Evaluation and significance of circulating epithelial cells in patients with hormone-refractory prostate cancer

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OBJECTIVE

To determine the feasibility of using flow cytometry fluorescence-activated cell sorting (FACS) analysis for detecting circulating epithelial cells (CECs) in patients with hormone-refractory prostate cancer (HRPC), and to determine whether CECs can be used to predict survival in these patients.

PATIENTS AND METHODS

Several prognostic models that include routinely used clinical and laboratory variables for predicting survival in men with HRPC have been reported; the presence of CECs measured by reverse transcriptase-polymerase chain reaction for prostate-specific antigen (PSA) in patients with hormone-refractory prostate cancer (HRPC) is an independent prognostic factor for survival. CECs detected by FACS analysis correlate with advanced stage and poor survival outcome. A retrospective study was conducted to assess the presence of CECs by FACS analysis in metastatic HRPC patients initiating systemic chemotherapy with a taxane-based regimen. The association between clinical variables previously described and the presence of CECs along with the effect of the magnitude of CECs on survival was calculated, in 41 patients with HRPC, all of whom had peripheral blood collected for FACS analysis.

RESULTS

Except for four patients, all those with metastatic HRPC had detectable CECs. Among these patients, the number of CECs/mL was correlated with age, serum PSA level and serum alkaline phosphatase (ALP). Higher serum levels of PSA and ALP predicted a poor survival outcome. Similarly, patients with \( \leq 1.8 \) CECs/mL had a significantly longer survival than those with more CECs/mL \((P = 0.02)\). With a median follow-up of 15.4 months, the median overall survival for all patients was 18.4 months.

CONCLUSIONS

The presence of more CECs in patients with metastatic HRPC was associated with a poorer survival outcome; levels of \( \geq 1.8 \) CECs/mL were associated with a shorter survival in patients with metastatic HRPC.
INTRODUCTION

Prostate cancer remains the most common cancer among men in the USA, accounting for >32% of all male malignancies. It is estimated that >234 000 men will be diagnosed with prostate cancer during 2006, and 27 350 will die from the disease. Virtually all deaths are due to the development of hormone-refractory prostate cancer (HRPC) [1]. Several prognostic models predictive of survival in men with HRPC have been reported [2–5]. Numerous reports have suggested that early-stage cancers have the potential to begin shedding cancer cells into the circulation early in their development. Unfortunately, the natural history of these cells, their ability to establish metastases, and their role in disease recurrence remains unclear. Detection of micrometastases, or circulating tumor or epithelial cells (CECs) has become an attractive technique that can be used to assess the prognosis in patients with cancer. Several authors showed that levels of CECs in patients parallel the tumor burden and response to therapy [6–12]. Indeed, the number of circulating tumour cells before treatment was recently found to be an independent predictor of progression-free and overall survival in patients with metastatic breast cancer [13]. CECs can be detected in 0–72% of patients with prostate cancer that is clinically organ-confined and in 25–100% of patients with distant metastatic disease. The presence of CECs at the time of primary therapy has also been associated with early disease failure and poor long-term outcome [14,15]. Various groups also showed that the presence of CECs measured by reverse transcriptase (RT)-PCR for PSA in patients with HRPC receiving cytotoxic chemotherapy correlated with survival outcome [16–20]. Positive RT-PCR for PSA is an independent prognostic factor for survival in men with HRPC [21]. Halabi et al. [22] confirmed that RT-PCR for PSA is a statistically significant predictor of overall survival for patients treated once with previous hormonal therapy.

