Universal Marker and Detection Tool for Human Sarcoma Circulating Tumor Cells

Arun Satelli1, Abhisek Mitra1, Jeffry J. Cutrera1, Marcos Devarie1, Xueqing Xia1, Davis R. Ingram2, Denada Dibra1, Neeta Somaiah3, Keila E. Torres2, Vinod Ravi3, Joseph A. Ludwig3, Eugenie S. Kleinerman1, and Shulin Li1,4

Abstract

To date, no specific marker exists for the detection of circulating tumor cells (CTC) from different types of sarcomas, though tools are available for detection of CTCs in peripheral blood of patients with cancer for epithelial cancers. Here, we report cell-surface vimentin (CSV) as an exclusive marker on sarcoma CTC regardless of the tissue origin of the sarcoma as detected by a novel monoclonal antibody. Utilizing CSV as a probe, we isolated and enumerated sarcoma CTCs with high sensitivity and specificity from the blood of patients bearing different types of sarcoma, validating their phenotype by single cell genomic amplification, mutation detection, and FISH. Our results establish the first universal and specific CTC marker described for enumerating CTCs from different types of sarcoma, thereby providing a key prognosis tool to monitor cancer metastasis and relapse.

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Introduction

Sarcoma constitutes approximately 10% of different cancer types (1). These are a rare group of malignant tumors that develop in the soft tissue and bone. There are several kinds of sarcomas, with soft tissue sarcomas that occur in fat, nerves, blood vessels, muscles, and deep skin tissues, whereas osteosarcomas occur in the bone and Ewing sarcomas are associated with bone and soft tissue. Despite the low incidence of these tumors, their occurrence is more common in adolescents and young adults in comparison with other cancers thus causing a loss of substantial years to the treatment of this disease and affects the quality of life. One way to detect the early spread of the localized disease to distant organs is to detect the circulating tumor cells (CTC) from the peripheral blood of the patients. CTCs are rare cells that detach themselves from primary tumor and enter into blood stream, from where they are carried to distant organs to metastasize. These CTCs are considered to be the seeds of metastases (2) and are emerging as promising targets for early detection and monitoring therapeutic efficacy of anticancer drugs (3). At present, the primary markers for detection of CTCs are EpCAM and cytokeratins that can be used to detect CTCs from epithelial cancers only (4) and lack the capability to detect CTCs from sarcoma tumors because these are mesenchymal in origin and do not express epithelial-specific markers. Although there are new technologies that are enriching the CTC based on size and density of CTC (3), none of these studies are applied for CTC enumeration from patients with sarcoma. CTCs have been isolated and identified in a wide range of malignancies and it has been well demonstrated that CTCs are associated with metastasis and play a key role in cancer progression and relapse (5); however, due to the limitations of existing epithelial markers of CTCs and the absence of a specific marker for detecting sarcoma CTCs, the research in this direction remains hampered. Therefore, identification of a new marker that can be useful in the enumeration of CTCs from patients with sarcoma will provide valuable information for patient care.

Vimentin overexpression is frequently associated with different cancers (reviewed in ref. 6) and single cell profiling of CTCs isolated from patients with cancer indicates the overexpression of vimentin transcript (7); however, intracellular expression of vimentin in normal mesenchymal cells, including most of the white blood cells (WBC), limits its usage as a CTC marker. We and others have previously reported the detection of cell-surface vimentin (CSV) in cancer cells (6, 8, 9); however, it remains unknown whether CSV can serve as a marker for detecting CTCs from blood of patients with cancer. Here, for the first time, we report the discovery of CSV as a universal sarcoma CTC marker by using a monoclonal antibody (mAb) 84-1 that was generated for detection of CSV on CTC. The data reported here holds great promise for the detection and enumeration of CTC from patient bearing any given type of sarcoma tumor irrespective of the origin, thus making CSV a universal sarcoma CTC marker.
Materials and Methods

