Detection of circulating tumor cells in patients with urothelial cancer

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Received 23 May 2008; revised 10 July 2008; accepted 7 August 2008

Background: Approximately 50% of patients with metastatic urothelial cancer (UC) respond to chemotherapy and several months of therapy is required to assess for radiographic response. Blood-based biomarkers may identify patients in whom a specific therapy provides clinical benefit, and this study sought to characterize circulating tumor cells (CTCs) in patients with metastatic UC.

Patients and methods: Peripheral blood from patients with metastatic UC was evaluated for CTCs using the CellSearch system. We assessed for associations between CTC counts and the number and sites of metastatic disease.

Results: CTC evaluations were carried out in 33 patients with metastatic UC. Fourteen of 33 patients (44%; 95% confidence interval 27% to 59%) had a positive assay (range 0–87 cells/7.5 ml of blood) with 10 patients (31%) having five or more CTCs. A significantly higher number of CTCs was seen in patients with two or more sites of metastases compared with those with less than one or one site of metastases (3.5 versus 0, \( P = 0.04 \)).

Conclusions: CTCs, detected by antibody capture technology, are present in 44% of patients with metastatic UC. Higher numbers of CTCs are seen in patients with a greater number of metastatic sites. One-third of patients have five or more CTCs providing a potential early marker to monitor response to chemotherapy.

Key words: bladder cancer, circulating tumor cells, urothelial cancer

Introduction

The presence of tumor-like cells in the peripheral blood was first reported by Ashworth [1] in 1869. Over the years, many technologies have been utilized to attempt to identify these circulating tumor cells (CTCs). The use of RT-PCR offered a sensitive technique that was also highly specific when the expression of target mRNAs was limited to the malignant cells [2]. Uroplakin markers are urothelium-specific markers that are often poorly expressed at the protein level but detectable at the messenger RNA level using RT-PCR. The identification of circulating urothelial cells expressing specific uroplakin heterodimers (UP1b/II) on the cell surface was highly predictive of recurrence. A recently reported approach using a microfluidic platform, mediated by the interaction of CTCs with epithelial cell adhesion molecule (EpCAM)-coated microposts under precisely controlled laminar flow conditions, detected CTCs in metastatic lung, prostate, pancreatic, breast and colon cancer with improved yield and purity compared with other techniques [3]. This approach remains investigational, and in this study we used immunomagnetic bead purification, the current standard technology employed to detect CTCs in the clinical setting.

The detection of CTCs has been well demonstrated in breast, colon, prostate and several other malignancies [4–6]. In patients with metastatic breast cancer, assessment of CTCs may represent an earlier and more reproducible indication of disease status than current imaging modalities [7]. This finding is important with the potential for monitoring response to treatment, particularly in this era of newer targeted agents where radiological response is not always easily defined. In prostate cancer, CTCs are inversely related to survival with higher CTC counts associated with poorer outcomes [8]. Patients with five or more CTCs have a worse survival than those with four or less [9]. CTCs also correlate with chemotherapy response much earlier than standard radiographic studies; benefit in terms of long-term survival for prostate cancer patients is seen when the CTC count decreases in response to chemotherapy just 2–5 weeks after starting chemotherapy [9].

The CellSearch system is a semiautomated technique using immunomagnetic capture to detect CTCs. An advantage of this system is that it is reproducible across different laboratories and
can identify CTCs in different cancer types [10]. To our knowledge, Naoe et al. [11] are the only investigators to report the detection of CTCs in UC patients using immunomagnetic capture. CTCs were present in 8 of 14 patients with metastatic UC but in 0 of 12 patients with nonmetastatic UC. If CTC detection in metastatic UC patients using this technology can be confirmed, and if CTC counts correlate with UC biology, then CTC counts may serve as a predictive UC marker to guide chemotherapy treatment decisions similar to patients with prostate cancer [9]. Therefore, we prospectively evaluated patients presenting to Memorial Sloan-Kettering Cancer Center (MSKCC) to determine the presence and incidence of CTCs detected using this system in a larger cohort of patients with metastatic UC and to assess for relationships between CTC number and clinical parameters including sites of metastases and the extent of disease burden.

patients and methods

study design

Patients with histologically confirmed UC and known metastases presenting to the Genitourinary Medical Oncology clinic at MSKCC between October 2007 and January 2008 were included. Blood was collected from patients with metastatic UC who were either newly diagnosed or had progressed on previous chemotherapy. A complete history, physical examination and laboratory studies, including complete blood count, chemistry panel and urinalysis, were carried out before obtaining fully informed consent. This study was conducted under an institutional review board-approved protocol allowing for the collection of tissue, blood and urine samples from patients with genitourinary cancers. All patients signed informed consent before phlebotomy. The Wilcoxon rank sum test was used to test for associations between CTC assay results, previous treatment and the sites and number of metastatic disease sites.

