from 0% to 100%, with a median of 56%. The distribution of percentages is shown in Fig. 3.

Discussion

In prostate cancer, the factors contributing to progression change over time (1). Any given molecular target that might be exploited therapeutically will be expressed in only a subset of patients at specific points in the illness. To optimize treatment selection requires serial acquisitions of tumor for molecular profiling with minimal patient discomfort. The present study showed that cells isolated from patients with progressive castration-resistant disease (25) with an automated CTC capture technology had molecular features of malignant prostate epithelial cells. We showed that two independent laboratories obtained similar cell counts for the same patient samples, and that the results were consistent whether the samples were processed within 24 or up to 72 and 96 h after phlebotomy. Anticipating the clinical application of this technology, all samples were obtained in the context of routine patient management and processed in a hospital-based clinical chemistry laboratory.

CTC number as quantified by the CellSearch methodology has been shown to have prognostic significance, and post-therapy decreases and increases in CTC number are associated with a superior and inferior survival, respectively (20, 26) in patients with breast cancer. Overall, five or more cells were isolated from 65% of patients in our study, whereas an additional 14% had three or four cells. It should be noted that

CTCs can be enumerated in all patients, even when cell counts are too low to allow molecular characterization by FISH or immunofluorescence (20). This rate of retrieval of cancer cells was significantly higher than the 10% rate we obtained historically using iliac crest bone marrow biopsies in a similar patient group (2). Although the diagnostic yield is increased when computed tomography-guided biopsies of fluorodeoxyglucose-avid lesions are done, the latter are invasive, costly, difficult to schedule, and much slower to provide results. Repetitive biopsy sampling is not feasible.

As there is no gold standard test for malignant CTCs, multiple techniques were used to confirm the malignant nature of the cells isolated. First, we looked at cell morphology and showed that the cells expressed cytokeratins and α -methyl CoA racemase, markers characteristic of malignant prostate epithelial cells (27, 28). Reasoning that the CTC numbers estimated using different techniques should be similar, we used flow cytometry to reanalyze the EpCAM antibody-enriched, immunofluorescently stained cells that remained after immunomagnetic selection. The rank order of samples showed a high concordance, although cell loss was noted when the samples were removed from the magnetic chamber.

By FISH, we showed a high frequency of aneusomy for chromosomes 17 and X. Amplification of the *AR* was observed in five patients with higher CTC counts among the nine analyzed. Such amplifications are seen in about 30% of advanced, hormone-independent tumors, but are extremely rare in tumors characterized at the time of diagnosis (29, 30). Although six patients had chromosome 17 gain in apparent tetraploid or near-tetraploid background, amplification of the *ERBB2/HER-2* region was not observed. This finding is consistent with various reports that, with disease progression, *HER-2* overexpression increases (31, 32) without gene amplification (33).

The molecular changes in specific metastatic sites vary significantly (34, 35). It is therefore possible that the tumor cells isolated from the blood may provide a better overall reflection of the biological heterogeneity of the illness than tumor from a site-directed biopsy. As an example, the proportion of cells positive for EGFR in the 20 samples analyzed ranged from 0% to 100% (Fig. 3). Similarly, FISH detected two different tumor cell populations in two of nine patients in our preliminary sample.

Whether the proportion of cells expressing a target molecule or the degree of expression is associated with clinical response to a targeted agent can only be addressed prospectively (36, 37). Ultimately, the validation of the molecular profiling methodology will come from additional studies that show that the markers identified predict for clinically relevant outcomes. This includes the likelihood of response to a treatment not yet given or evidence that a targeted therapy is affecting the target as predicted. These questions are currently undergoing prospective study.

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