RT-PCR for CECs has several limitations; the lack of specificity coupled with the lack of standardization of RT-PCR techniques has prevented this test from achieving widespread use. By contrast, fluorescence-activated cell sorting (FACS) analysis allows the detection of antigens in a heterogeneous mixture of cells, and offers several advantages over immunohistochemistry and RT-PCR. Cell sorting is easy to do and enables a high throughput of samples, quantification of results, and isolation of subpopulations of cells. The feasibility of using FACS assays for detecting micrometastases was reported in several cancers [12,13,20,23]. Compared with normal individuals there are significantly more CECs identified by FACS analysis in patients with prostate cancer. Also, the presence of CECs in patients with advanced prostate cancer appears to correlate with survival [24–27]. Unfortunately, limited sample sizes and the lack of clinical correlation make these results insufficient to assess the true clinical utility of this test. We report the results of a retrospective pilot analysis that evaluated patients with HRPC undergoing cytotoxic therapy, to determine the utility and feasibility of FACS analysis for detecting CECs, their change over time, and to assess whether or not the presence and number of CECs identified by FACS analysis was a predictor of outcome in men with HRPC.

PATIENTS AND METHODS

This was a retrospective study of 41 consecutively treated patients with metastatic HRPC who were starting systemic chemotherapy. All patients had peripheral blood collected before starting systemic cytotoxic chemotherapy with a taxane-based regimen. Subsequently, blood was collected at the start of each cycle of chemotherapy until therapy was discontinued. All 41 patients have had, and subsequently discontinued, second-line hormonal manipulations before entry to the present study. There were no uniform criteria applied for either the discontinuation of second-line hormonal therapy or the subsequent institution of systemic chemotherapy. For patients with measurable disease, progression was defined as a ≥20% increase in the sum of the longest diameter of target lesions or the appearance of one or more new lesions, as for the Response Evaluation Criteria in Solid Tumors system [28]. Patients with no measurable disease were required to have a positive bone scan and elevated PSA level. PSA evidence for progressive prostate cancer consisted of a PSA level of ≥5 ng/mL, which had risen above the minimum of the nadir and baseline on at least two successive occasions, at least 2 weeks apart. Response to therapy was assessed by Consensus Criteria [29]. There were no uniform criteria applied for the minimum or maximum number of peripheral blood collections required while patients were receiving systemic chemotherapy.

For the isolation and enumeration of CECs, blood samples were drawn into 10-mL EDTA-Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) to which a cell preservative was added [30,31]. Samples were maintained at room temperature and processed within 24 h after collection. All FACS analyses were performed at a central laboratory within our institution. For each sample the lymphocyte/monocyte fractions were separated using Ficoll-Hypaque density-gradient centrifugation. A positive-selection pre-enrichment step was used, by incubating the lymphocyte/monocyte fractions of each sample with ferrofluid particles coated with MJ37 (an anti-epithelial surface antigen encoded by the EGP2 or GA–733–2 gene, EpCAM) monoclonal antibody. The anti-EpCAM (EBA-1) antibody that recognises epitopes different from MJ37 was also added. The sample tube was then subjected to a magnetic field for 45 min in a magnetic separator and the sample blood aspirated from the tube. The sample tube was removed from the magnet and cells remaining in the tube were resuspended in 2 mL of cell buffer. The re-suspended cells were then transferred to one 12 × 75 mm polystyrene tube and subjected to magnetic separation for 5 min. The fluid material in the tube was aspirated and the pellet of cells was re-suspended in 150 μL of cell buffer. The antibody CD45 PerCP-Cy5.5 and a nucleic acid dye (ProCOUNT, Becton Dickinson) were added (20 μL). Fluorescently labelled monoclonal antibodies specific for leukocytes (CD45 PerCP-Cy5.5) and ECs (MJ-37 and EBA-1) are used to distinguish ECs from leukocytes. Samples were incubated in the dark for 15 min, and then fixed by adding 350 μL of 1% paraformaldehyde. Subsequently, samples were transferred to a TruCOUNT tube (Becton Dickinson) and then run on a FACS Calibur (Becton Dickinson) with four-colour option, until 35 000 bead events were acquired. Each sample was acquired with a threshold on both EpCAM (EBA-1) and nucleic acid dye (ProCOUNT). Circulating tumour cells were defined as nucleated cells, which are
Fisher's exact test for categorical variables, patient sample. Subsets were compared using statistics were used to characterize the entire of metastatic disease, details of previous including PSA level, Gleason score, extension time of entering the trial were collected, each patient's disease characteristics at the The data analysis was primarily descriptive; negative and CD45-negative [32,33].