CTC measurement

Blood was drawn at the time of presentation, before the administration of chemotherapy. One 7.5 ml sample was collected in a CellSave tube containing cell preservatives (Immunicon). Using the CellSearch methodology, CTC number was determined in the MSKCC clinical laboratory using immunomagnetic isolation and immunofluorescent staining [4]. Epithelial cells were captured using ferroparticles coupled to an antibody-targeting EpCAM. Cells expressing EpCAM were separated in a magnetic field and the enriched samples were then stained with fluorescent-labeled mAbs. Nucleic acids were stained with 4,6-diamidino-2-phenylindole (DAPI), and epithelial cells were stained with anti-cytokeratin–phycoerythrin. White blood cells were excluded by negative staining for CD45. Stained cells were then analyzed on a fluorescent microscope using the cell track analyzer II (Immunicon), and CTCs were enumerated by the operator.

results

Thirty-three patients (nine females and 24 males) with metastatic UC were evaluated. Patient characteristics are detailed in Table 1. The median age was 68 years (range 40–83). Median CTC count was 0, range 0–87. Primary sites of UC included bladder (20), renal pelvis (10), ureter and/or renal pelvis (two) and unknown (one). There was no significant difference in the distribution of CTCs based on site of primary disease.

Patterns of metastatic spread included lung in 15 patients (45%), liver in nine patients (27%), bone in 13 patients (39%), lymph nodes in 20 patients (61%) and other sites in eight patients including testis (one), prostate (one) and soft tissue (six). Seventeen patients had two or more sites of metastases and 26 patients had received one or more chemotherapy and/or an investigational agent (median = 1 prior treatment regimen; range 0–5). There was no statistically significant difference in median CTC counts between patients who received prior chemotherapy (0, range 0–87) and patients who never previously received chemotherapy (6, range 0–12, P = 0.35). Five patients received treatment perioperatively and 21 patients were previously treated for metastatic disease. If only the 21 patients who received chemotherapy for metastatic disease were compared with chemonaive patients, there remained no difference in median CTC number (0 versus 0, P = 0.49).

Fourteen [44%; 95% confidence interval (CI) 27% to 59%] patients with metastatic UC had CTC assays assessed as positive with at least one CTC. CTC counts ranged from 0 to 87 cells/7.5 ml of blood. Ten (31%) patients had five or more CTCs. Patients with two or more metastases had a higher CTC count compared with patients with only one site of metastasis (3.5 versus 0, P = 0.04)(Table 2). There were no significant differences in the number of CTCs based on the site of metastases (Table 3). A trend toward higher median numbers of CTCs was seen in patients with bone metastases compared with patients without bone metastases (3.5 versus 0, P = 0.19) and in patients with liver metastases compared with patients without liver metastases (8 versus 1, P = 0.20). However, neither of these differences was statistically significant. The median number of CTCs was higher in those without lung metastases compared with those with lung metastases (1 versus 0, P = 0.17). There was no difference in median CTC value for patients with and without lymph node involvement (0 versus 0, P = 0.78).

One patient receiving protocol therapy with gemcitabine, carboplatin and bevacizumab had progression of disease suggested by an increase in CTC value from 12 to 93, 1 month before progression of disease was detected on computed tomography (CT) scan.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>33</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>24 (73%)</td>
</tr>
<tr>
<td>Female</td>
<td>9 (27%)</td>
</tr>
<tr>
<td>Median age</td>
<td>68 (40–83)</td>
</tr>
<tr>
<td>Primary site</td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>20 (61%)</td>
</tr>
<tr>
<td>Renal pelvis/ureter</td>
<td>12 (36%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Metastatic site</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>15</td>
</tr>
<tr>
<td>Liver</td>
<td>9</td>
</tr>
<tr>
<td>Bone</td>
<td>13</td>
</tr>
<tr>
<td>Lymph node</td>
<td>20</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
</tr>
</tbody>
</table>
disease. Patients with two or more metastatic sites had a correlation between the number of CTCs and the extent of characteristics than disease characteristics [9].

