High detection rate of circulating tumor cells in blood of patients with prostate cancer using telomerase activity


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Background: Circulating tumor cells (CTCs) cannot be readily detected with currently available methods in the majority of patients with prostate cancer. Telomerase activation, one of the major immortalization events, is found in most cases of prostate cancer. We attempted to develop a method using telomerase activity to isolate CTCs in patients with prostate cancer.

Patients and methods: Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll–Hypaque. Immunomagnetic beads coated with an epithelial cell-specific antigen antibody (BerEP4) were used to harvest epithelial cells from PBMCs. Telomerase activity was detected in harvested epithelial cells using the telomerase–PCR–enzyme-linked immunosorbent assay method.

Results: Blood samples from 107 patients with prostate cancer were studied. CTCs were detected in 19 of 24 (79%) patients with advanced prostate cancer. In contrast, CTCs were not detected in blood samples from 22 healthy male volunteers. CTCs were even identified in patients with an undetectable (<0.1 ng/ml) serum prostate-specific antigen (PSA). CTCs were detected in 55 of 70 (79%) patients with localized prostate cancer before radical prostatectomy (n = 30) or brachytherapy (n = 40). CTCs were also detected in 3 of 13 patients (23%) with an undetectable serum PSA measured at least 1 year after radical prostatectomy, which is consistent with the expected relapse rate in this setting.

Conclusion: CTCs can be detected using telomerase activity in a large majority and a wide variety of patients with prostate cancer, including those with localized disease.

Key words: circulating tumor cells, prostate cancer, telomerase

introduction

Metastatic prostate cancer is the lethal form of the disease. Progression to metastases involves a series of molecular and physical events that result in the destruction of the extracellular matrix, intravasation, circulating tumor cells (CTCs) in the blood, extravasation, and eventually deposition at and proliferation to target sites, especially bones [1]. Detecting CTCs after definitive local therapy may have an impact on apparently localized prostate cancer by allowing better selection of patients for systemic treatments, provided CTC detection is feasible in this setting and is associated with the subsequent emergence of distant metastases. So far, this has not been formally proven. Obtaining tumor material in patients with metastases from prostate cancer has, however, been a challenge because most metastases involve the bone, which is not usually readily accessible and is sometimes painful to biopsy. Nowadays, however, the availability of tumor material is tremendously important since molecular or pathway-targeting agents are emerging as very promising treatments in oncology [1].

Although reports on the isolation of CTCs in prostate cancer patients are accumulating, one major weakness has been the relatively small series of patients, even with metastatic disease where CTCs can be readily detected with published methods [2]. Here, we report on a method based on telomerase activity which is able to detect CTCs with a high sensitivity and excellent specificity in a large series of patients with prostate cancer at various disease stages.

patients and methods

patients

One hundred and seven patients with histologically confirmed prostate cancer were included in this study. Blood samples were collected before any treatment of patients with localized prostate cancer and while under androgen deprivation therapy for patients with metastases. According to the clinical status and treatment, patients were divided into three groups: those with localized prostate cancer, those with undetectable prostate-specific antigen (PSA) after radical prostatectomy, and those with advanced prostate cancer. Twenty-two healthy male volunteers, aged 34–60 years, were selected as a negative control group. A Fischer’s exact test was used to

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evaluate the association between patients and tumor characteristics and the presence of CTCs.

blood samples and immunomagnetic separation of circulating epithelial cells
Blood samples (20 ml) were collected in heparinized tubes and stored at 4°C for a maximum of 2 h before experiments. Epithelial cells were separated, as previously described [3]. Briefly, peripheral blood mononuclear cells were isolated using Ficoll–Hypaque and resuspended in 1 ml of phosphate-buffered saline (PBS)–2% fetal calf serum (FCS). Then, 12.5×10⁴ prewashed immunomagnetic beads covalently coated with the BrefEp4 mAb (Dynal A.S., Oslo, Norway) were added. BrefEp4 mAb recognizes an epitope on the protein moiety of two glycopeptides (34 and 39 kDa) expressed at the surface of epithelial cells in normal and malignant tissues [4]. Following incubation at 4°C for 30 min, bead-bound cells were harvested using a magnetic field. Harvested epithelial cells (HECs) were then washed six times with PBS–2% FCS. Washing efficiency was controlled by microscopic examination to verify that the samples only contained immunomagnetic bead-coated cells. Cells were then stored at −80°C in two Eppendorf tubes for further experiments.

