Detection of Circulating Tumor Cells in Peripheral Blood of Patients with Renal Cell Carcinoma Correlates with Prognosis

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Abstract

Purpose: The aim of this study was to evaluate the clinical relevance of the presence of disseminated tumor cells in peripheral blood (so-called circulating tumor cells) for renal cell carcinoma patients.

Methods: Two hundred thirty-three peripheral blood samples from 154 renal cell carcinoma patients were investigated for the presence of disseminated tumor cells by autoMACS technique and immunocytochemical staining of cytokeratin. The frequency of circulating tumor cells was analyzed statistically for correlation with relevant clinical data.

Results: Two kinds of tumor cells were detected: those with expression of cytokeratin 8/18 (CK+) and cells without a detectable cytokeratin expression, which we called large blue-stained cells with a tumorlike morphology. After following the CD45 autoMACS depletion protocol, we identified circulating tumor cells in 96 (41%) of 233 peripheral blood samples, which originated from 81 (53%) of 154 renal cell carcinoma patients.

Conclusions: The presence of circulating tumor cells correlated with lymph node status and presence of synchronous metastases in renal cell carcinoma. It is important to evaluate CK+ and blue-stained tumor cells together to determine the role of circulating tumor cells in tumor behavior and disease progression. Detection of CK+ circulating tumor cells in peripheral blood is a significant and independent prognostic factor for renal cell carcinoma.

Introduction

Disseminated malignancy is responsible for most cancer-related deaths, whereas primary tumors account for only about 10% of cancer-related deaths (1). In contrast, metastases or recurrences of a previously diagnosed cancer are responsible for 90% of cancer-related deaths (2). During the tumor progression, disseminated tumor cells are generated, spread from the primary tumor, colonize distant organs, and lead to overt metastatic disease. Therefore, disseminated tumor cells are essential for establishing metastases. However, disseminated tumor cells are not sufficient because this process is highly inefficient and most cells will fail to grow in target sites (1). Numerous disseminated tumor cells die during migration, whereas others remain dormant for several years, and very few will grow into clinically detectable macrometastases (3). A better understanding of the biology of disseminated tumor cells, as well as the availability of more accurate tools to identify, quantify, and determine their capacity to establish metastases might lead to significant progress in risk assessment, and potentially provide novel therapeutic targets to prevent or treat metastases in cancer patients.

Disseminated tumor cells in the blood, so-called circulating tumor cells, are known to occur in the blood stream of cancer patients (4). However, the clinical relevance of their detection is still a subject of controversy, and their biology is poorly understood (1). Methods to detect circulating tumor cells have recently been extensively reviewed (4, 5). Briefly, there are two main methods: (a) immunologic assays using immunocytochemistry and (b) PCR-based assays, which investigate tissue-specific transcripts. Both methods include an enrichment of circulating tumor cells by immunomagnetic techniques (6), flow cytometry (7), Ficoll density centrifugation (8), or microchips (9, 10). Only the first method allows for the isolation and evaluation of circulating tumor cells by a pathologist. In this study, we did immunomagnetic circulating tumor cell enrichment by depletion of CD45-positive lymphocytes followed by epithelial cell–specific cytokeratin immunocytochemistry, as described previously (11-13).

Dissemination and implantation of tumor cells at distant sites is a complex multistep process that is affected by host and tumor molecular characteristics. Many questions about how this process is executed remain unanswered. There is a clear phenotypic and molecular heterogeneity...
The correlation of detection of circulating tumor cells in peripheral blood of renal cell carcinoma patients with prognosis suggests that circulating tumor cells can have an impact on detection of early metastases and may serve as a marker for monitoring therapeutic efficacy.

of disseminated tumor cells, which affects their efficiency in implanting in situ after dissemination and for the formation of micrometastases (14). Some groups have found that circulating tumor cells of inoperable breast cancer patients are associated with worse recurrence-free and overall survival (15–17). In contrast, others have reported that the presence of disseminated tumor cells is not convincingly associated with worse survival in operable breast cancer patients (8, 18, 19). In addition, the prognostic relevance of detection of disseminated tumor cells in the bone marrow of metastatic renal cell carcinoma patients has also been recently shown (20). However, the prognostic impact of circulating tumor cell detection in peripheral blood of renal cell carcinoma patients has not yet been determined.

In this study, we evaluated the presence of circulating tumor cells in peripheral blood samples of renal cell carcinoma patients after immunomagnetic cell enrichment with an automated magnetic cell separation. The rate of detection of circulating tumor cells was then correlated with the clinical data and impact on overall survival.