Cancers may be more a function of both technology and patient characteristics than disease characteristics [9].

A more recent study in prostate cancer reports a positivity rate of 62% using the same methodology as the prior study. Their study differed from the present study in that all four patients with nodal metastases had positive CTC assays in the prior study. This study confirms the ability of this methodology to detect CTCs in patients with UC. At first glance, the per cent of patients with positive assays in our study is lower that the 57% positivity rate reported by Naoe et al. [11]. However, this discrepancy across studies may simply reflect the smaller number of patients (14; 95% CI 33% to 79%) in the prior study with 95% confidence limits overlapping for both studies. Their study differed from the present study in that all four patients with nodal metastases had positive CTC assays in the prior study.

It has been suggested that CTCs are more readily detectable in UC than in other genitourinary cancers [12]. Using an older methodology, Meye et al. isolated CTCs in 53% of patients with UC (18 of 34 patients), compared with 42% (10 of 24 patients) with renal cell cancer and 37% (22 of 60 patients) with prostate cancer. A more recent study in prostate cancer reports a higher positivity rate of 62% using the same methodology as our study, suggesting that differences in CTC yields across diseases may be more a function of both technology and patient characteristics than disease characteristics [9].

This is the first study of CTC in metastatic UC to observe a trend toward higher median number of CTCs in patients with bone metastases compared with those without bone involvement (3.5 versus 0, \( P = 0.19 \)) and in patients with liver metastases compared with patients without liver metastases (8 versus 1, \( P = 0.20 \)). Although interesting, we believe that these observations are hypothesis generating due to the small sample size of this pilot study and provides the basis for further study. Although this observation suggests a correlation between CTC number and tumor burden [8], it is also possible that CTC number may more accurately correlate with tumor biology (find reference). In the study by Nagrath et al. [3], CTC number did not always correlate with tumor size, and it remains possible that other factors, such as tumor biology, are important. Only studies on a larger number of patients would clarify these correlations.

An intriguing finding in this study is that CTCs in UC patients may serve as a tumor marker to monitor early response to chemotherapy. Currently, UC patients undergoing chemotherapy are monitored for response using serial CT scans. Not only does this require months of therapy to assess response, no radiographic response criteria exist for patients with bone-only metastatic disease. In patients with metastatic breast cancer, assessment of CTCs is an earlier and more reproducible indication of disease status than current imaging methods [7]. We report that 31% of patients with metastatic UC disease have five or more CTCs and that 39% of patients with bone metastases have positive CTC assays. This threshold of five or more CTCs has been shown to be clinically useful in prostate cancer and holds potential promise in UC. In the study by Moreno et al. [9], prostate cancer patients whose CTC counts were less than five had a median survival twice that of patients with CTC five or more, 21.4 months versus 10.7 months. More importantly, patients in whom chemotherapy decreased CTC counts from ≥5 to <5 several weeks after starting chemotherapy had a median survival of 19.6 months compared with just 8.4 months in those patients whose CTC count did not drop below five. The five CTC threshold represents a similar, potentially useful response assessment in bladder cancer. There has been a recent case report that this phenomenon also exists for UC and that an increase in CTC level would provide an earlier indication of disease progression in patients with metastatic UC. In our study, one patient with progression of disease had an increase in CTC number before radiologically defined progression.

Immunomagnetic capture does have clinical limitations as a prognostic and predictive marker. This approach isolates only small numbers of cells with low purity (0.01–0.1) and low yield (20%–60% of cells per patient) [10, 14]. The required preparatory steps (centrifugation, washing and incubation) may also result in loss of cells. Clumped cells may be missed, further reducing the sensitivity of the assay. In addition, a statistically higher CTC count than those with a single site of disease. Using location of metastases alone is a crude measure of disease burden and may not accurately reflect the amount of metastatic cancer present. Nonetheless, this finding is in keeping with the correlation found between CTC counts and tumor burden in prostate cancer, as confirmed by prostate-specific antigen and bone scan index [8].

During the analyses, a number of cells that were dual positive for EpCAM and CD 45 were identified. Morphologically these cells did not resemble white blood cells. On the basis of the staining pattern, these cells were not considered as CTCs.