**Mean (range) haemoglobin, g/dL (40)**
- 12.7 (2.0)
- Median (range)
- 12.7 (9.1–18.4)
- n (%) with <12.0
- 11 (28)

**Mean (so) LDH, U/L (40)**
- 174.4 (64.1)
- Median (range)
- 152.5 (129–470)

**ECOG performance status, n (%)**
- 0
- 26 (64)
- 1
- 10 (24)
- 2
- 5 (12)

**Gleason sum, n (%)**
- 5–6
- 7 (17)
- 7
- 21 (51)
- 8–9
- 13 (32)

**No. of previous systemic therapies**
- 0
- 1 (2)
- 1
- 15 (37)
- 2–3
- 21 (51)
- 4–5
- 4 (10)

**Median blood volume/sample, mL**
- 20

**Mean (so) volume sampled**
- 128.1 (197.5, 0–1005)

**Median (range) CECs/mL**
- 1.8 (0.0–55.8)

**Mean (so)**
- 6.97 (10.66)

**N (%) with CECs/mL of**
- 0
- 4 (10)
- 0.1–5.0
- 20 (49)
- >5.0–10.0
- 10 (24)
- >15.0–30.0
- 6 (15)
- >30.0
- 1 (2)

The number of peripheral blood collections in the patients varied; half (51%) had only one collection for FACS analysis just before starting chemotherapy, 49% had more than one collection, and 15% had 7–15 collections. Most patients (66%) had 20 mL of blood collected, and no patient had <9.5 mL collected. When analysed by the volume of blood obtained (<20 vs 20 mL) for the first collection, there was no difference in the number of CECs/mL (data not shown).

There were no CECs in the peripheral blood in only four patients; all four had bone metastases only and their Gleason score was 7 in two and 8 in two. There were no significant differences between this small subset and the entire cohort. Overall, 49% of patients had a Gleason score of 7, while in 32% it was 8–10. As defined by the consensus criteria, all patients had castrate testosterone levels. More than half of the patients (61%) had received at least two previous systemic therapies that included androgen deprivation, immunotherapy on a clinical trial, and secondary hormonal manoeuvres with agents such as antiandrogens, oestrogens and ketoconazole.

The data analysis was primarily descriptive; each patient's disease characteristics at the time of entering the trial were collected, including PSA level, Gleason score, extension of metastatic disease, details of previous therapy, and laboratory variables. Descriptive statistics were used to characterize the entire patient sample. Subsets were compared using Fisher's exact test for categorical variables, ANOVA methods for continuous variables and the nonparametric Mann–Whitney U-test to compare distributions. The association between continuous variables was estimated by the Spearman rank correlation coefficient. The Kaplan–Meier product-limit method was also used to estimate the probability of survival, with the log-rank test used to compare distributions of subsets. Survival was measured from the start of chemotherapy until either death or the date of last contact. Multivariate analyses were done using Cox proportional-hazards model to identify independent predictors of survival. A forward stepwise approach was used, with significance determined by the likelihood-ratio test. Coefficients for significant predictors were tested using the Wald statistic.

**RESULTS**

FACS analysis data from 41 patients with metastatic HRPC who initiated systemic taxane-based chemotherapy at our institution between 1999 and 2001 were included; their characteristics are summarized in Table 1. All patients had radiographic evidence of metastatic disease in either soft tissue, bone or both (39%, 61% and 22%, respectively). The initial median (range) PSA level for all evaluable patients was 50.2 (0.9–3019) ng/mL; 30% had PSA levels of <20 ng/mL and 80% had an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1. The median (range) alkaline phosphatase (ALP) level was 111 (59–1160) U/L and the median haemoglobin level was 12.7 (9.1–18.4) g/dL. Overall, 51% of patients had a Gleason score of 7, while in 32% it was 8–10. As defined by the consensus criteria, all patients had castrate testosterone levels. More than half of the patients (61%) had received at least two previous systemic therapies that included androgen deprivation, immunotherapy on a clinical trial, and secondary hormonal manoeuvres with agents such as antiandrogens, oestrogens and ketoconazole.