Materials and Methods

Tumor Patients and Blood Sampling. The patients taking part in the study were informed, and oral consent was obtained from each. Altogether, 233 peripheral blood samples from 154 renal cell carcinoma patients from the Clinic of Urology at the University Halle-Wittenberg were investigated during the period 2000 to 2006. EDTA− peripheral blood samples (16 mL) were collected either at the time of diagnosis, 5 to 7 d after tumor surgery, during chemotherapy, or at later time points of follow-up control investigations. The peripheral blood samples were stored at 4°C for up to 5 h until separation. No clinical decision was made based on the results of the magnetic cell enrichment experiments.

Density Gradient Centrifugation and autoMACS Depletion. The mononuclear cells were isolated from 16 mL blood sample obtained by standard ficoll gradient separation, as described previously (11, 12). Briefly, the mononuclear cells were washed once in PBS (containing 2 mmol/L EDTA) and resuspended in 600 μL PBS. Afterward, the mononuclear cell population was magnetically labeled with 40 μL CD45-conjugated microbeads, according to the manufacturer’s instructions (Miltenyi Biotec). For the magnetic enrichment of tumor cells, we used an autoMACS unit in the sensitive separation modus (12). Besides the positive fraction (CD45-labeled cell population), the negative fraction with the tumor cells was eluted in a total volume of 3 mL directly from the autoMACS onto a cytosin slide. Slides were further stained by immunocytochemistry. The renal cell carcinoma–derived cell line Caki-1 was used as positive control, and mononuclear cells from healthy noncancer patients served as negative controls (data not shown).

Immunocytochemistry and Pathomorphologic Evaluation. The magmagnetically nonlabeled cell population containing the putative tumor cell population was eluted onto a cytosin. After mild cytosin centrifugation at 600×g for 7 min (Hettich), the surface-pretreated slides (Superfrost Plus, Menzel Gläser) were air dried and investigated immediately by immunocytochemistry.

Circulating tumor cells with expression of cytokeratin 8/18 (CK+) were detected with an anticytokeratin antibody directed against cytokeratins 8 and 18, as described previously (13). Labeled cells were detected with a biotinylated secondary antibody and a streptavidin–alkaline phosphatase complex–mediated color reaction following standard procedure (Dako). Slides were evaluated by light microscopy, and circulating tumor cells were evaluated by an experienced pathologist (U. Bilkenroth). The numbers of abnormal cells with no, weak, and distinct cytokeratin expression were recorded per peripheral blood sample.

Statistics. The correlation between the presence of circulating tumor cells and clinical parameters was evaluated by $\chi^2$ tests. The threshold of significance was $P < 0.05$. To study the effect of the presence of circulating tumor cells on overall survival, Kaplan-Meier analyses and Cox’s regression hazard analyses were done. The multivariate Cox’s hazard regression models were adjusted to tumor number, stage, age, sex, and presence of metastases. $P < 0.05$ was defined as significant, and the relative risk was calculated. The statistical analyses were carried out using SPSS software version 11.0 (SPSS, Inc.).

Results

The Detection of Cytokeratin-Negative Large Blue-Stained (Bl+) Cells. In our study, two kinds of tumor cells were detected: CK+ circulating tumor cells and cells without a detectable cytokeratin expression. These cells were larger than the CK+ tumor cells and seemed dark blue after staining with hemalaun. Interestingly, we detected these large Bl+ cells more frequently than the CK+ tumor cells. In contrast, in control experiments with peripheral blood samples from healthy volunteers ($n = 30$), no CK+ cells and no abnormal large Bl+ cells were identified. Detection of typical blood cell markers was negative for these large Bl+ cells. There were no positive reactions for megakaryocytes (factor VIII; CD61), preliminary stages of erythropoiesis (glysophorin C), for immature and mature granulocytes (myeloperoxidase), for B-lymphocytes and their preliminary stages (CD20; CD79a),

![Figure 1. Two examples for large Bl+ cells with tumorlike morphology are given. The cytoplasm shows no cytokeratin expression. The picture on the far right is an example of a CK+ tumor cell.](image-url)
for typical and atypical plasma cells (VS 38c), and for macrophages (MAK 387, CD68; data not shown). The negative staining reactions clearly revealed that the large Bl+ cells did not originate from the blood cell system and they were not of mesodermal origin. The large Bl+ cells showed a tumor cell-like morphology, including an enlarged nucleus compared with cytoplasm (Fig. 1). Therefore, these were considered tumor cells.