telomerase assay
HECs were re suspended in 100 μl of lysis buffer 3-[3-Cholamidopropyl]dimethylenammonio]-1-propanesulfonate as zwitterionic detergent (CHAPS) and incubated for 30 min at 4°C. The lysates were centrifuged at 16 000 × g for 20 min at 4°C, and the supernatants were transferred into fresh Eppendorf tubes and stored at −80°C until use. Protein concentration was measured in each extract using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). Telomerase activities were assessed using the TRAPeze enzyme-linked immunosorbent assay telomerase detection kit (Oncor, Gaithersburg, MD) according to the manufacturer’s instructions. Assays were carried out twice in independent experiments on 1 μg of lysates. In case of negative results, increasing amounts of proteins were tested up to 10 μg. First, we incubated lysates in the presence of a biotinylated telomerase substrate oligonucleotide (b-TS) at 30°C for 30 min. Then, the extended products were amplified by PCR using Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), b-TS, reverse primer (RP) primers, and a deoxynucleotide mix containing deoxycytidine triphosphate (dCTP) labeled with dinitrophenyl. The primer sequence was as follows: b-TS 5′-AATCCGCGACGACGAGTT-3′. The PCR conditions were 33 cycles at 94°C for 30 s and 55°C for 30 s. The telomerase repeat amplification protocol (TRAP) extension/amplification reaction is carried out with biotinylated primer and dNTP-labeled dCTP. Thus, the TRAP products are tagged with dinitrophenyl. The labeled products are immobilized on to streptavidin-coated microtiter plates via biotin–streptavidin interaction, and then detected by an anti-DNP antibody conjugated to horseradish peroxidase (HRP). The amount of TRAP products is determined by means of the HRP activity using substrate 3,3′,5,5′-tetramethylbenzidine and subsequent color development. Each sample was tested with a heat-inactivated (85°C for 10 min) or ribonuclease-treated aliquot. Reagent controls that lacked cell extracts were also systematically tested.

results
CTCs in patients with advanced prostate cancer
Overall, 24 patients with metastases of prostate cancer underwent tests to identify CTCs using telomerase activity. Their median age was 70 years (range 60–78). Median serum PSA was 2.5 ng/ml (range 0.1–88). Most of these patients (20 of 24, 83%) were on androgen deprivation therapy when the blood sample was obtained. CTCs were identified in 19 of 24 [79%; 95% confidence interval (CI) 58–93] of these patients. There was no association between the detection of telomerase-positive CTCs and serum PSA or tumor stage. CTCs were identified even in patients with a very low serum PSA level (<0.1 ng/ml). They were detected in both patients with androgen-sensitive and androgen-independent prostate cancer.

CTCs in patients with localized prostate cancer
A series of blood samples from 70 patients with localized prostate cancer treated at two different institutions was collected. Blood was obtained 2 days before local treatment, which consisted of radical prostatectomy (n = 30) or brachytherapy (n = 40). The interval between biopsies of the prostate and blood sampling was at least 1 month. Patient characteristics of this group are summarized in Table 1. CTCs were identified in 55 of 70 patients (79%; 95% CI 67–87) using telomerase activity. There was no association between CTC detection and patient or tumor characteristics [Gleason score, TNM (tumor–node–metastasis) classification (1997), and serum PSA].

CTCs in patients with undetectable serum PSA after prostatectomy
A series of 13 patients who had undergone a radical prostatectomy for localized prostate cancer at least 1 year earlier were studied. Serum PSA was undetectable (<0.1 ng/ml) in all these patients at the time of the study. Their characteristics are summarized in Table 2. CTCs were identified in 3 of 13
Table 2. Characteristics of patients with undetectable serum PSA after radical prostatectomy

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n = 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>65 (58–73)</td>
</tr>
<tr>
<td>pTNM classification</td>
<td></td>
</tr>
<tr>
<td>pT2a</td>
<td>4</td>
</tr>
<tr>
<td>pT2b</td>
<td>7</td>
</tr>
<tr>
<td>pT3a</td>
<td>2</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
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<tr>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Median serum PSA before prostatectomy (ng/ml) (range)</td>
<td>6.5 (2.2–13.6)</td>
</tr>
<tr>
<td>Detection of CTCs (%)</td>
<td>3/13 (23%)</td>
</tr>
</tbody>
</table>