Among all patients the number of CECs/mL obtained at the time of first collection was significantly correlated with PSA level, age (inversely) and ALP levels, with a Spearman rank correlation, r, of 0.53 (P < 0.001), −0.33 (P = 0.04) and 0.38 (P = 0.02), respectively. At the time of the first collection the association was strongest between the number of CECs/mL and PSA level (P = 0.01). If a patient had a PSA level of <20 ng/mL, then 83% also had <1.8 CECs/mL (the median). There was more variability in range for the CECs/mL if the patient had a PSA level of >20 ng/mL but most (61%) had >1.8 CECs/mL. The decreasing concentration of CECs with increasing age

<table>
<thead>
<tr>
<th>Characteristic (n in sample)</th>
<th>Value</th>
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<tbody>
<tr>
<td>Median (range) age, years (40)</td>
<td>70.1 (44–89)</td>
</tr>
<tr>
<td>Median (range) initial PSA level, ng/mL (40)</td>
<td>50.2 (0.9–3019)</td>
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<tr>
<td>n (%) with PSA level of:</td>
<td></td>
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<tr>
<td>&lt;10.0</td>
<td>10 (25)</td>
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<td>10.0–100.0</td>
<td>17 (42)</td>
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<tr>
<td>&gt;100.0</td>
<td>13 (33)</td>
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<tr>
<td>Mean (so) ALP, IU/L</td>
<td>184.8 (211.8)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>111.0 (58.0–1160)</td>
</tr>
<tr>
<td>Mean (so) haemoglobin, g/dL (40)</td>
<td>12.7 (2.0)</td>
</tr>
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reflects that those patients aged <65 years (the lower age quartile) more often had more than the median value of 1.8 CECs/mL (67%), whereas those aged ≥75 years usually had fewer than the median (67%). Of all patients, 80% with ALP levels of >200 U/L (the upper quartile) had >1.8 CECs/mL ($P = 0.02$). By contrast, patients with ALP levels of <110 U/L (the median) were more likely to have <1.8 CECs/mL (65%), resulting in the increasing correlation. For the first collection there was no association between the concentration of CECs and lactate dehydrogenase (LDH), haemoglobin, ECOG performance status or the number of previous therapies. Using the overall median (1.8 CECs/mL) to dichotomize the patients, those with ≤1.8 CECs/mL had significantly longer survival than those with >1.8 CECs/mL. The median survival of patients with metastatic HRPC with >1.8 CECs/mL was 13 months; that for patients with ≤1.8 CECs/mL has not been reached ($P = 0.02$; Fig. 1).

Moreover, there were no associations between changes in serum PSA level, serum ALP and the number of CECs/mL with disease response while on therapy. Nevertheless, when several measurements were available, there were often similar patterns over time for CECs/mL, PSA and ALP levels; Fig. 2 shows an example of this relationship.

Additional univariate analyses indicated that having a PSA level of <20 ng/mL, ALP of ≤110 U/L, a Gleason score of ≤7 or having had only one previous therapy resulted in a more favourable survival outcome ($P = 0.01, 0.03, 0.05$ and $0.02$, respectively). Multivariate analyses using a Cox proportional-hazards model were used to identify significant independent predictors of survival from among those significant factors determined by univariate methods. This included CECs/mL (≤1.8 vs >1.8), PSA and ALP levels, Gleason score (≤7 vs 8–10) and the number of previous therapies (1 vs >1). Both CECs/mL and the number of previous therapies were independent predictors of survival (likelihood-ratio test, $P = 0.02$ for each factor; Table 2). The median survival for all patients was 18.4 months; 19 of the 41 patients died, all within 20 months of starting chemotherapy, and 10 survived beyond that time for up to 65 months from diagnosis.