Cell lines

Human umbilical vein endothelial cell (HUVEC) cells were obtained from Dr. Lee Ellis (MD Anderson Cancer Center, Houston, TX), LM7, SAOS-2, K7, K7M3, LM-8, and DUNN cells were kindly provided by Dr. Eugenie S. Kleinerman (MD Anderson Cancer Center). HOS, MG-263, OS-D, OS-O, LM7-GFP, and OS-25 cells were kindly provided by Dr. Dennis Hughes (MD Anderson Cancer Center). Primary cell cultures from patients with osteosarcoma were kindly provided by Dr. Dina Lev (MD Anderson Cancer Center). HUVEC, human fetal osteoblast (HFOB), and SAOS-2 cell lines were obtained directly from American Type Culture Collection. Authenticity for LM7, K7, K7M3, LM-8, HOS, MG-263, OS-D, OS-O, SKNBE-2, LM7-GFP, and OS-25 cells were validated using short tandem repeat DNA Fingerprinting before experimentation at characterized cell line core facility, MD Anderson Cancer Center.

Blood collection and processing

Human blood samples for CTC analysis were obtained after informed consent, per Institutional Review Board protocol at MD Anderson Cancer Center. Healthy blood samples were obtained from Gulf Coast Blood Center (Houston, TX). A maximum of 8 mL of blood was obtained at any given blood draw, using CPT Vacutainer tubes (BD Biosciences). Single nucleated cells were isolated as per manufacturer’s recommendation. Cells were then washed in PBS and used for further analysis.

Spiking assay

For sensitivity assay demonstration, approximately 2, 5, and 10 Calcein AM labeled (EMD Bioscience) LM7 cells were spiked into 10 million peripheral blood mononuclear cells (PBMC) containing sample in triplicate. For specificity demonstration, approximately 5 cells of Calcein AM labeled LM7 cells were spiked into 10, 20, and 25 million PBMC containing samples in triplicates. All cells used for spiking experiments were subjected to 84-1-positive selection a day before the analysis to increase the fraction of 84-1-positive cells. For cell counting, cells were harvested in culture medium and then serially diluted to achieve the required counts, which were then confirmed in a series of 5 μL spots under a microscope. Under circumstances where in lower/higher numbers of cells were observed, calculations were performed to mix and obtain the necessary counts of cells required to be spiked. Spiking experiments were performed in triplicates to ensure the sensitivity and specificity of the method. For negative controls, CSV-negative cells were spiked into blood and analyzed for positive selection.

84-1-positive cell selection

First, CD45-positive cells were depleted using EasySep Human CD45 Depletion Kit (Stem Cell Technologies) according to manufacturer’s recommendation. To minimize nonspecific binding, antibody against human Fc receptor was added to the cocktail. Second, the CD45-negative cell fraction was subjected to 84-1-positive selection. Briefly, cells were labeled with 84-1 antibody and later mouse immunoglobulin G (IgG) binding microbeads (Miltenyi Biotec) were added to the mixture. 84-1-positive cells were then extracted using the magnetic column from Miltenyi Biotec according to manufacturer’s recommendation.

Antibody production

Recombinant human vimentin (rhVim) with 6-His (R&D Systems) was used as an antigen for antibody production. Anti-vimentin titer was determined using ELISA assay. Briefly, rhVim was used as solid antigen in ELISA. Serum at serial 2-fold dilutions was incubated for 2 hours in ELISA wells coated with rhVim. After washes and incubation with peroxidase-coupled anti-mouse IgG antibody, a color reaction was performed and analyzed with ortho-phenylene-diamine. Antibodies that showed that higher optical density at lower dilutions was considered for further screening. Antibodies selected for screening were incubated with osteosarcoma cell lines with and without vimentin on the cell surface. Antibodies that have very high affinity for CSV were selected using flow cytometry and analyzed further. 84-1 was the best clone available to detect cell surface vimentin with high affinity and sensitivity. This antibody was then further characterized for vimentin binding by ELISA. Western blotting, immunoprecipitation, immunocytochemistry, and immunohistochemistry. Also, from the screening analysis, we identified 12-1, a different mAb that also showed binding affinity to vimentin; however, this antibody was used only for validation purposes.