PSA, prostate-specific antigen; CTCs, circulating tumor cells.

discussion

Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric repeats on to chromosomal ends using a segment of its own RNA component as a template. Telomerase activity is involved in telomere maintenance and is one of the major immortalization events [5]. Telomerase activation has been described in many neoplasms. In prostate cancer, telomerase activation has been reported in up to 92% of cases [6, 7]. Moreover, the PCR-based TRAP assay evaluates telomerase activity with a high sensitivity. Taken together, this makes the detection of telomerase activity an excellent candidate for identifying immortalized cells from the blood of patients with cancer. We have developed a test in which epithelial cells are first harvested from the blood mononuclear cell fraction using epithelial cell antibody-coated magnetic nanoparticles, and immortalized cells are identified among epithelial cells by measuring telomerase activity. This technique was previously used in a smaller series of patients with breast cancer and bladder cancer with a detection rate of 21 of 25 (84%) and 27 of 30 (90%), respectively [3, 8, 9]. Not only does telomerase activity identify immortalized cells (CTCs) but it also has the added advantage of insuring that these cells are alive, since enzymatic activity is measured (and not a PCR transcript that could arise from cellular debris). When tested using MCF7 breast cancer cells, this method was able to identify as low as 12 cells/ml [3].

We demonstrate here that CTCs can be identified and isolated with this method in >75% of patients with prostate cancer. It is noteworthy that this method proved valid, not only in advanced cases but also in patients with untreated localized cancer, in contrast with most other techniques [10, 11]. This technique was very cancer specific since none of the 22 healthy male controls had a positive test. Interestingly, and in contrast with some previous reports using different techniques to isolate CTCs [12], our method allows to identify CTCs in a variety of prostate cancers since CTCs detection was not related to the serum PSA level, the Gleason score, or the TNM classification. Of note, this is perfectly consistent with previous studies on prostatectomy specimens which showed that telomerase activity is quite universal in prostate cancer and that it is not linked to the tumor burden, tumor differentiation, and preoperative serum PSA [7, 13]. To our knowledge, the high CTCs detection rate achieved both in patients with localized prostate cancer and those with advanced disease with our technique in this large series far exceeds the detection rates reported with other techniques, including mostly PSA and prostate-specific membrane antigen amplification by RT-PCR. For example, the detection rate of CTCs from the blood in large studies using RT-PCR directed against PSA is typically in the 40% and 50% range in patients with localized and advanced prostate cancer, respectively [14–20]. Moreover, the association between the detection of CTCs using RT-PCR and PSA messenger RNA as a target and the prognosis in patients with localized disease is debatable [21–24], although such an association was found, at least in some studies, in patients with metastatic prostate cancer [25, 26] and those with a rising serum PSA level after definitive local treatment [27]. Alternative techniques to identify CTCs in prostate cancer patients by coupling a BerEP4 and PSA staining [28], targeting the cell adhesion molecule [29], or using automated fluorescent microscopy [12] are currently under study.
Potential applications of this method for detecting CTCs using telomerase activity in prostate cancer patients may include early diagnosis, prognosis assessment in localized disease, and treatment monitoring, and this may be an easier way to obtain tumor material rather than carrying out a bone biopsy in patients with metastases, this last application being tremendously important to guide targeted therapy. Future developments of this technique may include a study of the expression of selected genes on isolated CTCs. Protocols which allow the extraction of both proteins (for telomerase assay) and RNA (to study gene expression by RT-PCR) have been developed. In a recent study, we previously showed that it was possible to amplify HER-2/neu on CTCs isolated from blood using magnetic nanoparticles coupled with the BerEP4 mAb in 6 of 11 patients with metastases from prostate cancer [30]. Ideally, a study of a large number of genes using microarray technology could be attempted. The small amount of RNA extracted from CTCs using our technique, however, is a potentially limiting factor and a technological challenge.

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References

22. de la Taille A, Olsson CA, Buttryn R et al. Blood-based reverse transcription polymerase chain reaction assays for prostate 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: 360–364.