Results for Peripheral Blood Samples of Renal Cell Carcinoma Patients. After CD45 autoMACS depletion, we identified circulating tumor cells in 96 of 233 peripheral blood samples (41%), which originated from 81 (53%) of 154 renal cell carcinoma patients (Table 1). In detail, seven peripheral blood samples (3.0%) from seven patients (4.5%) possessed CK+ circulating tumor cells, and in 72 peripheral blood samples (31%) from 59 patients (38%), large Bl+ tumor cells without a detectable cytokeratin expression were detected. Altogether, in 17 peripheral blood samples (7.3%) from 15 patients (9.7%), CK+ and Bl+ circulating tumor cells were identified (Table 1). The number of circulating tumor cells were in the range of 1 to 51 tumor cells (on average, 6 cells) per peripheral blood sample. Of 81 patients with circulating tumor cells, 36 patients had one peripheral blood samples analyzed (44%) and 45 (56%) patients had at least two peripheral blood samples analyzed.

The results indicate a significant correlation between the detection of circulating tumor cells and lymph node status ($P = 0.001$; $\chi^2$ test) and the presence of metastases at the time of primary tumor resection ($P = 0.014$; $\chi^2$ test; Table 2). Circulating tumor cells were identified in 42% of N0 patients (51 of 120) and in 43% of M0 patients (47 of 105) but in 86% of N1 patients (19 of 22), 91% of N2 patients (10 of 11), and 67% of M1 patients (33 of 49). Notably, occurrence of Bl+ circulating tumor cells was also significantly correlated with lymph node status ($P < 0.001$; $\chi^2$ test) and the presence of metastases ($P = 0.003$; $\chi^2$ test; Table 2).

Altogether, 14 (24%) of 59 renal cell carcinoma patients with large Bl+ tumor cells and 9 (41%) of 22 renal cell carcinoma patients with CK+ circulating tumor cells died. On the other hand, only 13 (18%) of 73 renal cell carcinoma patients without circulating tumor cells died. In a multivariate Cox's regression hazard model (adjusted to tumor size, tumor metastases, age, and sex), the presence of CK+ circulating tumor cells was associated with poor overall survival for renal cell carcinoma patients. Renal cell carcinoma patients with CK+ circulating tumor cells in their peripheral blood had a 2.3-fold increased risk for tumor-related death compared with patients without these cells ($P = 0.048$, Table 3; Fig. 2). In contrast, the detection of Bl+ circulating tumor cells did not affect survival of renal cell carcinoma patients. When patients were separated into those with CK+ circulating tumor cells before or after primary tumor resection (keeping the lower number of patients in mind), there was still a significant correlation between detection of CK+ circulating tumor cells before and after primary tumor resection and prognosis [relative risk, 2.7 ($P = 0.049$) and 4.3 ($P = 0.036$); Table 3].

Altogether, for renal cell carcinoma patients, the presence of circulating tumor cells was associated with the existence of lymph node and distant metastases and significantly correlated with an increased risk for tumor-related death.

Discussion

In this study, we investigated 233 peripheral blood samples from 154 renal cell carcinoma patients. The median follow-up time was 15 months (1-56 months). Most peripheral blood samples (140 of 233) were collected before primary tumor resection. We detected circulating tumor cells in 53% of the renal cell carcinoma patients and in 41% of the peripheral blood samples investigated. In two previous studies where we applied a semi-automated CD45 depletion protocol using an autoMACS, CK+ circulating tumor cells were identified in the peripheral blood of 32% and 42% of renal cell carcinoma patients (11, 12). In this study, we analyzed large Bl+ tumor cells without any expression of cytokeratin and CK+ circulating tumor cells together, which has not been previously done. The moderately higher positive rate of these circulating tumor cells in the postoperative (45%) peripheral blood samples compared with the preoperative ones (37%) is possibly due to a dissemination of circulating tumor cells into the blood during tumor resection because Oefelein and colleagues (21) reported that operations can cause a low hematogenous dissemination of tumor cells.

The presence of circulating tumor cells in peripheral blood did not correlate with either tumor size or tumor grade. Remarkably, circulating tumor cells were found in all four tumor sizes (pT1-pT4) and in all tumor grades (G1-G4) in comparable levels in about 50% of the patients. The detection of Bl+ and CK+ circulating tumor cells was significantly correlated with the presence of lymph node metastases ($P < 0.001$; $\chi^2$ test) and long-distance metastases.