Mutation analysis

Whole-genome amplification from small numbers of CTC cells or single cells was performed using the REPLI-g Mini Kit (Qiagen).

Flow cytometry

Indirect method of detection was used to detect 84-1 binding. Data acquisition was performed using Attune Flow cytometer (Applied Biosystems) and data analysis was performed using FlowJo software (Treestar).

Sodium orthovanadate treatment

LM8 cells plated on 8-well chamber slides and WBC in suspension were subjected to sodium orthovanadate (SOV; 100 μmol/L) or PBS as a control.

FISH

Cells were exposed to hypotonic treatment (0.075 mol/L KCl) for 20 minutes at room temperature. Cells were fixed in a methanol and acetic acid (3:1 by volume) mixture for 15 minutes, and washed three times in the fixative. The slides were air-dried and stored at −20°C. FISH was performed on these slides using K-RAS and MDM2 FISH probes (Agilent Technologies Inc.) according to the manufacturer’s protocol with slight modifications. Images were captured using a Nikon 80i microscope with a UV source using 4’,6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate, and Spectrum orange filters.

Microscopic image capture and analysis

All microscopic images were captured and analyzed using LEICA DM IRB microscope (Leica Microsystems).
**Statistical analysis**

Data reported here are as mean ± SEM as noted. Differences were considered significant at the 95% confidence level ($P < 0.05$). Data analysis was performed using GraphPad Prism software.

More detailed materials and methods can be found in the Supplementary Materials and Methods.

**Results and Discussion**

To date, detection of CSV on cancer cells has been shown by only using vimentin-specific peptide (8) or virus particles (10), because none of the commercial antibodies bind to CSV (Supplementary Fig. S1A). To generate CSV-specific antibody that binds only to cancer cells while excluding the normal cells, a cell differential expression screening strategy was used (Fig. 1A). Briefly, we generated a large number of hybridoma clones against full-length human vimentin (NCBI: NP_003371.2) and screened vimentin binding antibodies for CSV binding utilizing flow cytometry by targeting CSV on human metastatic osteosarcoma (LM7) cell line, which showed positive binding for CSV-specific peptide (Supplementary Fig. S1B; ref. 7). For negative binding, normal cell lines HFOB and NCM-336 (normal colon) were utilized. Utilizing this strategy, we identified a monoclonal antibody, or a CSV-binding IgG thereof, from hybridoma 84-1. The specificity of 84-1 toward vimentin was further confirmed using Western blotting, immunoprecipitation, and immunofluorescence (Supplementary Fig. S1C, S1D, and S1E). Furthermore, other sarcoma cell lines were evaluated for CSV expression using flow cytometry (Supplementary Table S1).

Cell-surface screening for different cancer and normal cell lines using flow cytometry indicated the presence of vimentin only on the surface of cancer cells (Fig. 1B). Importantly, 84-1 did not show any binding toward macrophages, endothelial cells, neutrophils, platelets, and apoptotic T lymphocytes that are abundantly present in the blood stream (Supplementary Fig. S1F), indicating the specificity of 84-1 toward cancer CSV.

Immunocytochemistry analysis of human metastatic osteosarcoma cells LM7 indicates the presence of vimentin only on the surface of cancer cells (Fig. 1C) that colocalizes with wheat germ agglutinin (WGA), a cell-surface marker.

