

Original Article

Circulating tumor cells using hTERT-specific replication-selective adenovirus in patients with soft tissue sarcoma

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Abstract: The presence of circulating tumor cells (CTCs) in peripheral blood offers a useful prognostic factor and tool for measuring the effects of treatment for various carcinomas. We attempted to detect viable CTCs in peripheral blood from sarcoma patients using a telomerase-specific viral agent. We examined correlations between numbers of CTCs and other clinical features of sarcoma. For CTC analysis, 20 blood samples were obtained from 10 patients with soft-tissue sarcoma of the trunk or extremities before and after surgery. Five patients were diagnosed with grade 2 sarcoma and five were diagnosed with grade 3 sarcoma according to the French Federation of Cancer Centers Sarcoma Group System. Mean postoperative follow-up was 36.6 months (range, 12-49 months). Oncological prognosis was as follows: 6 patients remained continuously disease-free, 2 patients showed no evidence of disease, and 2 patients died of the disease. Three patients developed lung metastases after surgery. We incubated 7.5-ml blood samples with a telomerase-specific, replication-selective, oncolytic adenoviral agent carrying the green fluorescent protein (GFP) gene, which allowed for the detection of viable CTCs. GFP-positive cells were counted using fluorescence microscopy. The mean number of CTCs showed no significant difference between preoperatively (1.9; range, 0-6) and postoperatively (2.6; range, 0-18; $P=0.704$). All patients with increased numbers of CTCs postoperatively compared to preoperatively displayed lung metastases. The number of postoperative CTCs correlated significantly with metastasis ($P=0.022$) and life prognosis ($P=0.014$). A significant relationship was found between the number of CTCs and both occurrence of lung metastasis and prognosis in patients with sarcoma. Detection of CTCs using telomerase-specific viral agent may prove useful for prognostic evaluation.

Keywords: Circulating tumor cells, human telomerase reverse transcriptase, adenovirus, sarcoma

Introduction

Soft-tissue sarcomas are relatively rare neoplasms arising from mesenchymal tissues. Sarcomas exhibit specific molecular characteristics and poor prognosis due to the tendency to spread to distant organs, particularly the lungs, via the vascular system [1, 2]. Circulating tumor cells (CTCs) in peripheral blood may thus be very important for analyzing the metastatic biology of sarcoma [3, 4]. Recently, the presence of CTCs in the blood has been shown to offer a clinically useful biomarker for early detection, prognosis, and assessment of aggressiveness. CTCs also serve as a surrogate marker of treatment effects in various car-

cinomas [5-8]. Distinguishing normal from neoplastic tumor tissues with selective labeling of tumor cells is necessary for detecting CTCs. In humans, telomeres are present at the ends of chromosomes and comprise specialized structures containing (TTAGGG)_n repeats [9]. Telomerase contains an RNA-dependent DNA polymerase, which is a cellular reverse transcriptase that contributes to telomere maintenance by adding new DNA repeats onto telomeric ends [10], enabling cellular immortality [11]. Telomerase holo-enzyme core components include human telomerase reverse transcriptase (hTERT), a catalytic reverse transcriptase. Quantification of hTERT mRNA expression

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Table 1. All clinical data and CTCs

Age	Gender	Site	Pathology	Grade	Pre.-CTC	Post.-CTC	Metastasis	Prognosis	Period (months)
86	Femal	Thigh	Myxofibrosarcoma	G2	1	1	None	CDF	26
84	Male	Chest	UPS	G3	3	0	None	CDF	45
82	Male	Chest	Myxofibrosarcoma	G2	0	0	None	NED	48
71	Male	Lower leg	Myxofibrosarcoma	G3	0	0	None	CDF	48
77	Femal	Thigh	Pleomorphic liposarcoma	G3	0	3	Lung	DOD	12
64	Femal	Lower leg	Myxofibrosarcoma	G3	4	18	Lung	DOD	28
82	Male	Chest	Pleomorphic liposarcoma	G3	0	4	Lung	NED	25
67	Femal	Lower leg	Myxofibrosarcoma	G2	0	0	None	CDF	49
44	Male	Chest	Fibrosarcoma	G2	5	0	None	CDF	45
70	Femal	Iliopsoas	Leiomyosarcoma	G2	6	0	None	CDF	40