<table>
<thead>
<tr>
<th>Table 1. Detection rates of circulating tumor cells in peripheral blood samples of renal cell carcinoma patients</th>
</tr>
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<tbody>
<tr>
<td><strong>Patients</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>CK+</td>
</tr>
<tr>
<td>7 (4.5 %)</td>
</tr>
<tr>
<td>7 (3.0 %)</td>
</tr>
<tr>
<td>1 (0.7 %)</td>
</tr>
<tr>
<td>4 (5.2 %)</td>
</tr>
<tr>
<td>Immunotherapy</td>
</tr>
<tr>
<td>Care</td>
</tr>
<tr>
<td>Postoperative</td>
</tr>
<tr>
<td>Immunotherapy</td>
</tr>
<tr>
<td>Care</td>
</tr>
<tr>
<td>Positive/total</td>
</tr>
</tbody>
</table>

Abbreviation: CTCs, circulating tumor cells.

<table>
<thead>
<tr>
<th>Table 2. Statistical results for clinical data of renal cell carcinoma patients</th>
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</thead>
<tbody>
<tr>
<td><strong>Presence of tumor cells</strong></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Presence of Bl+ CTCs correlates with pN</td>
</tr>
<tr>
<td>Presence of CK+/Bl+ CTCs correlates with cN</td>
</tr>
<tr>
<td>Presence of Ck+ /Bl+ CTCs correlates with cM</td>
</tr>
<tr>
<td>Presence of Bl+ CTCs correlates with cM</td>
</tr>
</tbody>
</table>

Abbreviation: CTCs, circulating tumor cells.
Table 3. Cox’s regression hazard analyses for overall survival of renal cell carcinoma patients with/without detection of CK+ circulating tumor cells

<table>
<thead>
<tr>
<th>Multivariate Cox’s regression hazard analysis</th>
<th>P</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK+ CTCs before primary tumor resection</td>
<td>0.049</td>
<td>2.7</td>
</tr>
<tr>
<td>CK+ CTCs after primary tumor resection</td>
<td>0.036</td>
<td>4.3</td>
</tr>
<tr>
<td>CK+ CTCs before and after primary tumor resection</td>
<td>0.048</td>
<td>2.3</td>
</tr>
</tbody>
</table>

NOTE: Detection of CK+ circulating tumor cells includes patients with only CK+ cells and those with CK+ and Bl+ cells in their peripheral blood. Abbreviation: CTCs, circulating tumor cells.

(\( P = 0.014; \chi^2\) test). Notably, only the detection of large Bl+ cells was significantly correlated with lymph node status (\( n = 153 \)) and the presence of metastases (\( n = 152 \)). Only a few studies to date have presented data to correlate the presence of circulating tumor cells or transcripts of epithelial cells in peripheral blood of renal cell carcinoma patients with their prognostic relevance (Table 4). Two studies showed circulating tumor cells in 47% and 49% of renal cell carcinoma patients (22, 23), and similar to this study, they also did not find any correlation between the presence of circulating tumor cells and tumor grade or tumor size. Shimazui and colleagues (24) detected circulating tumor cells in 45% of renal cell carcinoma patients by reverse transcription-PCR (RT-PCR), and again, no correlation between the presence of circulating tumor cells in peripheral blood and tumor grade and size was noted. In contrast to our findings, however, the presence of circulating tumor cells in this study did not significantly correlate with the presence of metastases. Two other groups have reported that 37.5% to 45.7% of renal cell carcinoma patients have circulating tumor cells in their peripheral blood (25, 26). However, the prognostic relevance of the presence of these tumor cells was not evaluated.

In addition, one study on bone marrow samples detected disseminated tumor cells in 25% of renal cell carcinoma patients (27). At the follow-up (median, 40 months) 14% of patients with CK+ disseminated tumor cells in their bone marrow suffered from tumor progression in contrast to 11% of patients without CK+ disseminated tumor cells. There is only one report to date, by Buchner and coworkers (20), that shows that detection of disseminated tumor cells in metastatic renal cell carcinoma patients is an independent prognostic factor. They detected CK+ circulating tumor cells in bone marrow samples of 42% of M1 patients and in 25% of M0 renal cell carcinoma patients. No CK+ circulating tumor cells were detected in the control group. In multivariate analysis, the detection of three or more CK+ cells in the bone marrow was found to be an independent prognostic factor (\( P < 0.001 \)). In the current study, we showed that detection of CK+ circulating tumor cells before and/or after primary tumor resection correlated with a poor prognosis. Detection of Bl+ circulating tumor cells was not associated with overall survival. Bl+ circulating tumor cells are probably less aggressive in their behavior, could accompany inflammation reactions, or may stay longer in the bloodstream (dormancy) until they form metastases. However, more studies, especially prospective ones, are needed before the impact of the presence of circulating tumor cells/disseminated tumor cells in renal cell carcinoma patients on prognosis and clinical decisions can be stated conclusively.