![Figure 1. Isolation and analysis of CSV-specific 84-1 monoclonal antibody. A, schematic representation of screening antibodies for CSV-specific binding. Pools of monoclonal antibodies from different hybridoma supernatants were analyzed for CSV detection using flow cytometry and antibodies that bound only cancer cells were selected and characterized for vimentin binding using immunocytochemistry (IFC) and Western blotting. B, immunologic assessment of CSV expression in different cancer and normal cell lines using flow cytometry. CSV is detectable only in the cancer cell lines SKNBE-2 (neuroblastoma), RH-41 (rhabdomyosarcoma), and LM7 (osteosarcoma) cells, whereas the nonepithelial normal cells HUVEC, HFOB, and PBMCs were negative for CSV expression. Isotype controls were used for negative controls. C, cell surface staining analysis for CSV in LM7 (osteosarcoma) cancer cell line using confocal microscopy. Cells were stained for CSV (green), WGA (red), and nuclear stain DRAQ5 (blue). 84-1 colocalizing with WGA indicates cell surface vimentin. Scale bars, 10 μm.](attachment:figure1.png)
of the primary cancer cell lines generated from human osteosarcoma patient samples (Supplementary Fig. S1G) shows expression of CSV restricted to metastatic cancer cells, whereas primary cancer cells were negative. These results indicate that CSV expression is associated with metastatic phenotype of the cells.

On the basis of the detection of CSV in a range of sarcoma cell lines, we hypothesized that CSV could serve as a marker to detect sarcoma CTCs. To test this hypothesis, we performed a blood spiking assay using labeled LM7 cells. Known concentrations of Calcein AM labeled cells were spiked into blood and after CD45 depletion and 84-1-positive selection, the cells recovered were subjected to immunofluorescence staining (schematic representation in Fig. 2A). From micrographs, it is evident that single labeled cell isolated from whole blood using 84-1 antibody is detectable utilizing fluorescence microscopy (Fig. 2B). Because sensitivity (limit of detection) and specificity (no background/unwanted cells) of detection are important parameters for using an antibody for CTC enumeration, we evaluated these parameters in this spiking assay using 84-1-positive LM7 cells. Linear regression of the number of detected tumor cells versus the number of tumor cells spiked yielded $R^2$ of 0.976 (Fig. 2C) and indicated that the recovery rates were >60% with approximately 100% specificity (Supplementary Fig. S1H). We also performed spiking assay by spiking higher numbers of cells that yielded similar results (Supplementary Fig. S1I). Furthermore, the isolated tumor cells were negative for CD45 staining, a leukocyte marker (Fig. 2D). As control, normal HFOB cells were spiked (100 cells) and were not detectable by 84-1 antibody. These spiking assays were further corroborated by in vivo studies wherein we utilized LM8 cells for osteosarcoma mouse model. These mice were monitored for changes in CTCs over a period of time (Supplementary Fig. S2A), and at the end of the study, the cells were isolated and confirmed for CSV+, CD45− staining (Supplementary Fig. S2B), and the CTCs were isolated and cultured in vitro (Supplementary Fig. S2C). We also utilized a spontaneous tumor model of p53-mutated mice that showed the presence of CTC in different types of sarcoma tumors that developed spontaneously over a period of time (Supplementary Fig. S2D). Furthermore, we tested for CTCs in the blood of a canine with spontaneous sarcoma tumors, and 84-1 antibody was able to detect CTCs in this model (Supplementary Fig. S2E). These results indicate a high sensitivity and specificity of 84-1 antibody toward spiked cancer cells as well as CTCs that are derived from spontaneous mice and canine sarcoma models.