UPS; undifferentiated pleomorphic sarcoma. CDF; continuous disease free. NED; no evidence of disease. DOD; dead on disease.

can be used as an alternative measure of telomerase activity [12, 13]. Expression of hTERT is not seen in most somatic cells [14], but is evident in more than 85% of carcinomas [15, 16] and more than 90% of sarcomas [17-19]. OBP-401 and OBP-1101 are telomerase-specific replication-competent adenovirus variants in which the hTERT promoter drives expression of E1A and E1B genes linked to an internal ribosome entry site and GFP, which is inserted into the E3 region under control of the *cytomegalovirus* promoter [20, 21]. Almost all carcinoma and sarcoma cells express hTERT mRNA, and this system therefore selectively labels carcinoma and sarcoma cells, but not normal cells, with green fluorescence. The present study aimed to detect CTCs in the peripheral blood of sarcoma patients using this novel method, then looked for a correlation between numbers of CTCs and other clinical features of sarcomas to determine whether these factors can be used to assess tumor progression or prognosis.

Materials and methods

Patients and blood samples

We investigated 20 blood samples obtained before and after surgery from 10 patients (5 men, 5 women; mean age, 73 years; range, 44-86 years) scheduled to undergo surgery for soft-tissue sarcoma of the trunk or extremities. Underlying pathology was myxofibrosarcoma in 5 patients, liposarcoma in 2, and undifferentiated pleomorphic sarcoma, fibrosarcoma, and leiomyosarcoma in 1 each. Tumors were locat-

ed in the chest wall (4 patients), lower leg (4 patients), thigh (1 patient) and iliopsoas muscle (1 patient). Histological grades were assigned according to the French Federation of Cancer Centers Sarcoma Group system based on tumor differentiation, mitotic count, and necrosis [22]. Sarcoma was categorized as grade 2 in 5 patients and grade 3 in 5 patients. Tumor size was evaluated by measuring the largest diameter on magnetic resonance imaging. Clinical data are shown in **Table 1**. All patients with these sarcomas underwent tumor resection and/or chemotherapy between 2012 and 2014 at our institute. After conservative surgery for the soft-tissue sarcoma, we performed external radiation therapy for patients who underwent marginal resection. Mean postoperative follow-up was 36.6 months (range, 12-49 months). Chemotherapy comprised multi-agent systemic chemotherapy in patients with metastasis. High-dose ifosfamide and doxorubicin were used. No patients received chemotherapy before blood specimens were collected. After surgery, 6 patients remained disease-free, 2 patients showed no evidence of disease, and 2 patients had died of the disease. Three patients developed lung metastasis without local recurrence. Two of those three patients with metastasis had died, while one remained alive after pulmonary metastasectomy. Participants in the current study were recruited from our hospital and provided written consent for blood collection in accordance with a protocol approved by the ethics committee at our institution (approval number COI2402-24-28).

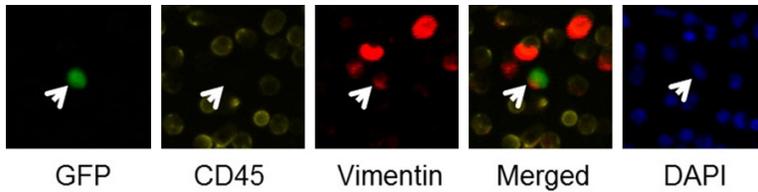


Figure 1. After 7.5-ml peripheral blood samples were infected with viral agent, each sample was treated with a surface-active agent to degrade red blood cells. The remaining cells were scraped from slides. Glass slides were then immunostained with CD45, vimentin and DAPI. The number of CTCs (GFP+, CD45-, vimentin + cells) was counted under fluorescence microscopy.

Abcam, Cambridge, UK). For signal amplification, fluorescently-labeled secondary antibodies (1:200, A21235, A11-046, A21093; Invitrogen, Waltham, US) or an *in vitro* labeling kit (Z25005; Invitrogen) were used. Cells were resuspended in 2% FBS/(phosphate buffered saline (PBS) and dispensed onto 96-well plates. Fluorescence signals were detected under fluores-

cent microscopy (IX71; Olympus, Tokyo, Japan), and cell images were acquired on Metamorph (Molecular Devices; Sunnyvale, US) using each filter set (DAPI/FITC/RFP/CY5; Olympus) (**Figure 1**).