For the evaluation of the prognostic impact of circulating tumor cells, molecular characterization will especially be of importance. Klein and colleagues (14) were the first to show that disseminated tumor cells before primary tumor resection are heterogeneous in their genetic alterations, whereas this heterogeneity was strictly reduced with the emergence of clinically evident metastases. For molecular characterization of these cells, receptors, such as ERBB2, uPAR, and CXCR4, and stem cell markers, such as ALDH1, CD44, and BM11, could be promising candidates (reviewed in ref. 4). It would be of interest if genes associated with (cancer) stem cell self-renewal, which also have prognostic impact for cancer patients (28), play a role in disseminated tumor cell/circulating tumor cell dormancy. Meng and colleagues (29) showed that uPAR and ERBB2/HER-2 gene status in breast cancer cells from blood and tumor tissue are concordant. Furthermore, Stoecklein et al. (30) found that a gain of the HER-2 gene in disseminated tumor cells correlates with a high risk for early death for esophageal cancer patients. Molecular analysis of circulating tumor cells will not only potentially affect prognosis but may also allow for the possibility of monitoring changes in epithelial tumor genotypes during the course of treatment, as was recently suggested for lung cancer patients (31).

Altogether, our results suggest that detection of CK+ circulating tumor cells in peripheral blood of renal cell carcinoma patients is an independent prognostic marker. Further studies are needed to identify, characterize, and target circulating tumor cells, which have the ability to form metastases, escape the immune response, and resist cancer therapies.
Circulating Tumor Cells and Renal Cell Carcinoma

Table 4. Survey of detection rates of disseminated tumor cells/circulating tumor cells in renal cell carcinoma and correlation to clinical data

<table>
<thead>
<tr>
<th>CTCs/DTCs</th>
<th>Method</th>
<th>Marker(s)</th>
<th>Detection rates (%)</th>
<th>Clinical factors</th>
<th>Association to prognosis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCs</td>
<td>RT-PCR</td>
<td>CK19</td>
<td>47</td>
<td>Stage</td>
<td>n.d.</td>
<td>20</td>
</tr>
<tr>
<td>CTCs</td>
<td>RT-PCR</td>
<td>MN/CA9†</td>
<td>47</td>
<td></td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>CTCs</td>
<td>RT-PCR</td>
<td>VHL gene mutation</td>
<td>21.7</td>
<td></td>
<td>n.d.</td>
<td>23</td>
</tr>
<tr>
<td>CTCs</td>
<td>ICC</td>
<td>panCK, CK18</td>
<td>28.5</td>
<td>G</td>
<td>n.d.</td>
<td>11</td>
</tr>
<tr>
<td>CTCs</td>
<td>ICC</td>
<td>panCK, CK18</td>
<td>34.5</td>
<td></td>
<td>n.d.</td>
<td>12</td>
</tr>
<tr>
<td>DTCs</td>
<td>RT-PCR</td>
<td>Cadherin-6</td>
<td>25</td>
<td>Stage</td>
<td>n.d.</td>
<td>13</td>
</tr>
<tr>
<td>CTCs</td>
<td>RT-PCR</td>
<td>Cadherin-6</td>
<td>45.7</td>
<td></td>
<td>n.d.</td>
<td>26</td>
</tr>
<tr>
<td>CTCs</td>
<td>ICC</td>
<td>panCK, CK18</td>
<td>29</td>
<td>Stage</td>
<td>n.d.</td>
<td>20</td>
</tr>
<tr>
<td>DTCs</td>
<td>ICC</td>
<td>panCK, CK18</td>
<td>43</td>
<td>Stage</td>
<td>n.d. CK+ indep. prog. factor</td>
<td>20</td>
</tr>
<tr>
<td>CTCs</td>
<td>ICC</td>
<td>panCK, CK18</td>
<td>41</td>
<td></td>
<td>M ≥3 CK+ indep. prog. factor</td>
<td>This study</td>
</tr>
</tbody>
</table>

Abbreviations: n.d., not determined; indep. prog. factor, independent prognostic factor; CK, keratin; CTCs, circulating tumor cells; DTCs, disseminated tumor cells.
† A45-B/B3 antibody against common cytokeratin epitope (cytokeratin 8, 18 and 19).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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