Next, we tested human blood samples from healthy volunteers and sarcomas that include osteosarcoma, Ewing...
sarcoma, angiosarcoma, leiomyosarcoma, and undifferentiated pleomorphic sarcoma (Supplementary Table S2). No CTCs were detectable in blood samples from healthy donors. Collectively, an increase in CTC count was observed in patients who were diagnosed with metastasis in comparison with patients with localized disease only at the time of first presentation of the clinical disease (Fig. 3A). Also, patients who have undergone prior chemotherapy had lower CTC counts. The CTCs isolated from sarcomas were further characterized by either single cell mutation analysis or FISH using specific probes or by specific markers. Angiosarcoma-derived CTCs were tested for mutations in TP53 and FLI1 genes that were detectable in primary tumors; however, only TP53 mutation was detectable in CTCs (Fig. 3B), indicating the heterogeneous nature of CTCs (11). Osteosarcoma-derived CTCs were evaluated for MDM2 and KRAS amplification using FISH and from the results, it is evident that there is increasing amplification of these genes in patient OS7 when compared with patient OS8 that did not show any amplification (Fig. 3C). OS7 patient was diagnosed with metastasis in lung, whereas OS8 had localized osteosarcoma. Because MDM2 and KRAS have been previously shown to be amplified in patients with metastatic osteosarcoma (12, 13) and from our results, amplification in the CTC is associated with metastasis, it is tempting to speculate that the detection of this amplification in the CTC can predict the onset of metastatic lesions at distant sites and also can prove to be an important tool to predict the therapeutic efficacy of the anticancer drugs. Furthermore, the isolated 84-1 + CD45 + CTCs were validated by utilizing specific markers for a given tumor; CD99 was used as a marker for Ewing sarcoma (14), α-SMA for leiomyosarcoma, and CD31 for angiosarcoma (Fig. 3D). To our knowledge, this is the first study to enumerate and validate CTC from different types of sarcomas using a single specific marker.

Although the mechanism of cell-surface transport of vimentin remains unclear, previous reports have suggested that the translocation of vimentin to cell-surface is phosphorylation dependent (reviewed in ref. 6). We hypothesized that inhibiting specific phosphatases can enhance phosphorylation of vimentin, thereby increasing vimentin translocation to cell surface. To test this hypothesis, we used SOV, an inhibitor of tyrosine phosphatases. SOV increases CSV expression in cancer cells (Fig. 4A, middle); however, there is no change in the CSV of normal leukocytes (Fig. 4A, bottom). Furthermore, we tested the translocation of vimentin to the surface of the 84-1 + CD45 + CTC isolated from osteosarcoma patient sample by treating with SOV or control PBS, and the results indicated a considerable amount of vimentin on the surface of the cancer cells treated with SOV when compared with control treated sample as detectable by fluorescence microscopy (Fig. 4B). Also, we stained the CTC isolated from blood of patient with gastrointestinal stromal tumor (with and without SOV treatment) for CD31 marker to prove that these are not endothelial cells (Supplementary Fig. S3). SOV can therefore be used as a CSV boosting agent, thus increasing the yield of CTCs, a major requirement in the detection of CTCs.

CTC detection using CSV as a marker provides several advantages. First, because there are no literature reports for a universal specific marker to detect sarcoma tumor-derived CTCs, this CSV marker together with 84-1 antibody fills this gap in the field of CTC detection. Second, isolation of viable CTCs for further molecular characterization, not just from human, but also from canine and mouse models highlights its usage as both preclinical and clinical research tool.
be followed up with larger validation studies with clinical endpoints such as relapse and survival. In summary, isolation of CSV-positive CTCs will provide an understanding about the metastatic precursor subpopulation and also help in providing novel diagnostics, treatment and prognostic options based on therapeutic monitoring in patients with sarcoma, and will play a potential role in clinical decision-making.

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

Authors' Contributions
Conception and design: A. Satelli, J.A. Ludwig, S. Li
Development of methodology: A. Satelli, A. Mitra, X. Xia
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Satelli, J.J. Cutrera, D.R. Ingram, D. Dibra, N. Somaiah, K.E. Torres, V. Ravi, J.A. Ludwig, E.S. Kleinerman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Satelli, J.J. Cutrera, S. Li
Writing, review, and/or revision of the manuscript: A. Satelli, J.J. Cutrera, E. S. Kleinerman, S. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Satelli, S. Li

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