Statistical analysis

Correlations between clinical factors and numbers of CTCs were calculated with unpaired t-tests. Each prognostic factor was divided into two groups based on average values. Data are presented as mean ± standard deviation. In all analyses, values of $P < 0.05$ were considered significant. All analyses were performed using the Statview version 5.0 statistical package (Abacus Concepts, Berkley, CA).

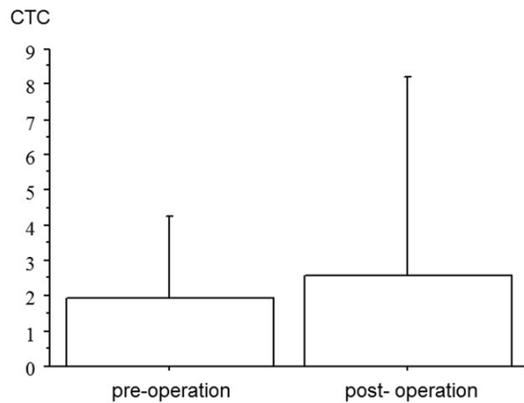


Figure 2. The mean number of CTCs was 1.9 in pre-operative patients and 2.6 in postoperative patients. No significant difference in number of CTCs was evident between pre- and postoperatively ($P=0.704$).

Measurement of viable CTCs

Peripheral blood (7.5 ml) was obtained from each patient before surgery and more than 2 weeks after surgery. Blood samples were drawn into collection tubes containing citrate-phosphate-dextrose solution and incubated with lysis buffer containing ammonium chloride to remove erythrocytes. After centrifugation, the remaining white blood cells were washed twice with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS). White blood cell pellets were mixed with 2.3×10^8 OBP-401 or 1×10^9 OBP-1101 viral particles and incubated for 24 h at 37°C with gentle rotation. Following centrifugation, cells were incubated with primary antibodies at room temperature for 30 min, fixed with 4% paraformaldehyde, and permeabilized with 0.15% Triton X-100. The following primary antibodies were used: anti-CD14 (1:200 325601; BioLegend, San Diego, US), anti-CD45 (1:100 304002; BioLegend), and anti-vimentin (1:200 ab45939;

Results

The mean number of CTCs was 1.9 (range: 0-6) preoperatively and 2.6 (range: 0-18) postoperatively (**Table 1**). No significant difference in numbers of CTCs were evident between pre- and postoperatively ($P=0.704$; **Figure 2**). All patients showing increased numbers of CTCs postoperatively compared to preoperatively also displayed lung metastases. The number of preoperative CTCs did not appear significantly related to each clinical factor (sex, $P=0.714$; tumor location, $P=0.254$; metastasis, $P=0.650$; age, $P=0.153$; histological grade, $P=0.538$; tumor size, $P=0.882$; life prognosis, $P=0.952$). The number of postoperative CTCs appeared unrelated to following clinical factors (sex, $P=0.338$; tumor location, $P=0.338$; age, $P=0.603$; histological grade, $P=0.190$; tumor size, $P=0.243$). However, the number of postoperative CTCs was significantly related to metastasis ($P=0.022$; **Figure 3**). The number of CTCs postoperatively was significantly higher in patients with metastasis (8.3 ± 8.4) than in

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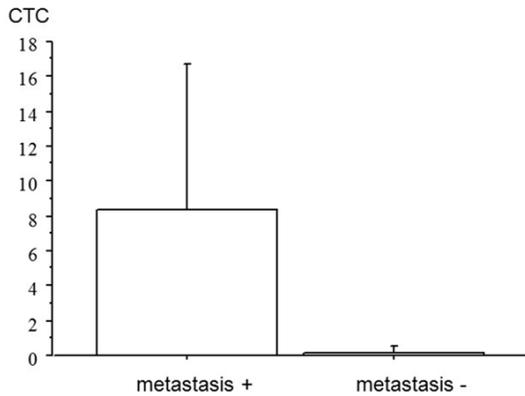


Figure 3. Patients with metastasis (8.3 ± 8.4) showed significantly higher CTC numbers than those without metastasis (0.14 ± 0.38).

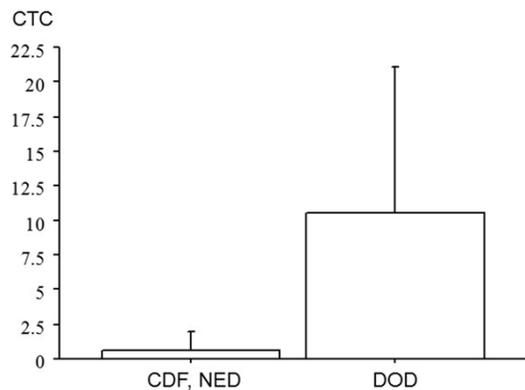


Figure 4. CTC numbers were significantly higher for dead patients (10.5 ± 10.6) than for living patients (0.63 ± 1.41).

those without metastasis (0.14 ± 0.38). The number of postoperative CTCs correlated significantly with life prognosis ($P=0.014$; **Figure 4**). The number of postoperative CTCs was significantly higher in dead patients (10.5 ± 10.6) than in living patients (0.63 ± 1.41).

Discussion

Cancer metastasis is the process of cancer cell dissemination to establish new growth in different organs from the primary lesion via the blood circulation [23, 24]. CTCs can be detected in peripheral blood, may play an important role in the biology of metastasis, and offer clinically useful biomarkers for several carcinomas [5-8, 25-27]. Detection of CTCs in the blood of patients with carcinoma offers a less-invasive method for detecting cancer in the early stages, assessing tumor progression, and deter-

mining prognosis. CTCs also serve as a biomarker of therapeutic efficacy.

In contrast to carcinoma, little data has been published on CTCs in sarcomas. CTCs may be detectable in sarcomas, but their clinical value is unknown [3, 4]. The spread of sarcoma cells to the lungs occurs mainly via the vasculature, although angiosarcoma, clear cell sarcoma, and epithelioid sarcoma more frequently spread to lymph nodes [2]. CTCs represent an important event in metastatic relapse via the vascular system and may have the potential to form metastases. CTCs may therefore be of great importance for the analysis of metastasis in almost all types of sarcoma. However, the prognostic significance of CTCs remains controversial. In some reports, detection of CTCs has indicated oncological aggressiveness and correlated with metastasis of the sarcoma [28-32]. In contrast, another report found that CTC detection did not correlate with tumor progression or metastasis [33]. In the present study, all patients with an increased number of CTCs postoperatively compared to preoperatively had lung metastases. The number of CTCs postoperatively correlated significantly with both metastasis ($P=0.022$) and life prognosis ($P=0.014$).

Tumor cell dormancy may be due to the micro-environment, response to treatments, and the immune system [34-36]. CTCs are present in peripheral blood; this cell type present in bone marrow is referred to as a disseminated tumor cell (DTC). CTCs and DTCs can persist as dormant cells in the absence of cell division and apoptosis [37-39], or may persist due to a balance between proliferation and apoptosis [40, 41]. DTCs may exist in specific organ niches with enhanced survival, and may remain dormant before growing into clinically detectable metastases [42, 43]. We therefore hypothesized that sarcomas with DTCs in the course of metastatic growth may exhibit a time lag before metastatic relapse, as indicated by an increase in the number of CTCs after surgery in relation to metastasis.

Previous reports have shown that CTCs can be shed into the vasculature during tumor resection, and manipulation has also been shown to correlate with micrometastases [44-47]. A previous report demonstrated that CTCs are increased during surgery, but the numbers

recover 7 days after surgery [44]. In the current study, peripheral blood was obtained from patients more than 2 weeks after surgery, removing the influence of surgical manipulation. Our data showed no significant difference in numbers of CTCs between pre- and postoperatively ($P=0.704$).

In conclusion, we demonstrated that an increase number of CTCs postoperatively compared to preoperatively was related to lung metastasis, although the small sample size represents a limitation of this study. The number of postoperative CTCs correlated significantly with metastasis and prognosis. This assay is extremely simple and uses an *ex vivo* method able to detect viable human CTCs in peripheral blood. Detection of CTCs using this method may be useful for prognostic evaluation of sarcoma patients.

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Disclosure of conflict of interest

None.

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