**Table 2.** CTCs and number of metastatic sites

<table>
<thead>
<tr>
<th>No. of metastatic sites</th>
<th>No. of patients (%)</th>
<th>CTC no. (range)</th>
<th>CTC no. (range) for 1 metastasis versus 2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12 (36)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>2</td>
<td>11 (33)</td>
<td>6 (0–87)</td>
<td>3.5 (0–87)</td>
</tr>
<tr>
<td>3</td>
<td>7 (21)</td>
<td>0 (0–51)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1 (3)</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1 (3)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

CTCs, circulating tumor cells.

**Table 3.** CTC number and metastatic sites

<table>
<thead>
<tr>
<th>n (%)</th>
<th>CTC no. with metastasis present (range)</th>
<th>CTC number with metastasis absent (range)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>15 (45)</td>
<td>0 (0–87)</td>
<td>1 (0–62)</td>
</tr>
<tr>
<td>Liver</td>
<td>9 (27)</td>
<td>6 (0–87)</td>
<td>0 (0–62)</td>
</tr>
<tr>
<td>Bone</td>
<td>13 (39)</td>
<td>3.5 (0–62)</td>
<td>0 (0–87)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>20 (61)</td>
<td>0 (0–62)</td>
<td>0 (0–87)</td>
</tr>
<tr>
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<td>9 (27)</td>
<td>0 (0–51)</td>
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</tr>
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CTCs, circulating tumor cells.

This is the largest study to date evaluating CTCs in patients with metastatic UC using immunomagnetic capture technology. Fourteen of 33 (44%) patients in this prospective study had detectable CTCs. This study confirms the ability of this methodology to detect CTCs in patients with UC. At first glance, the per cent of patients with positive assays in our study is lower that the 57% positivity rate reported by Naoe et al. [11]. However, this discrepancy across studies may simply reflect the smaller number of patients (\( n = 14; 95\% \) CI 33% to 79%) in the prior study with 95% confidence limits overlapping for both studies. Their study differed from the present study in that all four patients with nodal metastases had positive CTC assays in the prior study.

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while in the magnetic chamber cells can tear apart thereby impairing detection of cells, denying access to CTC DNA and preventing subsequent molecular analyses. While it is likely that cells detected by this assay represent circulating UC cells, EpCAM is a general epithelial marker. A more specific molecular target would allow greater certainty about the nature of the detected cells. The uroplakins represent more specific markers for UC with the addition of magnetic-labeled uroplakin antibodies potentially increasing the specificity for the detection of circulating UC cells [2]. One of the limitations of immunomagnetic bead purification is its use only as a gross prognostic tool that divides patients into high- and low-risk categories [5]. Using a more sensitive technique, such as microchip technology, may be of greater clinical impact: more efficient capture of bladder cancer T-24 cells using this approach has been reported [15].

We detected a number of cells that had dual positivity for both CD45 and EpCAM. Morphologically these cells did not resemble white blood cells and their origin remains unclear. The tumor stroma is composed of a variety of cells and one can speculate that these cells also circulate in the blood of patients with cancer. It is well known that the bone marrow contributes a variety of different cell types to the tumor stroma, including hematopoietic cells and vascular endothelial cells [16–18]. DePalma et al. [19] identified CD45-positive bone marrow-derived cells in mammary tumor allografts grown subcutaneously in mice. These cells homed to the tumor and interacted with endothelial cells at the tumor periphery. It is certainly possible that similar cells are required for metastases to develop in humans and that better cell capture techniques will enable identification of other circulating cells required for cancer metastases.

This prospective pilot study is limited by the relatively small number of patients. A larger prospective study would help to confirm the detection rate, assess possible relationships with specific sites of disease and determine the utility of CTCs as a marker of chemotherapy response in patients with UC. Moreover, a biomarker in UC is not currently used to help guide therapy in the metastatic setting but also may have utility in earlier stage disease to potentially select patients undergoing cystectomy for perioperative chemotherapy and to optimize patient selection for possible organ preservation. More sophisticated correlations of CTCs value in muscle-invasive disease can be envisioned to assess the need for chemotherapy beyond the current use of vascular invasion, extravesicle extension into fat or the status of regional lymph nodes.

In conclusion, we have confirmed that the immunomagnetic capture methodology can identify CTCs in a substantial proportion of patients with metastatic UC and that CTC count appears to correlate with disease burden. Future studies will determine the prognostic and predictive value of CTCs in patients with UC and further refinement of detection methods will allow for a better understanding of CTC biology.

funding

Work supported in part by the Gary Comer and the Wiener Funds.

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