**DISCUSSION**

This retrospective analysis evaluated the feasibility of using FACS analysis for detecting CECs in patients with HRPC; we also evaluated the correlation between the level of CECs and other clinical variables, e.g. PSA, ALP, LDH, and haemoglobin, all clinical features previously shown to affect the outcome in such patients.

Although the analysis was limited by being retrospective and including relatively few patients, CECs were present in the vast majority of the patients. There was no reference point to relate the time of collection.
with the course of disease, and hence any of the differences noted in this analysis only reflect the data at one point in time and not necessarily a common point for all patients. Therefore, these results require validation in a prospective trial, and cannot be universally applied to all patients with HRPC.

In the present analysis there were very strong correlations between the concentration of CECs, and serum PSA and ALP levels; hence, >1.8 CECs/mL, a serum PSA level of ≥20ng/mL and serum ALP levels of >110 U/L (the median values) were each strong predictors of a poorer outcome ($P = 0.02$, 0.01 and 0.03, respectively). Unfortunately, this limited study could not define an association between changes in serum PSA and ALP levels, and number of CECs/mL, with disease response. However, it was suggestive that the pattern of CECs/mL measured over time appeared to mirror the PSA pattern (with an increase or plateau) in an individual patient while on chemotherapy. Also, when several measurements were available, there were often similar patterns over time for CECs/mL and ALP levels. This reflects the correlation between these factors that was identified at the initial collection, and might suggest that in addition to clinical symptoms, serum PSA level, and imaging studies, CECs could potentially be used for predicting and assessing the response to systemic chemotherapy in patients with HRPC.

Similar to our data, Moreno et al. [34] reported their experience using FACS analysis for evaluating CECs in patients with advanced prostate cancer. Among their 26 patients with HRPC, the presence of ≥5 CECs/7.5 mL of blood was a strong predictor for survival outcome (hazard ratio 7.18, $P = 0.002$). After a multivariate Cox analysis the presence of CECs was of borderline significance in a model for predicting the survival in patients with HRPC (hazard ratio 4.18, $P = 0.056$). Similarly, their study showed that patients with <5 CECs/7.5 mL of blood had a median overall survival time of 2.5 years, compared with 0.5 years in patients with >5 CECs/7.5 mL ($P = 0.003$).

In the present study there were similar associations between the number of CECs/mL and overall survival. We also dichotomized the patient sample based on the overall median number of CECs/mL. With a median follow-up of >36 months, the overall median survival for all metastatic patients with >1.8 CECs/mL was 13 months, and the median for patients with ≤1.8 CECs/mL, overall or with metastases, has not been reached ($P = 0.02$).

Our multivariate analysis also indicated that CECs/mL and the number of previous therapies (which probably represents the extent of the disease process, and later stages in treatment) were each independent predictors of survival ($P = 0.02$ for each).

In summary, we showed that in addition to previously described clinical variables, measuring CECs in patients with HRPC can be used as a prognostic tool to predict the outcome. Having more CECs/mL appears to correlate with shorter survival in patients with metastatic HRPC. Our findings, combined with the results from others, suggest that CECs might be relevant and could be used to predict the outcome in patients with HRPC. Future clinical trials with chemotherapy or novel therapeutics in patients with HRPC should consider the prospective collection of peripheral blood for CEC analyses.

REFERENCES

15 Israeli RS, Miller WH Jr, Su SL et al. Sensitive nested reverse transcription


18 de la Taille A, Olsson CA, Buttyan R et al. Blood-based reverse transcriptase polymerase chain reaction assays for prostatic specific antigen: long term follow-up confirms the potential utility of this assay in identifying patients more likely to have biochemical recurrence (rising PSA) following radical prostatectomy. Int J Cancer 1999; 84: 360–4


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Abbreviations: HRPC, hormone-refractory prostate cancer; CEC, circulating epithelial cell; RT, reverse transcriptase; FACS, fluorescence-activated cell sorting; ALP, alkaline phosphatase; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase.